Studies on Opisthobranchia.

Marshall, Helen Caroline

HELEN CAROLINE MARSHALL B.SC. (HONS.) M.SC.

STUDIES ON OPISTHOBRANCHIA.

Submitted to the University of Wales Swansea in fulfillment of the requirements for the Degree of Doctor of Philosophy of Marine Biology.

University of Wales Swansea
2006

Declaration & Statements

DECLARATION

- This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

  Signed:.....(Candidate)

  Date:....24/4/06.....

STATEMENT 1

- This thesis is entirely the result of my own investigations, except where otherwise stated. All sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

  Signed:.....(Candidate)

  Date:.....24/4/06.....

STATEMENT 2

- I hereby give my consent for this thesis, if accepted, to be made available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organizations.

  Signed:.....(Candidate)

  Date:....24/4/06.....

Summary

Several species of nudibranch mollusc considered rare can be found at Mumbles, Swansea, UK. Collections were made during every spring low tide, weather permitting, for three years. During the course of this investigation several aspects of nudibranch ecology were investigated.

Oogenesis and spermatogenesis were studied using light microscopy in *Thecacera pennigera* (Montagu), *Ancula gibbosa* (Risso), *Facelina auriculata* (Müller) and *Palio nothus* (Johnston). Significant differences were found in oocyte length in relation to animal size, and nucleolus diameter in relation to the oocyte stage of development. It was concluded that oogenesis in all four species occurs in discrete waves; this is probably attributable to the lack of space within the female follicles once vitellogenesis has occurred. Spermatogenesis was uniform among *T. pennigera*, *F. auriculata* and *A. gibbosa*. However, a difference in the orientation of the developing spermatids was observed in *P. nothus*. Data presented in this chapter for *P. nothus* contradict previous studies investigating other *Palio* spp.

Veliger development was investigated using *T. pennigera*, *P. nothus*, *Onchidoris bilamellata* (L.), *A. gibbosa*, and *Cuthona gymnota* Couthouy. No veliger development or shell growth were observed in *A. gibbosa* or *C. gymnota*. Shell growth was observed in *P. nothus*, but the veligers did not structurally develop further towards metamorphic competency. *O. bilamellata* and *T. pennigera* showed significant development; both exhibited shell growth and developed eye spots. The latter species also developed a larval heart and underwent mantle fold retraction. However, when introduced to *Bugula plumosa* (Pallas) *T. pennigera* failed to undergo metamorphosis.

The population genetics of *T. pennigera* was investigated using mtDNA techniques. Three populations were sampled from South Wales; all three showed genetic homogeneity which was attributed to the long-term planktonic stage, and the extensive flushing which occurs within the Bristol Channel.

Light microscopy was used to investigate the internal morphology and histology of *A. gibbosa*. Many features were similar to that reported for other members of the Goniodorididae. However, *A. gibbosa* was discovered to have a diaulic reproductive system, suggesting that *Ancula* may be a primitive member of this family. Several of the *A. gibbosa* sectioned were parasitized with *Splanchnotrophus willemi* Canu. The effects of this parasite on *A. gibbosa* varied from no internal damage to complete disintegration of the visceral mass. Despite infection, all animals had copulated at least once as evidenced by the presence of allosperm within their receptaculum seminis. It was considered that the individuals suffering severe internal damage due to parasitism would have been unlikely to produce viable spawn. *S. willemi* was implemented as the cause for its sporadic occurrence.

The lecithotrophic anaspidean *Akera bullata* (Müller) was reared through metamorphosis on a variety of algal substrata, biofilm, and a control. Metamorphosis could be initiated within 24 hours of hatching, and was promoted by the presence of *Chondrus*.

Contents

Acknowledgements

There are many people who helped me during my time as a Ph.D. student at the University of Wales, Swansea. Without them this work would not have been possible; every opinion, criticism and suggestion I received has helped shaped this thesis and therefore I am eternally grateful.

Firstly, I have to thank my supervisor Dr P. J. Hayward for his suggestions, encouragement, financial aid and humor. It has been a great honour to work with such a distinguished Marine Biologist. He allowed me to reign freely over this project, albeit a few pointers; something which many Ph.D. students are never allowed to do. Thank you!

Many thanks go to Prof. D. O. F. Skinbinski who helped me understand genetics in a way which I never thought possible; Paul Facey for the hours and hours of help with my genetics, and (of course) the endless entertainment; and Dr S. Conlan for making space available in the genetics lab. At this point I have to mention the wonderful lab technicians "angels" who made things really easy for me: Jean Leamon who gave me lots of encouragement and support when things inevitably went pear-shaped, Cherrill who did all my washing up and made me feel a part of the "genetics family", Betty Griffiths who turned me into a whiz at algal culture, Keith Naylor for helping with the (horrific) water collecting from R.V. *Noctiluca* and for sorting out my sea water filter, Sue Jones for turning my PCR product into readable sequences, and Sarah Walmsley who made my histology stains (messy job) and countless cups of tea whilst I was demonstrating to the undergraduates! Extended thanks go to Dr Jo Porter for her advice on histology and genetics; Dr P. Dyrynda for collecting *Akera bullata* spawn from the Fleet and giving me the opportunity to work with the wonderful critters; Dr J. Lucas Cervera-Currado for the helpful advice on veliger culture; and Dr R. Huys at the Natural History Museum London for identifying the splanchnotrophid and for allowing me to copy all the literature for Chapter 6 without charging me a penny. I must make a special mention to Dr F. Glen for her unwavering enthusiasm, advice on chapter content and statistics ☺. Another special mention goes to Dr R. Callaway, who now knows everything there is to know about nudibranchs (much to her

delight), having translated the vast majority of Schmekel and Portmann's book! Thanks also to Prof. Dr H. Wägele and Prof. Dr A. Klussmann-Kolb for sending copies of their papers. Thanks also to Prof. A. Rowley, Prof. J. Ryland, Dr J. Lancaster, Dr N. Yonow and Dr E. Roberts.

Other thanks are extended to the following people who prevented me going insane over the four years and provided me with many hours of chat and humor: Hazel Loaring, Maggie Amui, Andy Woolmer, Thanos Roussos, Tristan Bantock, Anton Tsyganov, Emma Wootton, and Andrea Waschenbach. Apologies if I have missed anyone who feels they should be on this list, it was not intentional!

And of course, last but by no means least, all my love and heartfelt thanks goes to every member of my family: Mum, Dad, Graham, Joshua, Elle and Daisy (and my fish), for the infinite love and support you have given me over my whole life, not just in these past four years!! I would not have achieved this without you...well Mum, at least you now have two book ends☺ !!

Chapter 1

Chapter 1

An Introduction to Nudibranchiate Mollusca

1.1 *NUDIBRANCH ECOLOGY, DEFENSE, AND PHYLOGENY*

Opisthobranch research is an exciting but largely unexplored area of marine biology. The first major publication regarding the ecology of nudibranchs was produced by Joshua Alder and Albany Hancock during 1845-1855. The book pioneered opisthobranch research by introducing the scientific community to these beautiful molluscs. It is exquisitely illustrated and is the pinnacle of nudibranch literature. A century later, the late Tom Thompson rekindled interest and began a surge of research during the late 1950s, which continues to the present day. The advent of SCUBA has enabled many otherwise inhospitable habitats to be explored; this has lead to the discovery of many new nudibranch species and still does so today.

For further details regarding the ecology of nudibranchs, see reviews by Todd (1981) and Harris (1973). Excellent guides on the identification of European species include: Alder & Hancock (1845-1855), Schmekel & Portmann (1982), Thompson & Brown (1984), Thompson (1988), and Picton & Morrow (1994).

The Order Nudibranchia is an extremely successful and highly adaptable group of gastropod molluscs. Their representatives can be found occupying a diverse range of habitats from polar regions to the tropics, on hard substrata intertidally and sublittorally, in sediments and in the open ocean. Increased transoceanic shipping has assisted the distribution of many opisthobranch molluscs. Many species prey on common fouling organisms i.e. Bryozoa, thus the transfer of adult opisthobranchs over long distances to habitats which would otherwise be inaccessible, is now occurring. One example of this is *Thecacera pennigera* (Montagu) (Thompson & Brown, 1984), its distribution has widened to include Pakistan, Australia, Japan, New Zealand, Korea, and more recently Woods Hole,

Massachusetts. Bill Rudman's website, The Sea Slug Forum (http://www.seaslugforum.net), contains extensive information on the worldwide species of Opisthobranchia.

It has been estimated that there are approximately 3000 species (Willan & Coleman, 1984; cited in Wägele & Willan (2000)). Of these, 108 species have been recorded from the United Kingdom (Picton & Morrow, 1994). This figure is almost certain to increase, and may have done so already.

The phylogeny of the Nudibranchia is constantly under review (Wägele & Willan, 2000). However the current proposed classification by Wägele & Willan (2000) is as follows:

ORDER NUDIBRANCHIA
Anthobranchia
BATHYDORIDOIDEA
Family Bathydoridiidae
DORIDOIDEA
Family Hexabranchidae
Onchidoridiidae
Goniodoridiidae
Corambidae
Gymnodoridiidae
Polyceridae
Triophidae
Aegiridae
Doridiidae
Chromodoridiidae
Conualeviidae
Dendrodoridiidae
Phyllidiidae
Cladobranchia
DENDRONOTOIDEA
Family Tritoniidae
Dendronotidae
Lomanotidae
Dotidae
Bornellidae
Hancockiidae
Phylliroidae
Tethydidae
Scyllaeidae
ARMINOIDEA
Family Arminidae
Goniaeolididae
Heterodoridiidae
Charcotiidae
Doridomorphidae
Leminididae
Dironidae
Zephyrinidae
Madrellidae
Pinufiidae
AEOLIDOIDEA
Family Notaeolidiidae
Flabellinidae

Calmidae
Aeolidiidae
Facelinidae
Embletoniidae
Fionidae
Glaucidae
Pseudovermidae
Eubranchidae
Tergipdidae
Phylogenetic position is currently uncertain:
Family Doridoxidae

For the majority of nudibranch species, life is short, typically lasting one year. They are semelparous, hence die after a period of spawning, however for some species senescence is innately determined. Some nudibranchs are subannual or biennial. The former have several generations per year, the latter survive longer than a year (Todd, 1981). They hatch from a spawned egg mass as a typical gastropod veliger. The veligers can be planktotrophic i.e. they must feed (obligate planktotrophs) in order to develop the necessary organs required for metamorphosis; or lecithotrophic. The lecithotrophic veligers may or may not feed (facultative planktotrophs) depending on the species; however feeding is not necessary for metamorphosis. Often the first sign of nudibranch activity is the presence of an obvious spawn mass. Certain species have been observed to pass though the veliger stage and metamorphose within the egg capsule, hatching out as miniature adults, "direct developers". A few species exhibit poecilogony, where one egg mass releases direct developers and lecithotrophic veligers (see Chapter 3). The veligers spend several hours (typically the lecithotrophic species) to several months (typically the planktotrophic species) within the plankton. During this time the planktotrophic veligers undergo significant development and detorsion, the extent and timing of which is dependent on the species. Lecithotrophic species spend longer within the egg capsule and therefore, on hatching, are more developed than the planktotrophic veligers. The time spent in the plankton, hence the time taken to reach competency is far less (Todd & Doyle, 1981; Hadfield & Miller, 1987). Settlement is often stimulated by cues released from the adult prey species. During metamorphosis they discard their veliger shell and assume the adult vermiform shape. A period of rapid growth is followed by copulation, spawning, and death.

The majority of species are specialist feeders and their life cycles are intimately associated with the prey species. The types of prey consumed vary. Members of the

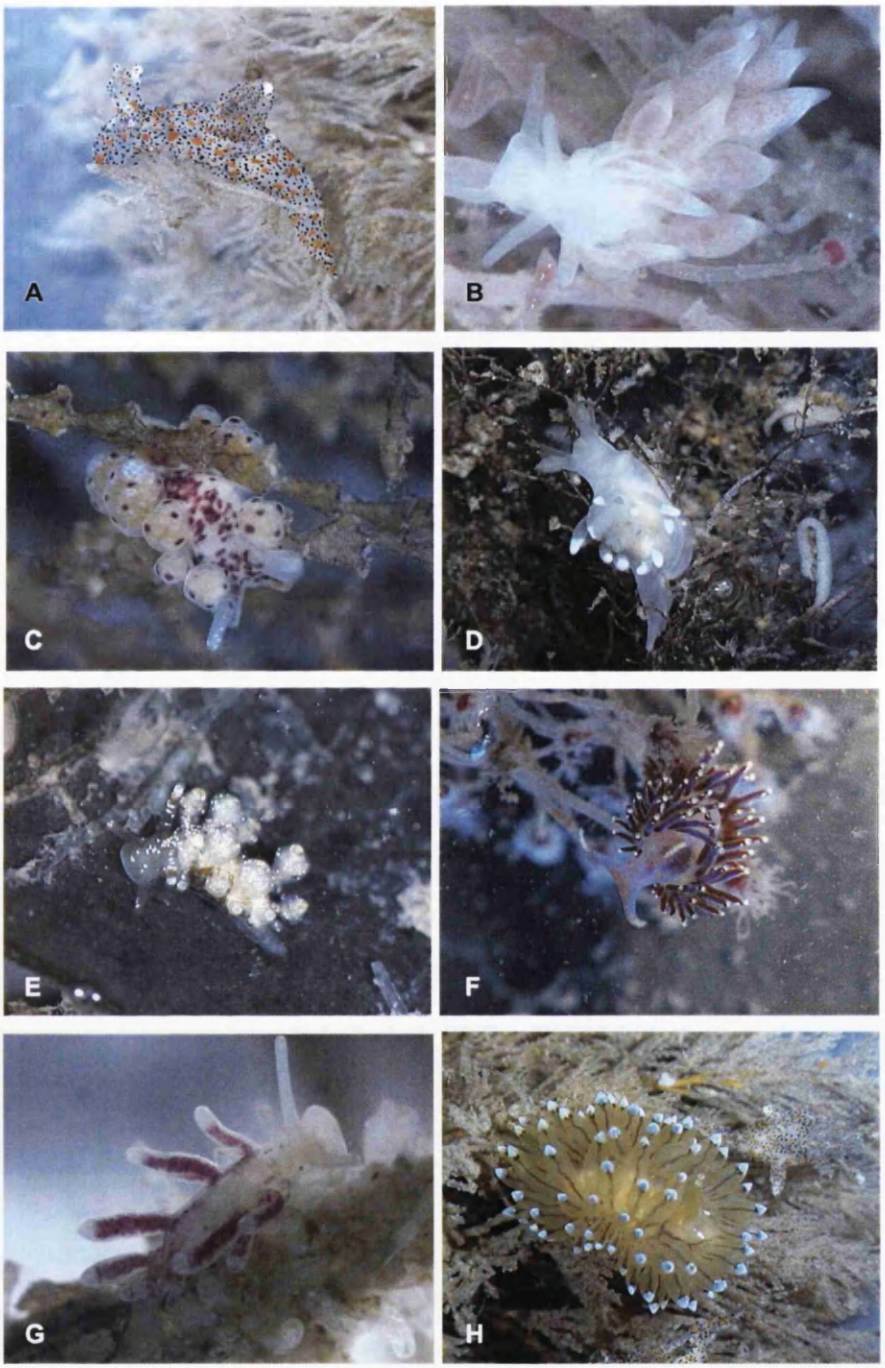

Figure 1.1. Eight species of nudibranch found at Mumbles pier, Swansea. **A.** *Thecacera pennigera*. **B.** *Cuthona gymnota*. **C.** *Doto coronata*. **D;** *Ancula gibbosa*. **E.** *Eubrandnus exiguus*. **F.** *Facelina auriculata*. **G.** *Tenellia adspersa*. **H.** *Janolus cristatus*.

Aeolidoidea typically prey on hydroids (*Doto* and *Facelinia*), anemones (*Aeolidia*), pelagic *Physalia physalis* (L.) (*Glaucus*), and pelagic *Velella velella* (L.) (*Fiona*). Members of the Doridoidea consume sponges (*Archidoris* and *Jorunna*), Bryozoa (*Polycera* and *Thecacera*), barnacles (*Onchidoris bilamellata* (L.)), ascidians (*Goniodoris* and *Okenia*), and possibly entoprocts (*Ancula*). Some members of the Dendronotoidea and Arminoidea prey on soft corals (*Tritonia* and *Armina*). *Calma glaucoides* A. & H. preys exclusively on fish eggs (Todd, 1981).

The evolutionary loss of the gastropod shell in all species of the Nudibranchia has enabled them to evolve without the physical constraints imposed by the shell (Todd, 1981). As a result however, they have lost their protection against predators. Hence, the differences in morphology within the Nudibranchia can often be attributed to the adoption of a particular defense mechanism. Their morphology is highly variable ranging from the inconspicuous flattened members of the Doridoidea, to the elaborate Aeolidoidea with their characteristic cerata (Fig. 1.1). As well as gaining nutrition from their prey, thereby enabling somatic growth and reproduction, many nudibranchs also acquire defense mechanisms. Some species, typically the sponge predators, sequester secondary metabolites obtained from their prey. These in turn are used as defensive allomones (Schulte & Scheuer, 1982; Thompson, Walker, Wratten & Faulkner, 1982; Faulkner & Ghiselin, 1983; Fontana, Ciavatta, D'Souza, Mollo, Naik, Parameswaran, Wahidulla & Cimino, 2001). The types of chemicals vary widely, but include scalaradial, desacetylscaladial and deoxoscalarin in *Glossodoris pallida* (Ruppell & Leuckart) and furanosesquiterpenoids in *Hypselodoris* (Avila & Paul, 1997). Many of these chemicals are known feeding deterrents. The main defensive mechanism of many members of the Aeolidoidea involves sequestering untriggered nematocysts of their coelenterate prey in cnidosacs located at the tip of their cerata. When threatened they are then expelled through the cnidopore. In the event of an sustained attack the cerata can autotomize causing no detrimental effects to the nudibranch. The mucus produced by *A. papillosa* inhibits nematocyst discharge (Mauch & Elliott, 1997). A specific component of the mucus is thought to offer protection to *A. papillosa* during feeding thus protecting the nudibranch's alimentary canal (Mauch & Elliott, 1997). It is likely that a similar property is present in all aeolidoidean nudibranchs. The dorid species possess cellular mechanisms which enable them to synthesize spicules i.e. *T. pennigera.* Acid secreting cells are common in the notum of some species of nudibranchs. The secretions (pH 2) have

been found to be distasteful to fish (Thompson, 1960a; Thompson, 1960b; Edmunds, 1968; Thompson, 1969). Other defensive mechanisms include the colouration of nudibranchs. Edmunds (1987) summarized the function of colour in opisthobranchs and concluded that most were anti-predatory in function. He listed camouflage e.g. cryptic colouration, special resemblance e.g. to that of their respective food, aposematic colouration e.g. a warning signal, batesian and müllerian mimicry, flash colouration, deimatic display e.g. frightening behaviour, and the deflection of attack e.g. through diverting predators to areas of the body which are expendable or noxious, as all possible predator defense adaptations in opisthobranchs. *Hexabranchus sanguineus* (Ruppell & Leuckart) can actively swim, this behaviour is associated with a deimatic display.

Nudibranchs are renowned for their bright pigmentation. Many of these pigments are obtained from their diet. Perhaps the most well known examples are members of the Genus *Rostanga*, these scarlet coloured nudibranchs feed on red sponges such as *Microciona atrasanguinea* Bowerbank. It is believed that the nudibranch assimilates the carotenoids (including astaxanthin) from the sponge on which it feeds (Harris, 1973). The pigmentation provides excellent camouflage when *Rostanga* is on the sponge.

Predators of nudibranchs are few, with the exceptions of other opisthobranchs e.g. *Navanax*, cannibalism in *Facelina auriculata* (Müller) (when kept together under artificial conditions), some species of echinoderms, Crustacea, and fish (Todd, 1981). Todd (1981) also documented the eider duck consuming *O. bilamellata* from pier pilings. During this study young *T. pennigera* were observed to be attacked by pycnogonids. It is unknown as to whether the pycnogonids actually ingested the *T. pennigera*, however the young *Thecacera* never survived these encounters. The larger *T. pennigera* were apparently ignored, possibly due to physical constraints of the pycnogonids.

Many species of Nudibranchia have symbiotic zooxanthellae within their tissues. Typically they are obtained from their cnidarian prey. Members of the Sacoglossa obtain chloroplasts are from their algal diet which are incorporated within their digestive system, "kleptoplasty". The symbionts are often found within the cells of the digestive gland which are located subepidermally to maximize light exposure. McFarland & Muller-Parker (1993) found zooxanthellae and zoochlorellae in *A. papillosa.* Prior to their study it was assumed that only tropical species assimilated zooxanthellae and zoochlorellae. The symbionts translocate carbon to the nudibranch, this extra source contributes to its nutrition and is

thought to be particularly beneficial when food is scarce. Harris (1973) postulated that the short generation time and the rapid growth rate of *Phestilla sibogae* Bergh compared to *P. melanobranchia* Bergh, is due to the former feeding upon coral containing zooxanthellae whereas the latter does not. However, McFarland & Muller-Parker (1993) found that if *A. papillosa* (L.) is starved the symbionts are expelled within 11 days. Wägele & Johnsen (2001) found that symbionts were absent from individuals from several species which in previous investigations had been reported to contain them. Thus symbiosis among the Nudibranchia is not a "hard and fast rule". It is likely that in the majority of instances, the presence or absence of zooxanthellae is dependent on diet, although there are exceptions. Wägele & Johnsen (2001) suggested that the assimilation of symbionts within *Piseinotecus gabinierei* (Vicente) which feeds on a hydroid that does not contain zooxanthellae, may occur as a result of the nudibranch inadvertently feeding on organisms known to possess zooxanthellae.

Bioluminescence has been reported in only a few species of nudibranch, there is dispute over the mechanisms involved and the source of the luminescence. Some authors claim bioluminescence is a defense mechanism which is under voluntary control by the nervous system, others however have suggested that it is generated by bacteria from within subepidermal sacs (Harris, 1973).

1.2 *NUDIBRANCHS IN THE UNITED KINGDOM*

For many years there have been sightings of nudibranchiate Mollusca under Mumbles Pier, Swansea (P. J. Hayward, pers. comm.). Out of the 108 species recorded from the United Kingdom (Picton & Morrow, 1994), 19 have been found under Mumbles Pier, Swansea, over the course of this Ph.D. (Table 1.1). Several other species were found at Carr's Rocks in Pembrokeshire and Porthcawl including *Jorunna tormentosa* Cuvier and *Polycera faeroensis* Lemche. All three sampling sites are sheltered, and are characterized by large boulders covered with *Fucus serratus* L. on the lower shore with patches of *Ascophyllum nodosum* (L.) higher up, and fine silty sediment (Fig. 1.2). Some of the species found have been recorded in the literature as sporadic, however in South Wales many thrive and return year after year; a phenomenon not typical for the majority of nudibranch species.

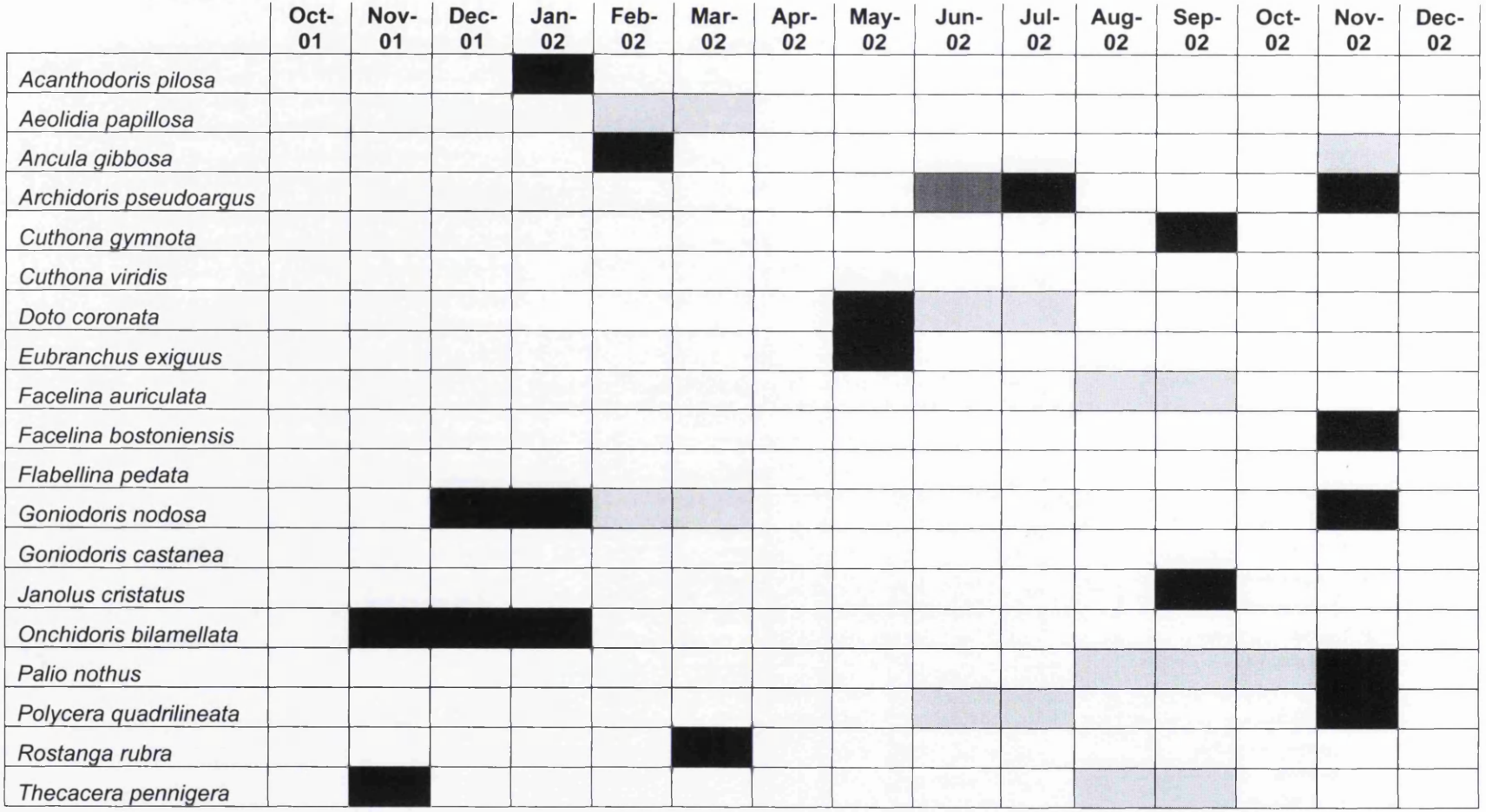

Table 1.1. Caption overleaf.

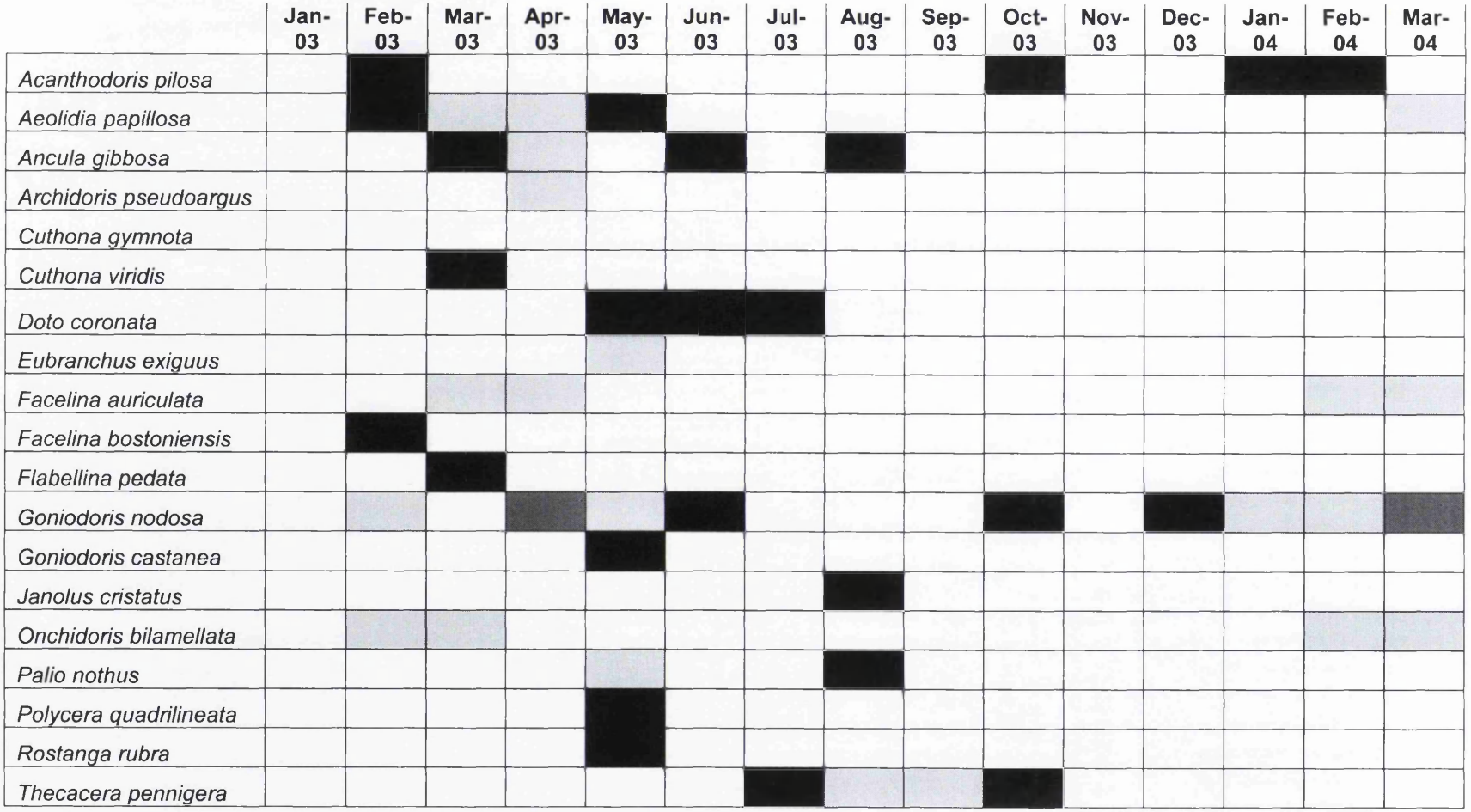

Table 1.1. All the species of nudibranch found in Mumbles, Swansea from October 2001 to March 2004. Black squares: only adults found; dark grey squares: only spawn found; light grey squares: spawn and adults found.

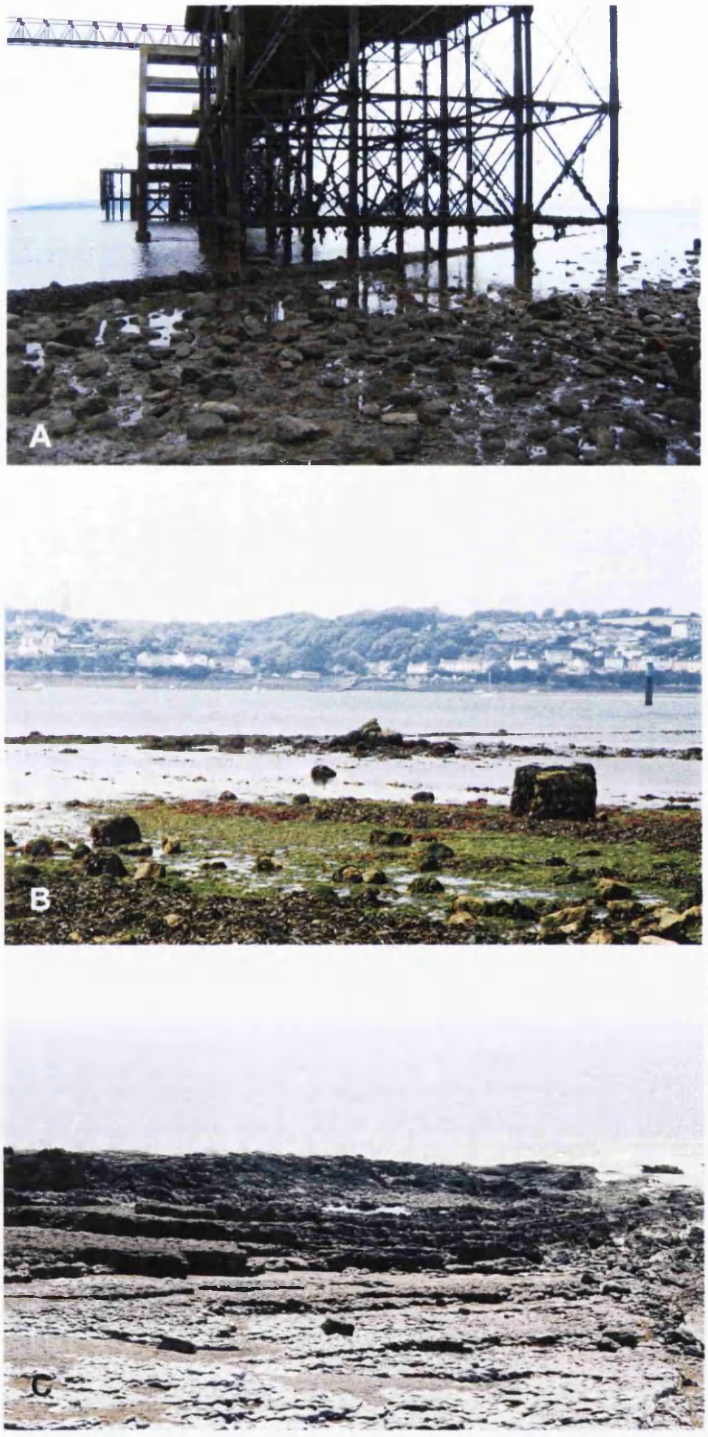

Figure 1.2. Sampling sites. **A.** Mumbles pier. **B.** Carr's Rocks at Pembroke Dock. **C.** Porthcawl.

There is a long history of nudibranch research in the United Kingdom started by Alder & Hancock (1845-1855), followed by Tom Thompson, and finally Chris Todd. However, the majority of opisthobranch research is now only conducted by a few scientists in Germany, North America and Australia. It is disappointing that in its founder country, opisthobranch research is now reduced to only a few scientists.

Despite the continuing widespread interest in the Opisthobranchia there is still much to be learned about these enigmatic little molluscs.

1.3 *CHAPTER SUMMARY*

This thesis has been divided up into seven chapters, excluding this introduction. Some of the chapters were planned prior to the start of this Ph.D.; other topics were discovered accidentally and deemed "too good an opportunity to miss". Due to the wide scope over which this thesis is based, there is very little overlap in any of the chapters; although some of the methods are common to more than one. The following paragraphs provide a brief overview of the objectives, content and results of each chapter.

Studies investigating gametogenesis in the Opisthobranchia are scarce; only two have covered gametogenesis in the Nudibranchia (Eckelbarger & Eyster, 1981; Medina, Garcia, Moreno & Lopez-Campos, 1986). In Chapter 2 the processes of oogenesis and spermatogenesis were investigated using light microscopy in *T. pennigera*, *F. auriculata*, *A. gibbosa*, and *Palio nothus* (Johnston). In the female acini three stages of oogenesis were identified: oogonia, previtellogenic and vitellogenic. The fourth and final stage was characterized by the breakdown of the nucleolus and the initiation of meiosis. This stage occurred after the oocytes had left the female acini, it was only observed in *T. pennigera* and *F. auriculata*. The sizes of the first three stages of oogenesis were significantly different in all species in different sized individuals. The size of the nucleolus was also significantly different in previtellogenic and vitellogenic oocytes in the four species. From the results it was concluded that vitellogenesis occurs in phases. One batch of previtellogenic oocytes starts to mature and only after spawning does vitellogenesis begin again. Spermatogenesis was classified into four stages: primary spermatocytes, secondary spermatocytes, spermatids and spermatozoa. Spermatogenesis within *T. pennigera*, *F. auriculata* and *A. gibbosa* was the same as that documented for other opisthobranch species. However, in *P. nothus* the morphology of the ovotestes and the arrangement of the male and female acini differed

from *T. pennigera*, *F. auriculata* and *A. gibbosa* and other *Palio* spp. Spermatogenesis occurred in long string-like clusters and no accessory cells were observed; this arrangement has never been reported in a nudibranch species.

Prior to studies attempting to rear veligers in the 1950s; the lifecycle of nudibranchs was largely misunderstood. Many authors have managed to rear veligers through metamorphosis, using the techniques outlined in Chapter 3 (Table 3.1). With the advent of new techniques, and perhaps more importantly an understanding of the necessary requirements of veliger dietary regimes and cleanliness, veliger culture through successive generations has become more successful. Despite this however, only a few planktotrophic species have been successfully reared; their long larval stage adds to the difficulty of culture. Over two years of this Ph.D. attempts were made to rear the planktotrophic veligers of *O. bilamellata*, *P. nothus*, *A. gibbosa*, *T. pennigera* and *Cuthona gymnota* Couthouy in order to study the morphological changes which occur at metamorphosis, and to investigate their settlement preferences. Despite efforts, none of the five species reached competency. *O. bilamellata* and *T. pennigera* did however show evidence of further development. *T. pennigera* underwent mantle fold retraction and may have reached competency, although (possibly) due to poor food quality metamorphosis was not stimulated. This work is presented in Chapter 3.

During the early stage of this Ph.D., two populations of *T. pennigera* were found, one at Mumbles and the other at Pembroke Dock. After further reading a fourth population at Porthcawl was sampled (Boyden, Crothers, Little & Mettam, 1977). Unfortunately attempts to obtain animals from a fourth "foreign" population failed, therefore only three Welsh populations were analyzed. The population genetics of *T. pennigera* was investigated during the third year of this Ph.D. in order to investigate larval dispersal. The mitochondrial COI gene was sequenced and analyzed from animals gathered from the three populations. The populations all exhibited a high degree of genetic homogeneity, supporting similar results found by Todd, Lambert & Thorpe (1998) investigating *Goniodoris nodosa.* There was no significant difference in the 16 haplotypes within and between the three populations. It was suggested that the teleplanic larval phase of *T. pennigera* and the tidal influences in the Bristol Channel result in extensive larval dispersal, thereby producing a common gene pool and a large effective population size. The results are presented in Chapter 4.

Sampling was undertaken every spring low tide (weather permitting) during the three years; *A. gibbosa* was found several times (Table 1.1). Due to the sporadic occurrence of this species and the consequent lack of literature regarding it, its internal morphology was investigated using light microscopy. The majority of features were similar to that of *G. castanea* Alder & Hancock (Wägele & Cervera, 2001). However, it was found to exhibit a diaulic reproductive system. *A. gibbosa* has no vagina, instead both the bursa copulatrix and receptaculum seminis branch off the proximal region of the oviduct. This is the first documented account of diaulie within the Goniodorididae. The results are presented in Chapter 5.

During the work presented in Chapter 5, it was discovered that several of the *A. gibbosa* were parasitized by a copepod of the Genus *Splanchnotrophus*. The discovery of the parasite *S. willemi* Canu, which is even rarer than its host, presented an ideal opportunity to study the effect of parasitism on *A. gibbosa*. Some individuals had no obvious internal damage, while in others the digestive gland had been destroyed. In an attempt to understand the influence *S. willemi* has on *A. gibbosa*, the chapter posed many new questions which require further research. It seems highly probable that in understanding the ecology *S. willemi*, we will further understand the ecology that governs populations of *A. gibbosa*. The results are presented in Chapter 6.

In June 2003 *Akera bullata* Müller adults and spawn were gathered from the Fleet, Dorset, by Dr P. Dyrynda for his Ph.D. student. I was allowed to use the spawn; this gave me the opportunity to study the veligers and rear them through metamorphosis. The veligers are lecithotrophic and were capable of metamorphosis within 24 hours of hatching. Three species of algae, four species of phytoplankton, a biofilm and a control were tested to determine whether metamorphosis was selective to one species, or general for a variety of species. Settlement was successful on all substrata provided. However, *Chondrus* and a biofilm initiated faster settlement, irrespective as to whether phytoplankton was added. The number of juveniles that died after metamorphosis was independent of the substratum provided. The results are presented in Chapter 7.

A general discussion is presented in Chapter 8.

Chapter 2

Chapter 2

Oogenesis and spermatogenesis in several species of nudibranch

2.1 *INTRODUCTION*

The majority of the members of the Opisthobranchia are hermaphrodites, except for a few of the Aocochlidioidea; thus when sexually mature they have both male and female germ cells: spermatozoa and oocytes respectively. The male gametes develop first, hence they are protandric hermaphrodites; this protandric period is often short in duration. Todd (1978) emphasized the importance of protandry in *Onchidoris muricata* (Müller). The production of male gametes is energetically less expensive than the production of female gametes. Thus by maturing into a functional male at a smaller size, more individuals are available to copulate with hermaphroditic individuals. This reduces the potential waste of gametes as a result of hermaphroditic individuals remaining unfertilized when the population is small. The morphology of the reproductive system will be discussed in a later chapter (Chapter 5). This chapter details the processes and stages of oogenesis and spermatogenesis in the Opisthobranchia.

The ovotestis is the organ common to all hermaphroditic opisthobranchs, in which the male and female germ cells develop. Often it is closely associated with the digestive gland. The situation in which the ovotestes surround the digestive gland is common to the Tritoniidae, and most Doridoidea, Arminoidea, and Aeolidoidea (Wägele & Willan, 2000). However, in *Godiva banyulensis* Portmann & Schmekel the ovotestis is found within the haemocoel and is unassociated with the digestive gland (Medina, Garcia, Moreno & Lopez-Campos, 1986). The ovotestes are surrounded by a basal lamina and subepidermally by a thin layer of muscle cells.

In the Nudibranchia the ovotestes can have one of two morphologies. In the plesiomorphic condition, the male and female gametes lie within the same follicle. Typically the developing oocytes are located in the distal region of the follicle and the spermatozoa

are located in the proximal region. This arrangement has been observed in a number of opisthobranch species, including *Tritonia hombergi* Curvier (Thompson, 1961b), *Flabellina* spp. (Schulze & Wägele, 1998) etc. In the apomorphic condition, the oocytes are located in separate female follicles called acini, often several adjoin one male acinus. This arrangement exists in *Ancula gibbosa* (Risso) (Chapter 5), *Thecacera pennigera* (Montagu), *Facelina auriculata* (Müller). *Palio zosterae* O'Donoghue and *P. dubia* (M. Sars) which copulate by the injection of the penis through the notum followed by the ejaculation of sperm into the male acini (Rivest, 1984). Both species lack a vagina. To facilitate successful penetration, the male acini are located at the periphery of the viscera immediately beneath the notum, and the female acini are located beneath the male acini adjacent to the digestive gland (Rivest, 1984). The complete separation of the male and female acini occurs within a few sacoglossan species. Within the female acini oogenesis occurs; the primordial oogonia develop into mature oocytes which are then fertilised post-copulation and consequently spawned. Within the male acini spermatogenesis occurs; the primordial spermatogonia develop into mature spermatozoa, after which they are stored in the ampulla in an inactive state ready for copulation.

2.1.1 *OOGENESIS*

There is only limited literature investigating oogenesis for the members of the Opisthobranchia. Yonow (1996), however, identified five stages of oogenesis in *Acteon tornatilis* (L.) These stages were identified by size and by their staining properties. The categories included: oogonia (5-10 μm), pre-vitellogenic oocytes (10-40 μm), early vitellogenic oocytes (40-60 μm), late vitellogenic oocytes (60-80 μm), and mature oocytes (80-210 μm). Thompson (1966) commented on the difficulties of obtaining accurate measurements of the oocytes in *Archidoris pseudoargus* (Rapp) due to their irregular shape. This was reiterated by Todd (1978); he warned against using size alone to classify the stages of oogenesis due to the distortion of the immature stages against the wall of the female acini. Kress (1986) classified oogenesis in *Runcina* into three stages; pre-vitellogenic and early vitellogenic oocytes (covering the development of oogonia to initial yolk deposition); late vitellogenic oocytes; and mature oocytes (covering the development after vitellogenesis just prior to ovulation).

Oogenesis begins with the differentiation of the primordial germ cells into oogonia. These germ cells also differentiate into the male spermatogonia and follicle cells. The

oogonia undergo mitosis thereby increasing in number. In some molluscs, meiosis may occur at this stage (de Jong-Brink, Boer & Joosse, 1983; Dohmen, 1983); however, this has not been reported for the Opisthobranchia (Thompson & Bebbington, 1969). The development of the oogonia into pre-vitellogenic oocytes has not been documented for any species of nudibranch. Medina *et al.* (1986) failed to comment on their existence in either *Hypselodoris tricolor* Cantraine or *G. banyulensis*; instead they postulated that the follicle cells develop directly into pre-vitellogenic oocytes. This does not occur in any other species of mollusc (de Jong-Brink *et al.*, 1983; Dohmen, 1983). The nucleus of the oogonia swells to form a germinal vesicle which acts as storage for cellular components. RNA is then synthesized from within the germinal vesicle where it is either stored or transported into the cytoplasm. Next, other components and organelles are synthesized, including mitochondria and RNA. After sufficient accumulation vitellogenesis occurs, in which yolk granules (either protein or lipid based) are synthesized along with the formation of cortical granules within the cytoplasm (de Jong-Brink *et al.*, 1983; Dohmen, 1983). Typically the pre-vitellogenic oocytes are located at the periphery of the acini and the vitellogenic oocytes at the centre. It was postulated by Medina *et al.* (1986) that as the pre-vitellogenic oocytes mature they are displaced into the centre of the acini by the younger oocytes arising from the periphery. In *O. muricata* the pre-vitellogenic oocytes are often angular in shape and have a homogeneous cytoplasm (Todd, 1978). Once vitellogenesis has begun the cytoplasm takes on a granular appearance as the yolk granules are deposited (Todd, 1978; Kress, 1986; Medina *et al.*, 1986; Yonow, 1996). The constitution of yolk depends on the species. In *O. muricata* and *H. tricolor* the yolk is composed of protein (Todd, 1978; Medina *et al.*, 1986 respectively); however in *G. banyulensis* the yolk contains both protein and lipid constituents (Medina *et al.*, 1986). Due to the yolk synthesis there is a substantial increase in oocyte volume. In *H. tricolor* and *G. banyulensis*, the nucleus also increases in size and the chromatin disbands (Medina *et al.*, 1986). Yonow (1996) identified three stages of vitellogenesis. The first stage was identified by staining with Mallory's, bromophenol blue, or Sudan Black B; the following two stages were only distinguished by size (60-80 μm and 80-120 μm, respectively). Medina *et al.* (1986) and Kress (1986) observed the formation of an irregular nuclear envelope caused by invaginations at regions where the number of nuclear pores was greatest. Medina *et al.* (1986) concluded that the indentations facilitated the exchange of components between the nucleus and the cytoplasm during vitellogenesis. After development in the acini is complete

the oocytes undergo one final stage of maturation in which the nucleolus breaks down (Thompson, 1961b; 1966; Todd, 1978). This typically occurs during the translocation of the oocytes out of the female acinus and along the hermaphroditic duct (Thompson, 1961b; Todd, 1978). During this stage meiosis was observed in *Aplysia* spp. (Thompson & Bebbington, 1969). In *Runcina*, between leaving the female acini and arriving at the fertilization chamber, the oocytes had gained another layer of albumen (Kress, 1986).

There are two mechanisms by which yolk is synthesized: autosynthetic or heterosynthetic. In the former, yolk is synthesized by the oocytes, this is typical in gastropods; in the latter the yolk and or precursors are synthesized by cells other than the oocytes, which are then transported into the oocyte. Despite their comprehensive study Medina *et al.* (1986) were unable to identify the processes by which yolk is synthesized in either *H. tricolor* or *G. banyulensis*. They suggested that it may form from multivesicular bodies which are derived from modified mitochondria. In *Runcina*, Kress (1986) observed yolk precursors originating from the Golgi within the oocyte. She also observed however, an uptake of constituents via pinocytosis across the oocyte membrane from the haemolymph, suggesting that both mechanisms of yolk formation were occurring. In other molluscs the endoplasmic reticulum, autophagous vacuoles, and multivesicular bodies have all been implicated in protein yolk formation (de Jong-Brink *et al.*, 1983). The synthesis of lipid yolk can appear to be unassociated with organelles; however the mitochondria, Golgi and Balbiani bodies have all been documented in lipid yolk synthesis (de Jong-Brink *et al.*, 1983). There are many ultrastructural changes that occur during vitellogenesis which will not be discussed here (Kress, 1986; Medina *et al.*, 1986).

Follicle cells are frequently not reported or even discussed in literature describing the ovotestis and/or oogenesis in the Opisthobranchia. The function of follicle cells during oogenesis, if any, is widely disputed. Jong-Brink *et al.* (1983) record seven documented functions of follicle cells in Mollusca: assisting in vitellogenesis, the production of the vitelline membrane, assisting in ovulation, undergoing phagocytosis, the production of hormones, influencing egg polarity and assisting in the transportation of the oocyte. In *Runcina*, prior to vitellogenesis the follicle cells began to develop "extensive rough endoplasmic reticulum"; as a result Kress (1986) suggested that the follicle cells may contribute to the vitelline membrane. The follicle cells surround the oocytes in such a way that they do not come into contact with the basal lamina of the acini (Kress, 1986). In *H.*

tricolor the pre-vitellogenic oocytes are found within the follicle cell layer and do contact the basal lamina (Medina *et al.*, 1986). The follicle cells are easily distinguished by the obvious condensation of chromatin within their nuclei. Within *Runcina* the arrangement of the chromatin is dependent on the degree of cellular activity (Kress, 1986).

2.1.2 *SPERMATOGENESIS*

There are several detailed accounts of sperm ultrastructure in the Opisthobranchia (Thompson, 1966; Thompson & Bebbington, 1970; Holman, 1972; Thompson, 1973; Healy & Willan, 1984; Wilson & Healy, 2002; Fahey & Healy, 2003). Some authors have tried to reconstruct phylogeny using sperm ultrastructure; however due to the structural differences between closely related species of Opisthobranchia, Healy & Willan (1984) and Fahey & Healy (2003) concluded that sperm morphology alone should not be used. Very few studies have investigated the processes involved in spermatogenesis (Beeman, 1970; Eckelbarger & Eyster, 1981).

The following description of spermatogenesis is a synopsis of Eckelbarger & Eyster's (1981) study on *Spurilla neapolitana* Delle Chiaje. Eckelbarger & Eyster (1981) identified four stages of spermatogenesis which were based on the morphology of the nucleus. They failed to identify spermatogonia, hence this stage of development was not included in their study. Beeman (1970) similarly failed to distinguish spermatogonia from primary spermatocytes within *Phyllaplysia taylori* Dall. The initial stage of spermatogenesis in *S. neapolitana* was termed the "precup". In this stage two "plaques" situated anteriorly and posteriorly polarize the nucleus. The mitochondria reside anteriorly to the nucleus and increase in size. The nucleus develops into an ellipsoidal shape as the axoneme starts to develop. The nucleus then becomes cup shaped, which characterizes the start of the second stage of spermatogenesis, the "cup stage". During this stage the chromatin begins to condense, and the Golgi begins to synthesize several unidentified secretions. The flagellum inserts into the fosse, and the axoneme is initially situated at an oblique angle to the nucleus. The mitochondria, which have undergone "extensive differentiation", start to fuse and become orientated next to the axoneme. A "putative acrosome" starts to form at the sperm tip. During the "postcup" stage the chromatin continues to condense and the "putative acrosome" continues developing. The last stage of spermatogenesis in *S. neapolitana* is termed the "elongate stage". During this stage the periaxonemal mitochondria synthesize two sheaths; one of which surrounds the axoneme, the other surrounds the axoneme and

the keel. The keel contains glycogen, and does not extend the whole length of the axoneme. Eckelbarger & Eyster (1981) failed to identify any stages of spermatogenesis beyond the "elongate stage" in *S. neapolitana.* Spermatogenesis in *S. neapolitana* is similar to that described by Beeman (1970) for *P. taylori.*

In *Acteon tornatilis* Yonow (1996) identified five stages of spermatogenesis using light microscopy: spermatogonia (5 μm), primary spermatocytes (7-7.5 μm), secondary spermatocytes (5 μm), spermatids (2 μm) and spermatozoa (2 μm). The spermatogonia were distinguished by their large densely staining nucleus. The spermatocytes had characteristic granular chromatin, and the spermatids and spermatozoa were identified by their elongate nucleus and flagellar tail (Yonow, 1996).

As in oogenesis, spermatogenesis occurs in close association to undifferentiated cells. Eckelbarger & Eyster (1981) termed these cells "accessory cells" as their function has not been determined, although they suggested that it may be involved in the phagocytosis of material released during spermatogenesis, and the translocation of nutrients to the developing spermatozoa. Beeman (1970) called these cells "nurse cells" in *P. taylori*, but failed to comment on their function. Other authors researching spermatogenesis in molluscs have termed these accessory cells Sertoli cells (Hodgson, Bernard & Lindley, 1991). A second type of cell was discovered by Beeman (1970) in *P. taylori*, which he called "gonial amebocytes" he tentatively suggested that they phagocytosed debris released during spermatogenesis.

2.1.3 *AIMS OF STUDY*

The aims of this study were to identify the stages of oogenesis and to investigate spermatogenesis in four species of Nudibranchia: *Thecacera pennigera*, *Facelina auriculata*, *Ancula gibbosa*, and *Palio nothus* (Johnston). The relationship between body size and oocyte diameter for oogonia, pre-vitellogenic and vitellogenic oocytes; and the relationship between nucleolus diameter in pre-vitellogenic and vitellogenic oocytes were investigated. The histology of the ovotestis was also examined.

2.2 *MATERIALS AND METHODS*

19 *Thecacera pennigera*, 13 *Facelina auriculata*, 20 *Ancula gibbosa* and eight *Palio nothus* were collected from Mumbles, Swansea (003°58'4"W 051°34'2"N) during 2002-2004. They were anaesthetized using a 7% $MgCl_2$ solution diluted 1:1 with aquarium sea water (see Chapter

three for an explanation of the source of sea water). Often anaesthesia would take several days, therefore during this time they were left in a fridge until they failed to respond to physical stimuli. The $MgCl_2$ solution was changed daily. A 3.7% formalin solution (Sigma, catalog number F-1635; 37% solution diluted using distilled water) was used to fix the animals. After one week they were transferred into 70% ethanol (Fischer Scientific analytical reagent grade, diluted using distilled water from absolute) for long term storage. All ethanol used hereafter was Fischer Scientific analytical reagent grade, and all dilutions were made with distilled water unless otherwise stated.

In some instances the animals needed to be anaesthetized quickly, consequently they were placed into aquarium carbonated sea water, generated using a SodaStream™. They were kept in this solution for up to two hours, and then fixed as described above.

The following embedding schedule was adapted from Grimstone & Skaer (1972):

Day 1: Samples placed into 90% ethanol, and left 12 hours.

Day 2: Samples transferred to 100% ethanol. The solutions were changed every two hours. After the third change, the samples were placed into a 1% Necoloidine solution (BDH laboratory supplies) dissolved 50:50 in ethanol:diethylether (BDH); changed after 24 hours.

Day 4: Samples placed into chloroform (Sigma, catalog number C-2432), and left over night.

Day 5: Samples removed from the chloroform and placed into molten paraffin wax (BDH Paramat extra). The specimens were then placed into an oven at 60°C, which was depressurised and left for two hours. The wax was then poured off and replaced, this was repeated four times. On the last wax change the specimens were transferred into TAAB™ silicon embedding moulds, and orientated for sectioning. They were left to cool at room temperature.

The wax moulds were fixed onto wooden blocks of the appropriate size using molten wax. The moulds were trimmed into a trapezoid profile around the specimen using a razor blade. A Beck Microtome (3880/A4) was used to slice the specimens at 7μm to 8μm. The sections were placed onto twin frosted microscope slides which had been damped with a water and glycerine/albumen mix. The slides were gently heated to ensure the sections adhered to the slide.

Cole's haematoxylin and eosin (Appendix pg. 229) and Mallory's were used as general histological stains. Cole's staining schedule:

10 minutes in Histoclear™ (5 minutes in two separate baths).

2 minutes in 100 % ethanol.

1 minute in 90% ethanol.

2 minutes in 70% ethanol.

10-15 minutes in Cole's haematoxylin (on average 14 minutes).

Stain washed off in running tap water.

2 minutes in Scott's solution.

Slides rinsed in running tap water.

The slides were examined under a microscope on low power to ensure the correct degree of staining. Care was taken to ensure the slides did not dry up:

- Overstained, slides were differentiated in acid alcohol.
- Understained, slides were placed back into the haematoxylin and then Scott's solution.

1 minute in 70% ethanol.

3-6 minutes in 0.05% alcoholic eosin (on average 4 minutes).

Few seconds in 70% ethanol until stain ceases to wash out.

Slides examined under low power to ensure adequate staining.

- Overstained, slides differentated in 70% ethanol
- Understained, slides placed back into eosin.

10-15 seconds in 90% ethanol.

5 minutes in 100% ethanol.

10 minutes in Histoclear™.

Mallory's staining schedule:

5 minutes in Histoclear™

1 minute in 100% ethanol.

1 minute in 90% ethanol.

1 minute in 70% ethanol.

1 minute in distilled water.

5 minutes in Mallory's stain.

15 second rinse in running tap water.

30 seconds in 70% ethanol.

30 seconds in 90% ethanol.

30 seconds in 100% ethanol.

5 minutes in Histoclear™.

After staining, the slides were mounted immediately using Histomount™. They were left on a hot plate for several hours to ensure even coverage of the mount. Air bubbles were gently forced out using a seeker. Subsequently, the slides were left to dry at room temperature.

The slides were examined under an Olympus BH2 compound microscope and a Wild Heerbrugg Makroskop M420. Photographs were taken using a JVC TK1270 digital camera using Image-Pro Plus version 4.1 for Windows 95/NT/98. Measuring of oocytes was performed using a function on Image-Pro Plus. Those oocytes which were severely distorted were not measured. The greatest diameter was always recorded, and only in sections where the nucleus and the nucleolus were observed.

2.3 *RESULTS*

The ovotestes of *Thecacera pennigera*, *Facelina auriculata*, and *Ancula gibbosa* were arranged with several female acini located around one central male acinus (Figs 2.1A, 2.2A, 2.4A). In *Palio nothus* the female acini were orientated beneath the notum on the dorsal and lateral sides, and the male acini were orientated immediately beneath the female acini (Fig. 2.3A). In *T. pennigera*, *F. auriculata* and *A. gibbosa* the location of the male and female acini varied between individuals. In *T. pennigera*, *A. gibbosa* and *P. nothus* the ovotestes were found in close association with the digestive gland; however the degree of association differed. In *A. gibbosa* the ovotestes were found embedded within the digestive gland. In *T. pennigera* and *P. nothus* the ovotestes were arranged peripherally to the viscera. In *F. auriculata* the ovotestes occupied the majority of the body cavity; they were separate to the digestive gland which was located between the anterior ovotestes and the posterior ovotestes. In *T. pennigera*, *F. auriculata* and *A. gibbosa* the lumen between the male and female acini was continuous. However in *P. nothus* the male and female lumens were separated by a distinct muscular sphincter (Fig. 2.3B). In all the four species studied the ovotestes were surrounded by a thin epithelium which lacked a muscular sub-epithelium.

From the animals sectioned in this study it was observed that *T. pennigera* became a functional protandric (with mature spermatozoa) at between 7 to 9 mm in length, and became a functional hermaphrodite (with mature oocytes) at approximately 10 mm in length. *F. auriculata* became a functional protandric at <9 mm in length, and became a functional hermaphrodite at approximately 15 mm in length. *A. gibbosa* became a functional hermaphrodite between a length of 4-9 mm. *P. nothus* became a functional protandric at between 4-5 mm, and became a functional hermaphrodite at approximately 5 mm in length (Table 2.1).

2.3.1 *OOGENESIS*

In all species of nudibranch investigated the female acini were arranged with the immature oogonia, pre-vitellogenic oocytes and the follicle cells at the periphery, and with the vitellogenic oocytes at the centre (Figs 2.1B, 2.2B, 2.3D, 2.4B). Four stages of oogenesis were identified:

- OOGONIA. These spherical cells were difficult to distinguish when stained with Mallory's, however they were easily identified using Cole's haematoxylin and eosin. The nucleus stained densely. The oogonia were always located at the periphery of the female acini in close association with the follicle cells. At this stage the nucleolus could not be observed using light microscopy (Figs 2.1B, C, 2.4B).
- PRE-VITELLOGENIC. These cells were distinguishable by their large nucleus, which filled the majority of the cell volume, and their obvious nucleolus (which stained red with Mallory's and dark purple with Cole's haematoxylin and eosin). The cells lacked yolk granules, and were always found at the periphery of the female acini in close association with the follicle cells (Figs 2.1B, 2.2B, 2.3C, 2.4B).
- VITELLOGENIC. These cells were distinguished from the pre-vitellogenic oocytes by their obvious yolk granules located within the cytoplasm. The yolk stained red with Mallory's and pink with Cole's haematoxylin and eosin. Typically, the cells were located in the central area of the female acini; however this was not always the case (Figs 2.1C, 2.2B, 2.3C, 2.4B). The nucleus and nucleolus were similar in appearance to those in the pre-vitellogenic oocytes, however the nucleolus was larger (see below).
- MATURE. This stage was only observed in *T. pennigera* and *F. auriculata.* In both species the mature oocytes were identified due to the absence of the nucleolus. They were

Table 2.1. Caption overleaf.

	SIZE (MM)	SPERMATIDS	SPERMATOZOA	OOGONIA	PRE-VIT	VIT
T. PENNIGERA	4	✓	×	×	×	×
	4	×	×	×	×	×
	4	✓	×	×	×	×
	5	✓	×	×	×	×
	6	×	×	×	×	×
	6	✓	×	×	×	×
	7	✓	✓	×	×	×
	7	✓	×	×	×	×
	8	×	×	×	×	×
	8	✓	×	×	×	×
	9	✓	✓	×	×	×
	10	✓	✓	✓	✓	×
	10	✓	✓	✓	✓	✓
	10	✓	✓	✓	✓	×
	10	✓	✓	✓	✓	✓
	11	✓	×	×	×	×
	13	✓	✓	✓	✓	✓
	15	✓	✓	✓	✓	✓
	18	✓	✓	✓	✓	✓
F. AURICULATA	9	✓	✓	✓	✓	×
	11	✓	✓	✓	✓	×
	13	✓	✓	✓	✓	×
	13	✓	✓	✓	✓	×
	15	✓	✓	✓	✓	✓
	16	✓	✓	✓	✓	×
	16	✓	✓	✓	✓	✓
	16	✓	✓	✓	✓	✓
	16	✓	✓	✓	✓	✓
	17	✓	✓	✓	✓	✓
	17	✓	✓	✓	✓	✓
	18	✓	✓	✓	✓	✓
	20	✓	✓	✓	✓	✓
A. GIBBOSA	4	✓	×	×	×	×
	9	✓	✓	✓	✓	✓
	9	✓	✓	✓	✓	✓
	9	✓	✓	✓	✓	✓
	10	✓	✓	✓	✓	✓
	10	✓	✓	✓	✓	✓
	11	✓	✓	✓	✓	✓
	13	✓	✓	✓	✓	✓
	13	✓	✓	✓	✓	✓
	14	✓	✓	✓	✓	✓
	14	✓	✓	✓	✓	✓
	15	✓	✓	✓	✓	✓
	15	✓	✓	✓	✓	✓
	15	✓	✓	✓	✓	✓
	15	✓	✓	✓	✓	✓
	15	✓	✓	✓	✓	✓
	15	✓	✓	✓	✓	✓
	17	✓	✓	✓	✓	✓
	17	✓	✓	✓	✓	✓

	SIZE (MM)	SPERMATIDS	SPERMATOZOA	OOGONIA	PRE-VIT	VIT
P. NOTHUS	4	✓	✗	✗	✗	✗
	4	✓	✗	✗	✗	✗
	5	✓	✓	✓	✓	✓
	5	✓	✓	✓	✓	✓
	5	✓	✓	✗	✗	✗
	9	✓	✓	✓	✓	✓
	10	✓	✓	✓	✓	✓
	15	✓	✓	✓	✓	✓

Table 2.1. The stages of sexual maturity in different sized individuals of *Thecacera pennigera*, *Facelina auriculata*, *Ancula gibbosa* and *Palio nothus*. ✓ indicates the presence of a particular germ cell and ✗ indicates the absence of a particular germ cell. "Pre-vit" is pre-vitellogenic and "Vit" is vitellogenic.

never seen within the female acini, it occurred during passage along the hermaphroditic duct after the vitellogenic oocyte had left the ovotestis (Figs 2.1D, 2.4C). In *F. auriculata* mitotic cell division was observed during passage of the mature oocytes along the hermaphroditic duct, prior to reaching the ampulla (Fig. 2.4D).

The relationship between the length of the adults and the size of the oocytes was investigated. The greatest diameter of oogonia, pre-vitellogenic oocytes and vitellogenic oocytes was recorded for six *F. auriculata*, six *A. gibbosa*, five *T. pennigera* and three *P. nothus* of different sizes. Only the pre-vitellogenic oocytes of *A. gibbosa* and *P. nothus*, and the oogonia of *T. pennigera* conformed to a normal distribution (F_{max} $P>0.05$ and Levene $P>0.05$), they were therefore tested using a one-way ANOVA. All the other measurements were not normally distributed (F_{max} $P<0.05$ and Levene $P<0.05$), and were therefore tested using Kruskal-Wallis. The smallest and largest median and mean diameters recorded for the different sized species are given in Figures 2.5A-D. There was a significant difference in the diameter of oogonia and the five sizes of *T. pennigera* (ANOVA, $F = 7.331$, $P<0.05$). A significant difference was also found in the diameter of pre-vitellogenic oocytes and vitellogenic oocytes and the five sizes of *T. pennigera* (Kruskal-Wallis test, $\chi^2 = 105.693$, $P<0.05$ and $\chi^2 = 33.092$, $P<0.05$ respectively). A significant difference was found in the longest diameter of oogonia, pre-vitellogenic oocytes and vitellogenic oocytes and the six sizes of *F. auriculata* (Kruskal-Wallis test, $\chi^2 = 36.032$, $P<0.05$; $\chi^2 = 11.732$, $P<0.05$; $\chi^2 = 74.868$, $P<0.05$ respectively). As in *F. auriculata*, a significant difference was found in the longest diameter of oogonia and vitellogenic oocytes and the six sizes of *A. gibbosa* (Kruskal-Wallis test, $\chi^2 = 17.207$, $P<0.05$; $\chi^2 = 67.990$, $P<0.05$ respectively), and in the longest diameter of pre-vitellogenic oocytes and the six sizes of *A. gibbosa* (ANOVA, $F =$

9.913, $P<0.05$), and in the longest diameter of oogonia, and vitellogenic oocytes and the three difference sizes of *P. nothus* (Kruskal-Wallis test, $\chi^2 = 7.440$, $P<0.05$; $\chi^2 = 303.56$, $P<0.05$ respectively), and in the longest diameter of pre-vitellogenic oocytes and the three different sizes of *P. nothus* (ANOVA, $F = 30.661$, $P<0.05$).

The relationship between the size of the nucleolus and the stage of oocyte development was also investigated (Appendix pg. 232). For each species, the nucleolus diameter was recorded in pre-vitellogenic oocytes and vitellogenic oocytes in at least three different individuals. There was a significant difference between the size of the nucleolus in pre-vitellogenic oocytes and vitellogenic oocytes (irrespective of size of the adult) for *T. pennigera*, *F. auriculata*, *A. gibbosa* and *P. nothus* (Mann-Whitney *U*-test, $U = 811.000$, $P<0.05$; $U = 2223.500$, $P<0.05$; $U = 1584.000$, $P<0.05$; and $U = 2696.500$, $P<0.05$ respectively).

2.3.2 *SPERMATOGENESIS*

The stages of spermatogenesis were similar for *T. pennigera*, *F. auriculata*, *A. gibbosa* and *P. nothus*. However, in *P. nothus* the developing sperm was arranged in long strings which ran the length of the acinus (Fig. 2.3D), instead of the usual clusters of spermatozoa (Figs 2.1F, 2.2E, 2.4F). The male region covered a greater area (personal observation) in *P. nothus* than in any of the other three species studied. Four stages of spermatogenesis were identified. The stages of spermatogenesis were not measured due to the poor resolution of the microscope, which would have resulted in inaccurate measurements. Spermatogonia could not be identified and therefore are not discussed in this report.

- PRIMARY SPERMATOCYTE. The primary spermatocytes were the largest germ cells in the male acini. They were circular, and could be easily identified by their large nucleus containing densely staining chromatin (Figs 2.1F, 2.2C,E, 2.3E, 2.4F). Diakenesis was identified at this stage (Fig. 2.2C).
- SECONDARY SPERMATOCYTE. The secondary spermatocytes maintained their spherical appearance but were distinguished due to the reduction in the size of the nucleus. The chromatin within the nucleus began to condense (Figs 2.1F, 2.2C, 2.3E, 2.4E, F).
- SPERMATID. The spermatids were identified due to the formation of the classic arrow-head shaped nucleus. The chromatin within the nucleus continued to undergo extensive condensation. At this stage a flagellum was not present (Figs 2.1E, 2.2D).

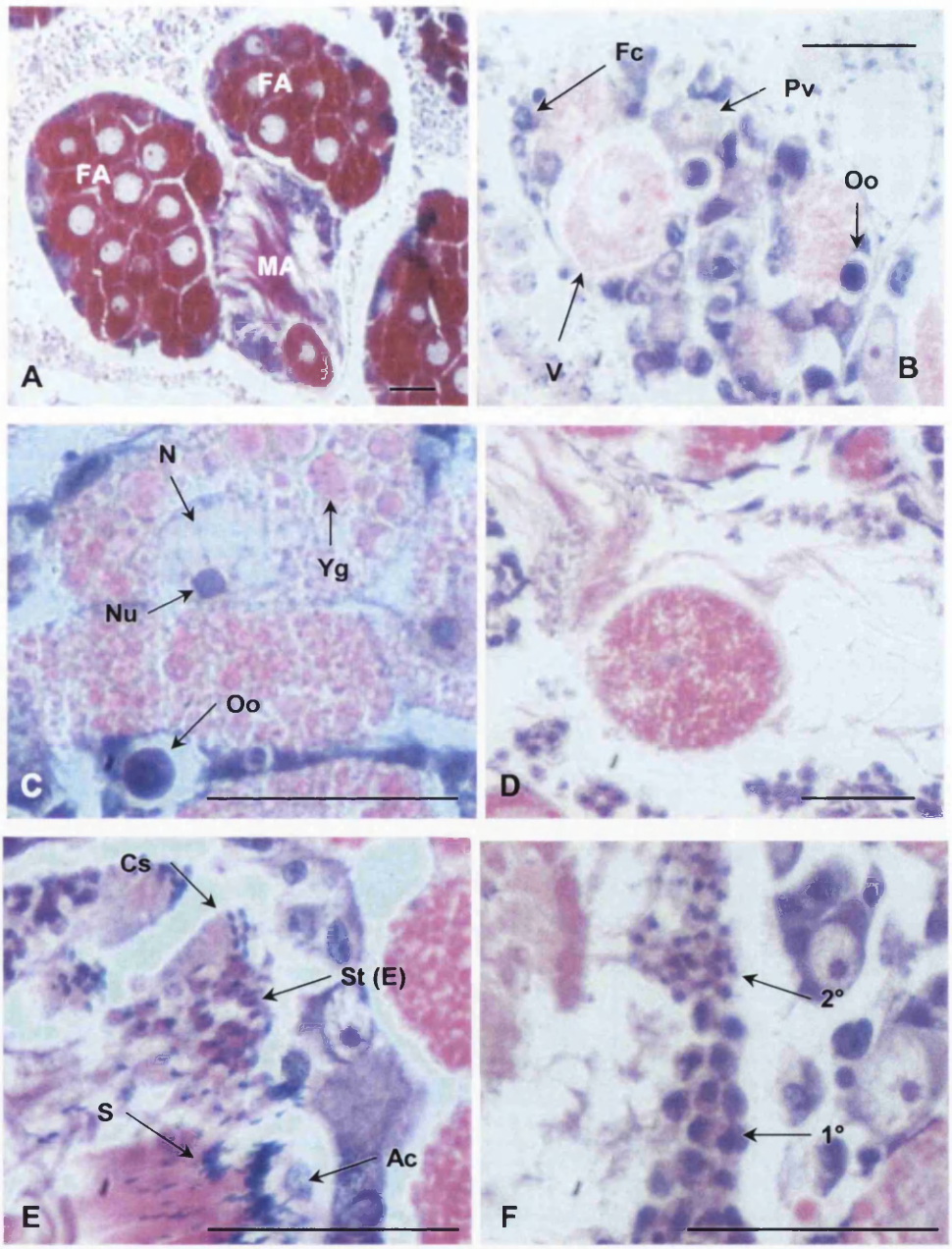

Figure 2.1. *Thecacera pennigera*. **A.** The ovotestis, showing the separate female and male acini. **B.** The stages of oogenesis. **C.** A vitellogenic oocyte showing yolk granules, with an oogonium **D.** The final stage of oogenesis, the breakdown of the nucleolus. **E-F.** The stages of spermatogenesis. Abbreviations of labels given on page 33. Scale bars: **A-F** = 50 μm.

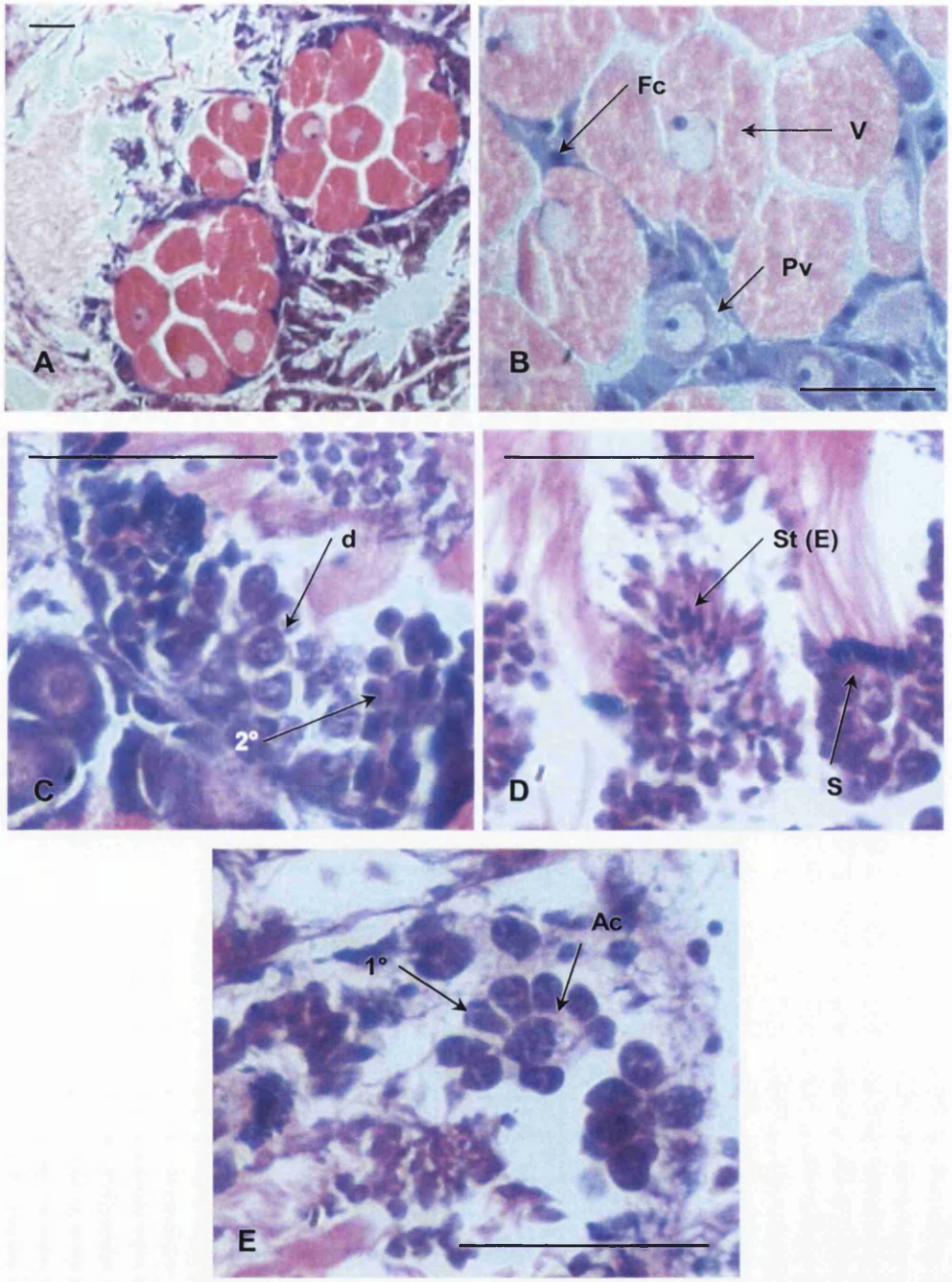

Figure 2.2. *Ancula gibbosa*. **A**. The ovotestis, showing the arrangement of the female and male acini. **B**. The stages of oogenesis within a female acini, showing the pre-vitellogenic oocytes at the periphery and the vitellogenic oocytes at the centre. **C**. Primary spermatogonia exhibiting diakenesis. **D-E**. The stages of spermatogenesis. Scale bars: **A-E** = 50 μm.

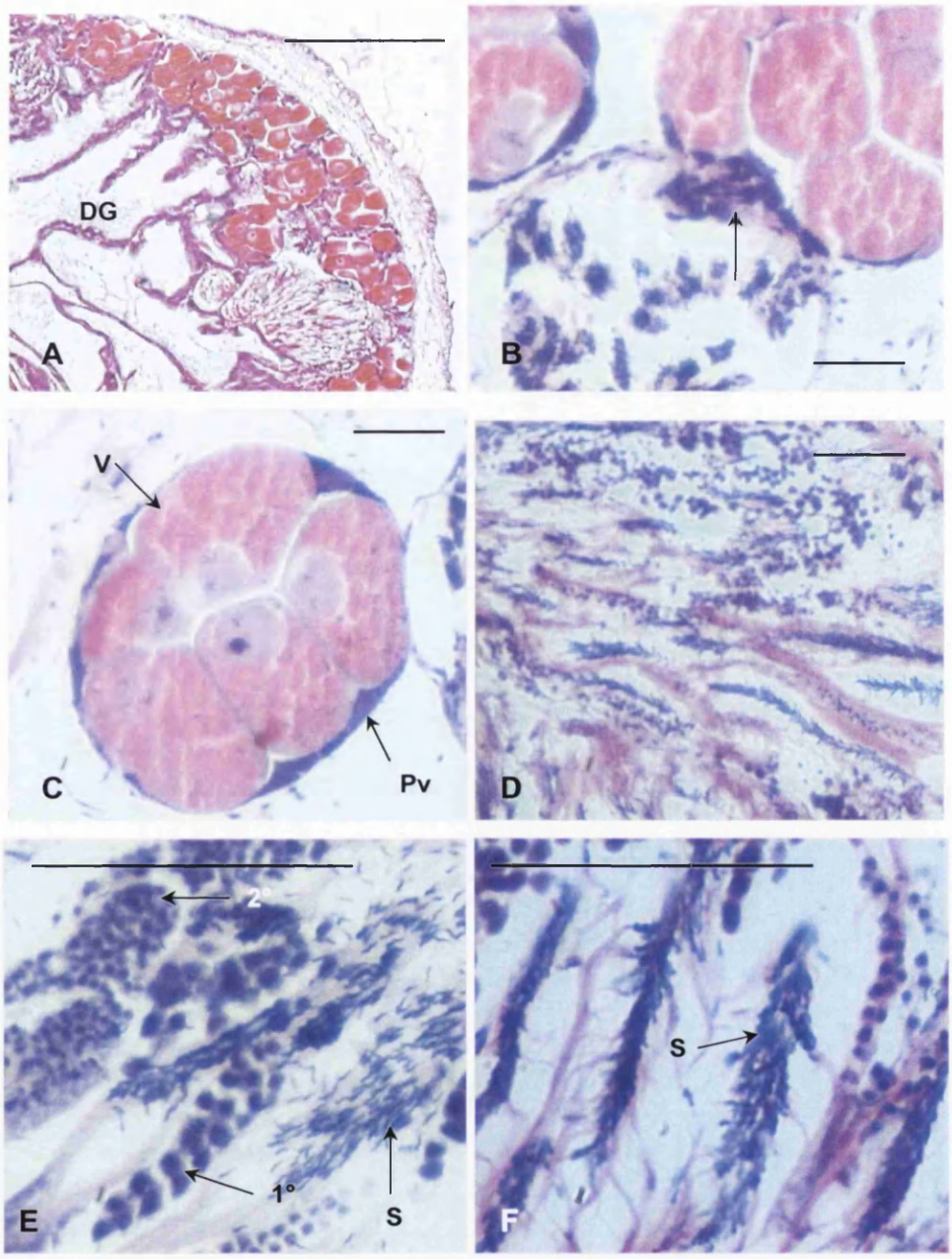

Figure 2.3. *Palio nothus*. **A.** The orientation of the male and female acini, note the location of the female acini at the periphery lying beneath the notum. Scale bar 500 μm. **B.** The ovotesis showing the join between the male and female acini, arrow. **C.** The stages of oogenesis. **D.** The orientation of spermatogenesis within a male acini, note the long chains of cells. **E-F.** The stages of spermatogenesis. Scale bars: **A-F** = 50 μm.

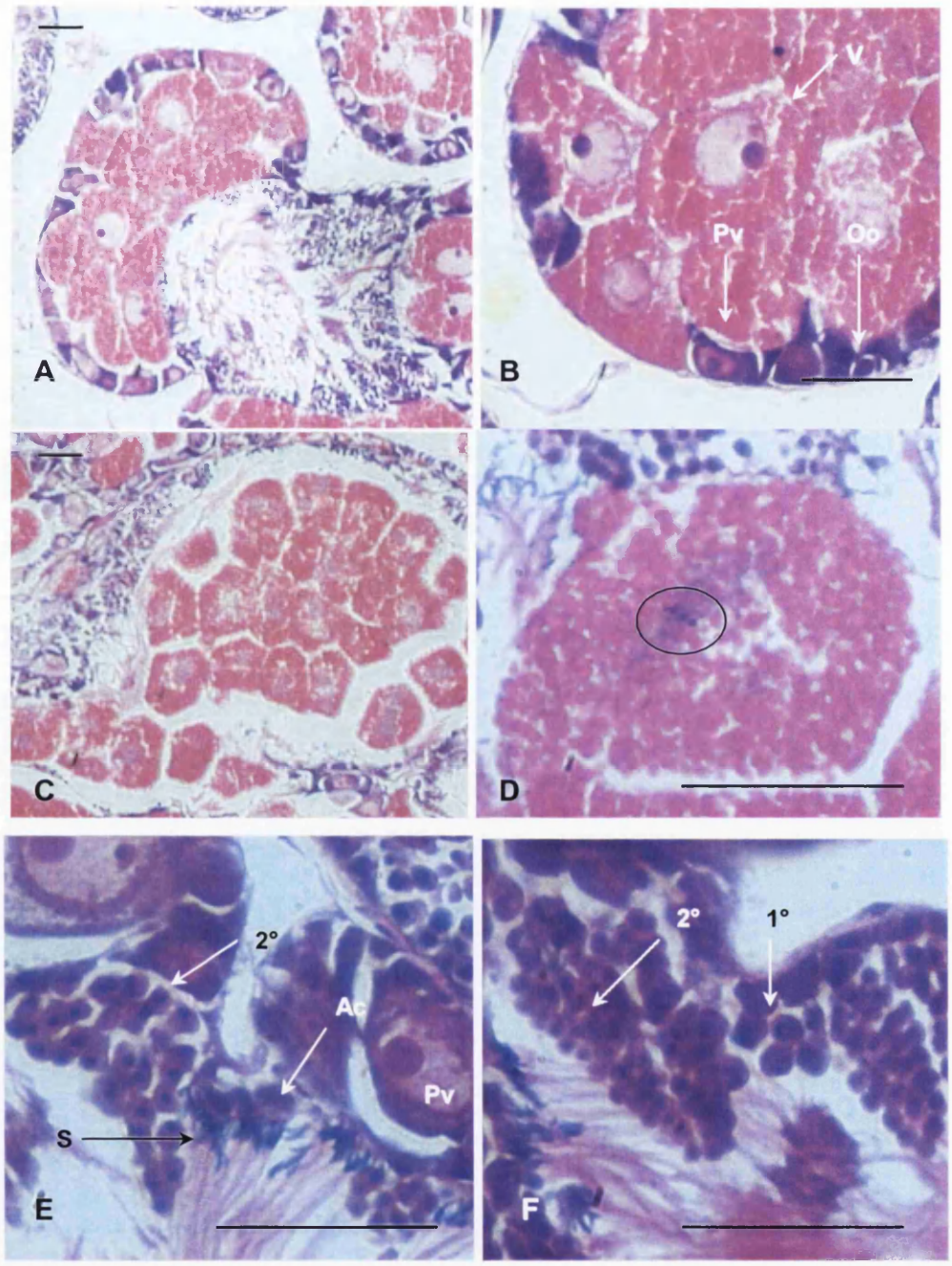

Figure 2.4. *Facelina auriculata.* **A.** The ovotestis, showing the orientation of the male and female acini. **B.** The stages of oogenesis. **C.** The final stage of oogenesis, the breakdown of the nucleolus. Some oocytes are undergoing meiosis. **D.** An enlarged image of an oocyte undergoing meiosis, the metaphase stage (circled). **E-F.** Stages of spermatogenesis. Scale bars: **A-F** = 50 μm.

Abbreviations to figures:

1°	Primary spermatocyte	MA	Male acinus
2°	Secondary spermatocyte	N	Nucleus
Ac	Accessory cell	Nu	Nucleolus
Cs	Cup stage	Oo	Oogonia
d	Diakinesis	Pv	Pre-vitellogenic
DG	Digestive gland	S	Spermatozoa
E	Elongate stage	St	Spermatid
FA	Female acinus	V	Vitellogenic oocyte
Fc	Follicle cell	Yg	Yolk granule

- SPERMATOZOA. The spermatozoa were readily identified due to the presence of the long flagella, and the final reduction and elongation in the shape of the nucleus (Figs 2.1E, 2.2D, 2.3E, F, 2.4E).

2.4 *DISCUSSION*

The morphology of the ovotestes in *Thecacera pennigera*, *Facelina auriculata* and *Ancula gibbosa* conformed to that previously described for other species of nudibranch. In *T. pennigera* the ovotestes are located adjacent to the digestive gland; the acini form a discrete layer surrounding the digestive viscera. In *A. gibbosa* and *Palio nothus* however, the ovotestes are found intimately associated with the digestive gland, the acini often penetrate into the viscera. This arrangement is similar to that described by Medina *et al.* (1986) for *Hypselodoris tricolor*. In *F. auriculata* the ovotestes occupies the majority of the posterior body cavity. The acini are not associated with the digestive gland; instead the ovotestes are located as two discrete pockets on either side of it. A similar arrangement was recorded for *Godiva banyulensis*, in which the ovotestes was described as "detached" from the digestive gland and extending "freely into the haemocoel" (Medina *et al.*, 1986). In the Nudibranchia there are two distinct arrangements of the ovotestes. The apomorphic state (Wägele & Willan, 2000) is found in *T. pennigera*, *F. auriculata*, *A. gibbosa* and *P. nothus*, in which several female acini are located as follicles off one central male acinus. This arrangement was observed in *G. banyulensis* (Medina *et al.*, 1986). The other condition, considered to be plesiomorphic (Wägele & Willan, 2000), is exhibited by *Tritonia hombergi* and *Spurilla neapolitana* in which the male and female germ cells are located within the same follicle (Thompson, 1961b; Eckelbarger & Eyster, 1981 respectively). The developing oocytes are located distally and the developing spermatozoa are located proximally. In *P. nothus* the join between the male

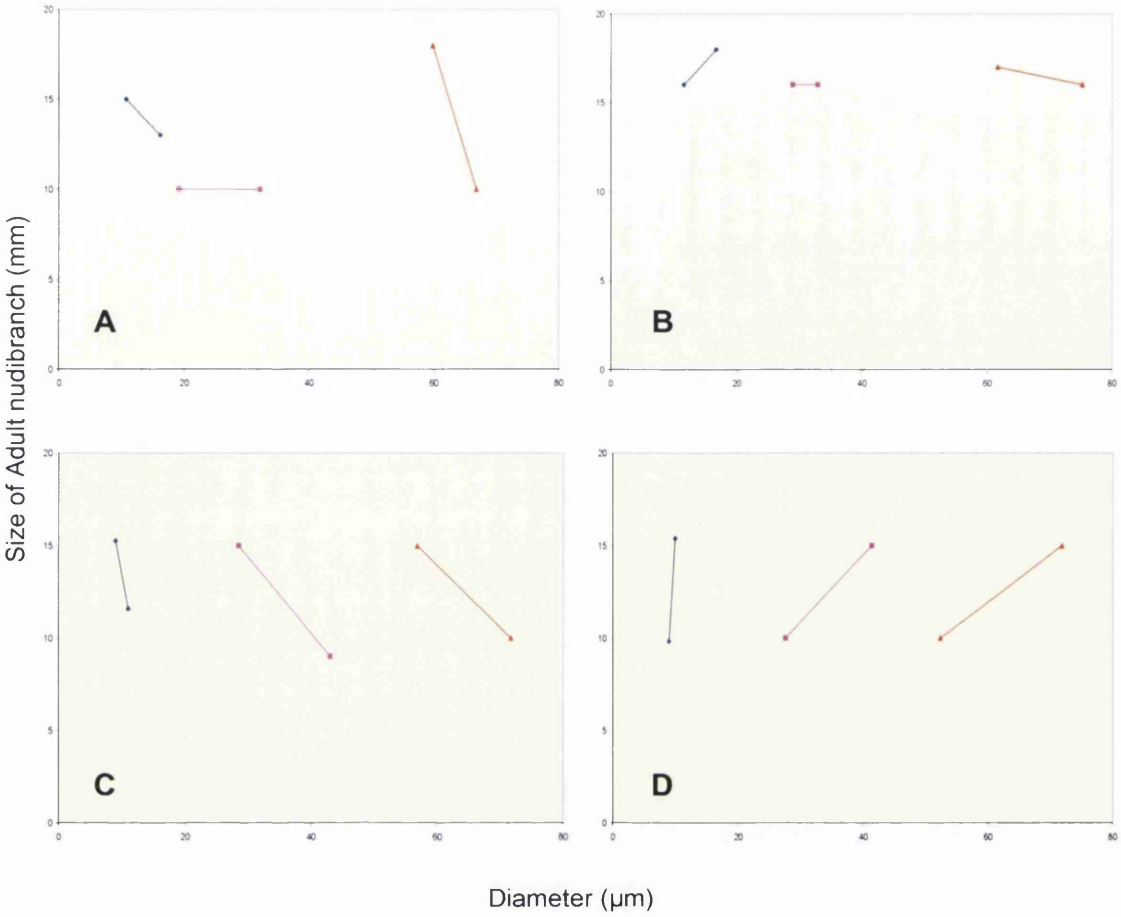

Figure 2.5. The smallest and largest median diameter of oogonia (dark blue), previtellogenic (purple), and vitellogenic oocytes (red). **A.** In *Thecacera pennigera* (oogonia represented by mean measurements). **B.** In *Facelina auriculata*. **C.** In *Ancula gibbosa* (previtellogenic oocytes represented by mean measurements). **D.** In *Palio nothus* (previtellogenic oocytes represented by mean measurements).

and female acini is diminutive, so much so that on first inspection they appeared to be completely separate. However, further investigation revealed that they are joined by a very narrow constriction which appears as a muscular sphincter (Fig. 2.3B). Similar to other nudibranch species, several female acini joined one male acinus. Rivest (1984) investigated copulation in *P. dubia* and *P. zosterae*, his findings are not consistent with the results of this report. Firstly, Rivest (1984) noted that the male acini were connected to the female acini by "short necks". No neck was apparent in the ovotestes of *P. nothus* (Fig. 2.3B). Secondly, Rivest (1984) observed the male acini located just beneath the notum, located beneath these were the female acini. He also stated "a few oogenic acini are found at the surface of the visceral mass, but not on the right lateral surface where penetration by a cirrus is most likely to occur". In *P. nothus* however, the female acini were almost always located immediately beneath the notum both laterally and dorsally (Fig. 2.3A). Thirdly, from the photograph of the paraffin section (fig. 4) included within Rivest's (1984) report; the orientation of the sperm in *P. zosterae* appears to be clustered. This was not observed in *P. nothus*, instead the developing spermatozoa were arranged into long strings (Fig. 2.3D-F). A possible explanation is that the members of the Genus *Palio* are not as closely related as originally assumed.

2.4.1 *OOGENESIS*

The female acini were morphologically similar in all four species of Nudibranchia. Situated at the periphery of the acini were the follicle cells, oogonia and pre-vitellogenic oocytes. Located at the centre were the vitellogenic oocytes, these were readily distinguished from the latter three due to the presence of yolk granules, which stained red with Mallory's and pink with Cole's haematoxylin and eosin. The arrangement was similar to *H. tricolor* and *G. banyulensis* (Medina *et al.*, 1986). In *H. tricolor* and *G. banyulensis* Medina *et al.* (1986) described the displacement of mature oocytes into the centre of the acini during vitellogenesis by the developing oocytes at the periphery. My observations suggest that the same displacement process occurs in *T. pennigera*, *F. auriculata*, *A. gibbosa* and *P. nothus*.

Three stages of oogenesis were identified in *T. pennigera*, *F. auriculata*, *A. gibbosa* and *P. nothus*. A fourth stage was observed in *T. pennigera* and *F. auriculata*. The oogonia were identified due to their spherical morphology and the densely staining cytoplasm. (Figs 2.1B, C, 2.4B). They were always located at the periphery of the female acini. The nucleolus could not be distinguished, due to inadequate differentiation by the stains. Kress (1986) failed to

classify the oogonia as a separate stage in *Runcina*, instead she classified "the time when germinal cells become recognizable as oocytes" as "pre-vitellogenic". Medina *et al.* (1986) also failed to identify oogonia in either *H. tricolor* or *G. banyulensis*. However, Yonow (1996) was able to distinguish them clearly in *Acteon tornatilis* by their size, nucleus and nucleolus morphology.

The pre-vitellogenic oocytes were identifiable by their obvious nucleolus and the absence of yolk granules. The large nucleus formed a germinal vesicle which occupied the majority of the cell's volume (Figs 2.1B, 2.3C, 2.4B). Medina *et al.* (1986) reported a similar situation in the previtellogenic oocytes of *H. tricolor* and *G. banyulensis*, and Kress (1986) for *Runcina*. Medina *et al.* (1986) suggested that the pre-vitellogenic oocytes arose from follicle cells in *G. banyulensis*. When investigating the function of the follicle cells in *Runcina*, Kress (1986) did not refer to them as the precursors to pre-vitellogenic oocytes. Oogonia were identified as the precursors of pre-vitellogenic oocytes in *A. tornatilis* (Yonow, 1996). Typically in molluscs, the gonadal stem cells (GSC) differentiate into germ cells and accessory cells (the male accessory cells and female follicle cells). During development the GSC forms a "germinal epithelial ring" within the ovotestes, from here germ cells are formed (de Jong-Brink *et al.*, 1983). The initial stage of oogenesis, when the germ cells become primary oogonia, is termed the premeiotic phase. These cells undergo a series of mitotic divisions to form secondary oogonia. The secondary oogonia develop a germinal vesicle which results in the increase of the nucleus' volume. RNA synthesis starts to occur, and the oocytes enter the pre-vitellogenic stage (see de Jong-Brink *et al.* (1983) and Dohmen (1983)). Thus it is likely that Medina *et al.* (1986) were wrong in their assumption; oogonia are likely to be present in *H. tricolor* and *G. banyulensis* and they give rise to the pre-vitellogenic oocytes rather than the follicle cells.

The vitellogenic oocytes were identified by the presence of yolk granules within the cytoplasm, and their consequential increase in volume (Figs 2.1B, C, 2.2B, 2.4B). The reported irregularity of the nuclear membrane in *Runcina* (Kress, 1986) and *H. tricolor* (Medina *et al.*, 1986), was not observed in this study for any of the four species investigated, nor was it determined whether the synthesis of yolk was hetero- or autosynthetic. The latter however is known to be typical in the Opisthobranchia (Kress, 1986; Medina *et al.*, 1986). The size of the nucleolus in the vitellogenic oocytes was found to be significantly different compared to the size of the nucleolus in the pre-vitellogenic oocytes in all four species

investigated. This supports observations by Medina *et al.* (1986) for *G. banyulensis*. Thus the oocytes in *T. pennigera*, *F. auriculata*, *A. gibbosa* and *P. nothus* probably undergo vitellogenesis in discrete groups, rather than as a continuous process throughout sexual maturity. It is likely that prior to spawning a group of pre-vitellogenic oocytes commence vitellogenesis, and once mature they are spawned (provided copulation has occurred). Until spawning, the process of vitellogenesis is suspended, if not the female follicles may become full and mature oocytes would be forced out into the male acini where they have been seen to degenerate (Medina *et al.*, 1986). The degeneration of mature oocytes would be energy expensive and counter productive to the individuals involved if they had previously copulated. After spawning the pre-vitellogenic oocytes at the periphery of the acinus have enough room to develop, hence vitellogenesis is initiated again. As in other species of molluscs, this process is likely to be governed hormonally (de Jong-Brink *et al.*, 1983). When investigating the greatest diameter of the different stages of oogenesis in different sized nudibranchs, a significant difference was discovered. The reasons for the significant difference in size of the individual and the oocyte length are most likely to be attributable to when the individual last spawned. The more recently an individual has spawned the more probable it is that the diameters of the oocytes will be smaller, as vitellogenesis would have only recently started in the remaining pre-vitellogenic oocytes. This is further evidence that oogenesis occurs in waves. Each of the individuals sectioned were at different stages of their vitellogenic cycle, and therefore the diameters of the oocytes would have been different. It is unlikely that oocyte diameter is different depending on individual size; Jones *et al.* (1996) found that the variation in egg diameter between individuals of *Adalaria proxima* (Alder & Hancock) was not significant. Other authors seem to have probably assumed this to be the case (Todd & Doyle, 1981; Hadfield & Miller, 1987).

The final stage of oogenesis in which the nucleolus breaks down, was only observed in *T. pennigera* and *F. auriculata*. In *T. hombergi* this stage is reached after the oocyte is transported away from the female acini (Thompson, 1961b). Thompson (1966), Thompson & Bebbington (1969) and Todd (1978) commented on the breakdown of the nucleolus within *Archidoris pseudoargus*, *Aplysia* and *Onchidoris muricata* respectively, but failed to specify when it occurred, although Todd (1978) suggested that the breakdown may occur a "few hours prior to ovulation". In *T. pennigera* and *F. auriculata* nucleolus breakdown was only observed after the oocytes had left the female acini (Figs 2.1D, 2.4C). Kress (1986) failed to

document the breakdown of the nucleolus in *Runcina*, despite documenting the passage of oocytes into the fertilization chamber, through fertilization and into the female gland mass. In *F. auriculata* meiosis occurred during passage along the hermaphroditic duct (Fig. 2.4D). The first meiotic division in members of *Aplysia* was also seen as the oocytes passed through the hermaphroditic duct; however, following the breakdown of the germinal vesicle and the orientation of the chromosomes onto the spindle equator, meiosis was suspended (Thompson & Bebbington, 1969). Thompson & Bebbington (1969) failed to explain why this occurred, and failed to state when meiosis recommenced following its suspension.

Jong-Brink (1983) recommended the used of "follicle cell" to describe those cells which support the developing oocytes within molluscs. Follicle cells were present in all of the four species of nudibranch investigated in this report. They were located at the periphery of the ovotestes, and were easily identified due to the condensed chromatin within the nucleus (Figs 2.1B, 2.2B). Follicle cells have been identified in *H. tricolor*, *G. banyulensis* (Medina *et al.*, 1986), *Runcina* (Kress, 1986), and *A. pseudoargus* (Thompson, 1966). In the latter species, they were called "nurse cells". The function of the follicle cells was not investigated in this study. However Kress (1986) suggested that they are involved in the formation of the vitelline membrane and they may assist in a "morphologically undetectable support of vitellogenesis and the maturation of the oocyte".

2.4.2 *SPERMATOGENESIS*

Spermatogenesis was similar in all four species of nudibranch studied. No measurements were taken of the stages due to poor resolution of the light microscope. As reported by Beeman (1970) and Eckelbarger & Eyster (1981), no spermatogonia could be identified in any of the species examined, although Yonow (1996) observed them in *A. tornatilis*. In this study four stages of spermatogenesis were identified in *T. pennigera*, *F. auriculata*, *A. gibbosa* and *P. nothus*. The primary spermatocytes were characterized by their spherical shape and the presence of a large nucleolus (Figs 2.1F, 2.2E, 2.3F, 2.4F). These cells were the largest observed in the investigation of spermatogenesis. Beeman (1970) observed two arrangements of the chromosomes within the primary spermatogonia: the pachytene stage in which the chromosomes were described as "thickened and easily visible"; and diakinesis in which the condensed chromosomes were found associated with the nuclear membrane. Diakinesis was only observed in *A. gibbosa* (Fig. 2.2C). Eckelbarger & Eyster (1981) identified the zygotene and pachytene stages of meiosis in *S. neapolitana*.

The primary spermatocytes undergo the first meiotic division, which results in the formation of secondary spermatocytes. These cells maintained their spherical shape but the nucleus reduces in size due to the initiation of condensation of the chromosomes (Figs 2.1F, 2.2C, 2.3E, 2.4E, 2.4F). During this stage the second meiotic division occurs. In *S. neapolitana* this division often results in incomplete cell separation, leaving the cells joined together by "intracellular bridges" (Eckelbarger & Eyster, 1981). The cells undergo a further reduction in size (Eckelbarger & Eyster, 1981; Yonow, 1996). After the second meiotic division the spermatid is formed. During this stage the nucleus begins to elongate. Eckelbarger & Eyster (1981) classified spermatid development in *S. neapolitana* into four additional stages based on the nucleus formation: precup, cup, postcup and elongate stages. Due to the restrictions in resolution of the light microscope, it was very difficult to distinguish the spermatid stages described by Eckelbarger & Eyster (1981) in the species studied here. However, the cup stage was seen in *T. pennigera* (Fig. 2.1E), and the elongate stage was visible in both *T. pennigera* and *A. gibbosa* (Figs 2.1E, 2.2D). During the cup stage in *S. neapolitana*, the flagellum inserted into the fosse and the axoneme becames closely associated with the mitochondria (Eckelbarger & Eyster, 1981). In the postcup stage the chromatin continued to condense and the acrosome begans to develop. In the elongate stage the nucleus lengthened, and the two sheaths which surround the axoneme, and the axoneme and the keel form (Eckelbarger & Eyster, 1981). Eckelbarger & Eyster (1981) were unable to identify spermatozoa in *S. neapolitana*; therefore their study failed to describe the transition of spermatids into the spermatozoa. In the initial stages of spermatogenesis in *Phyllaplysia taylori*, the nucleus was located at the anterior end of the cell. The nucleus began to lengthen into the typical arrow-shape, and eventually forming the spermatozoa (Beeman, 1970).

The only difference discovered in this study regarding spermatogenesis was that the orientation of the developing sperm in *P. nothus* was different to that described for other species of nudibranch, even for *P. zosterae* (Rivest, 1984). Instead of clusters of developing spermatozoa, they were arranged into long strings (Fig. 2.3D-F). These long strings were anchored to the wall of the ovotestis distally to the hermaphroditic duct (next to the point of attachment to the female acini). No accessory cells were observed, although this was probably due to the orientation of the spermatozoa. If they are present, they are far less

obvious and must be smaller than those observed in this study for *T. pennigera*, *F. auriculata*, and *A. gibbosa*.

Accessory cells were observed in *T. pennigera*, *F. auriculata*, and *A. gibbosa* (Figs 2.1E, 2.2E, 2.4E). They were closely associated with all stages of spermatogenesis, and were easily identified due to the large nucleus which contained densely staining chromatin. In *S. neapolitana* the head of the developing spermatid was observed to be embedded within the accessory cell (Eckelbarger & Eyster, 1981). Thompson & Bebbington (1969) reported a similar situation in *Aplysia*, in which the head of the developing spermatozoa were initially embedded within "nurse cells", on reaching maturity they became dissociated from them. This degree of association was not observed in this study. The function of the accessory cells during spermatogenesis in unknown; however Eckelbarger & Eyster (1981) suggested the function involved the phagocytosis of products released into the male acini, and the translocation of nutrients from the male acini into the developing spermatozoa. The "gonial amebocytes" observed by Beeman (1970) were not observed in this study.

2.4.3 *SUMMARY*

The ovotestis of *T. pennigera*, *F. auriculata*, *A. gibbosa* and *P. nothus* all exhibited the apomorphic condition, in which several female acini are joined to a single male acinus. In *P. nothus* the male and female acini are almost completely separate except for a small sphincter.

The stages of oogenesis in *T. pennigera*, *F. auriculata*, *A. gibbosa* and *P. nothus* are typical of those described for other opisthobranchs (Thompson, 1961b; 1966; Todd, 1978; Kress, 1986; Medina *et al.*, 1986; Yonow, 1996). Four stages were identified based on the morphology and the staining properties of the cells: oogonia, pre-vitellogenic oocytes, vitellogenic oocytes and mature oocytes. The final "mature" stage (which is often overlooked during investigations regarding oogenesis) was observed in *T. pennigera* and *F. auriculata* and was identified by the breakdown of the nucleolus and the initiation of meiosis. The results reported here indicate that oogenesis occurs in discrete waves; this was attributed to a lack of space within the ovotestes once vitellogenesis is complete. Following spawning, it is likely that a new batch of pre-vitellogenic oocytes commences vitellogenesis to replace those spawned.

The stages of spermatogenesis were similar to those described for other opisthobranchs (Beeman, 1970; Eckelbarger & Eyster, 1981; Yonow, 1996). Four stages were identified based on morphology: primary spermatocytes, secondary spermatocytes,

spermatids and spermatozoa. Spermatogonia were not observed in this study. The arrangement of spermatogenesis was atypical in *P. nothus*, in which the developing cells were arranged into long strings, these were anchored to the wall of the male acini distally to the hermaphroditic duct. This orientation has not been reported for any other species of nudibranch mollusc. Further investigation into *P. nothus* should be carried out to determine whether this species has been correctly classified. Studies on its copulatory behaviour would be of interest to allow comparison with the study by Rivest (1984) on *P. dubia* and *P. zosterae*.

Chapter 3

Chapter 3

Pre-metamorphic growth and development of several species of nudibranch

3.1 *INTRODUCTION*

Since the advent of opisthobranch research, there have been many studies on veliger growth and development. The ultimate goal of much of this research was to identify the stimulus to metamorphosis and the morphological changes which convert the free swimming veliger into the adult benthic form. Some of these studies have been successful in rearing veligers through metamorphosis, and to adulthood over several generations. Initially, success was limited to those species with lecithotrophic larvae. These veligers hatch out after an extended period within the egg mass almost fully competent to metamorphose. They only spend a short time within the plankton and settle soon after emerging. As they possess a yolk, they do not need to feed, although many can and do when provided with phytoplankton. As knowledge of culturing techniques improved, the number of planktotrophic species taken through metamorphosis has increased. Harris (1975) was the first to rear a planktotrophic species of nudibranch, *Phestilla melanobranchia* Bergh, to metamorphosis.

The techniques used to rear nudibranch veligers successfully are summarized in Table 3.1. Included in this table are the Anaspidea, as initial culturing success involved members of this Order, and as most of the species exhibit planktotrophy, like the majority of the members of the Nudibranchia.

3.1.1 *SPAWN STUDIES*

Hurst (1967) produced a comprehensive review of the types of spawn and details of veligers for 30 species of opisthobranch molluscs. She described four different

Table 3.1. Caption on page 49.

			Water used in veliger culture			Phytoplankton				
Species	**D. type**	**S. type**	**Source**	**Filtered?**	**Freq. of exchange**	**Species**	**Conc. cells ml^{-1}**	**Culture volume**	**Conc. of veligers**	**L:D**
Anaspidea										
Aplysia californica	1	1	Sea water	Millipore pre-filter	Every two days	*Isochrysis galbana*	10^4	4 litre	100 l^{-1}	?
Phyllaplysia taylori	3	1	?	?	?	None	N/A	N/A	N/A	?
Aplysia dactylomela *Aplysia juliana* *Dolabella auricularia* *Stylocheilus longicauda*	1 1 1 1	1 1 1 1	Sea water	Millipore pre-filter	Three days	*Monochrysis lutheri*	10^4	600 & 1000 ml	0.8-1.0 ml^{-1}	24:0
Aplysia parvula *Aplysia pulmonica*	1 1	1 1	?	?	?	Unknown	?	?	?	?
Aplysia brasiliana	1	1	Sea water 28-30	0.2 µm	Never	*Isochrysis galbana*	5000-10000	300 ml	50-150	?
Aplysia brasiliana *Bursatella leachii plei*	1 1	1 1	Artificial sea water	Prefiltered	5-7 days	*Isochrysis galbana*	10^4	3 litre	1 veliger 10 ml^{-1}	24:0
Aplysia californica	1	1	Sea water	1 µm	Four days	*Pavlova lutheri*	10^4	2 litre	?	24:0
Aplysia oculifera	1	1	Sea water	0.45 µm	Twice per week	*Isochrysis galbana*	10^5	1 litre	2000 (embryos)	Ambient
Akera bullata	2	2	Sea water	0.45 µm	Alternate day	Either: *Tetraselmis*, *Isochrysis*, *Chaetoceros* or *Rhinomonas*	50 cells μl^{-1}	60 ml Sterilin pots	20-30 per vessel	12:12
Nudibranchia										
Berghia verrucicornis	2 & 3	1	Seasoned artifical sea water-	0.45 µm	5-10 days unchanged then daily	None	None	Finger bowls	≈ 50 per dish	?
Rostanga pulchra	1	1	Sea water	Filtered	2-3 days	*Monochrysis lutheri* *Isochrysis galbana*	10^4	100 ml	5 ml^{-1}	?
Onchidoris bilamellata	1	1	Sea water	0.45 µm Millipore filtered UV sterilized	After 14 and 25 days	*Rhodomonas*, *Isochrysis*, *Pavlova* & *Tetraselmis*	1×10^5 then 5×10^4	200 litre or 10 litre	≈ 1 ml^{-1}	?
Eubranchus farrani	2	2	Sea water	?	?	Not fed	N/A	?	?	?
Eubranchus exiguus	2	2	Sea water	Millpore filtered	?	None	N/A	?	?	?
Tritonia diomedea	1	1	Sea water	Millipore pre-filtered/0.45 µm	Alternate days	*Monochrysis lutheri* *Isochrysis galbana*	1×10^4	500 ml	≈ 2 ml^{-1}	24:0
Adalaria proxima	2	1	Sterile/artificially enriched sea water	?	?	None	None	0.5-1 litre	?	?
Adalaria proxima	2	1	Sea water	0.22 µm	Every 5 days	*Pavlova*, *Isochrysis* & *Rhodomonas*	5×10^4	Various sizes	Various	12:12
Adalaria proxima	2	1	Sea water	0.45 µm	?	Not fed	N/A	?	?	?
Goniodoris nodosa	1	1	?	?	?	Unknown	?	?	?	?
Onchidoris bilamellata	1	1	Sea water	?	?	Mixes of: *Dunaliella*, *Isochrysis*, *Pavlova*, *Rhodomonas*, or *Phaeodactylum*	?	?	?	?
Hermissenda crassicornis	1	1	Sea water	0.2 µm	Weekly	*Isochrysis galbana* *Rhodomonas salina*	15×10^3	2 litre roller bottles	≈ 1 ml^{-1}	12:12

Table 3.1. Caption on page 49.

Species	D. type	S. type	Water used in veliger culture: Source	Filtered?	Freq. of exchange	Phytoplankton: Species	Conc. cells ml^{-1}	Culture volume	Conc. of veligers	L:D
Nudibranchia										
Doridella obscura	1	1	Sea water	1.0 µm or 0.22 µm	Daily	*Isochrysis galbana*	1×10^5	250 ml finger bowls	1 veliger 3 ml^{-1}	?
*Phestilla melanobranchia**	1	2	Sea water	Cuno filter	Daily	*Phaeodactylum tricornutum*	5-15 ml per day	250 ml beakers	?	?
Phestilla sibogae	2	2				*Dunaliella* sp.				
Phestilla sibogae	2	2	Sea water	Millipore filtered	Daily	Not fed	N/A	?	?	?
Phestilla sibogae	2	2	Sea water	0.22 µm or 0.45 µm	?	None	N/A	?	?	?
Doridella steinbergae	1	1	Sea water	Millipore prefilter	Daily	*Monochrysis lutheri*	10^6	100 ml finger bowls	2-3 veligers ml^{-1}	?
						Isochrysis galbana				
Hermissenda crassicornis	1	1	Sea water	Millipore 0.22 µm	Three times weekly	*Isochrysis galbana*	3×10^4	1 litre	3 veligers ml^{-1}	12:12
						Monochrysis lutheri	3×10^4			
						Chroococcus salina	7.5×10^3			
Hermissenda crassicornis	1	1	Sea water	Sterile & filtered	Daily	*Monochrysis lutheri*	Variable	?	1000 per 500 ml sea water	24:0
Aeolidia papillosa	1	1				*Isochrysis galbana*				
						Dunaliella tertiolecta				
						Chlorella clone				
						Phaeodactylum tricornutum				
Cadlina laevis	3	1	Sea water	?	Daily	None	N/A	N/A	N/A	0:24
Tritonia hombergi	2	1	Sea water	?	?	Do not feed	N/A	?	?	?
Onchidoris muricata	1	1	Sea water	0.22 µm	?	*Monochrysis lutheri*	50 cells $µl^{-1}$	100 ml	3 ml^{-1}	?
Archidoris pseudoargus	1	1				*Rhodomonas sp.*		5 l, 400 ml and 100 ml		
Adalaria proxima	2	1								
Armina tigrina	2	1	Sea water	Filtered	?	Not fed	N/A	N/A	N/A	?
Tenellia adspersa	2 & 3	2	12-20	Unknown	?	Not fed	N/A	N/A	N/A	?
Tenellia adspersa	2 & 3	2	Sea water	Filtered	Daily	Not fed	N/A	N/A	N/A	External light
Tenellia adspersa	2 & 3	?	Sea water	?	?	Do not feed	N/A	N/A	N/A	?
Melibe leonina	1	1	Sea water	Millipore filtered	1 or 2 days	*Monochrysis lutheri*	10^4	100 ml	2-3 ml^{-1}	?
Cuthona gymnota	?	?	?	?	?	Not fed	N/A	N/A	N/A	?
Tenellia adspersa										
Tergipes tergipes										
Hypselodoris infucata	1	1	Sea water	0.22 µm	Alternate days	Tahitian *Isochrysis*	$0.5\text{-}1 \times 10^5$	750-1900 ml	4-5 ml^{-1}	Ambient
						Ochromonas sp.				
Cuthona nana	Non-pelagic 2	2	Sea water	0.45 µm	Twice daily	Nothing	N/A	N/A	?	?
Cuthona nana	1 & 2?	2	Sea water	?	?	?	?	?	?	?
Eubranchus doriae	2	2	Sea water	?	?	Not fed	N/A	N/A	?	?
Aeolidiella alderi	3	2	Sea water	?	?	Not fed	N/A	N/A	?	?
Coryphella trilineata	1	1	Sea water	?	Alternate days	*Dunaliella tertiolecta*	Two drops of each per day	?	?	?
						Monochrysis lutheri				
Coryphella stimpsoni	3	?	Sea water	?	?	Not fed	N/A	N/A	N/A	?

Table 3.1. Caption on page 49.

Species	D. type	S. type	Water used in veliger culture: Source	Water used in veliger culture: Filtered?	Water used in veliger culture: Freq. of exchange	Phytoplankton: Species	Phytoplankton: Conc. cells ml^{-1}	Culture volume	Conc. of veligers	L:D
Nudibranchia										
Casella obsoleta	3	?	Sea water	?	?	Not fed	N/A	N/A	N/A	?
Discodoris erythraeensis	2	?	Sea water	?	?	Not fed	N/A	N/A	N/A	?
Trippa spongiosa	2	1	Sea water	?	?	Not fed	N/A	N/A	N/A	?
Dermatobranchus striatellus	3	1	Sea water	?	?	Not fed	N/A	N/A	N/A	N/A
Glossodoris sibogae	3	C	Sea water	?	Twice daily	Not fed	N/A	?	N/A	Dim light
Aeolidiella mannarensis	2	1?	33.53-34.18	?	?	Not fed	N/A	N/A	?	?
Cuthona adyarensis	2	2	24	?	?	Not fed	N/A	N/A	?	?

Table 3.1. Caption on page 49.

Species	°C	Rafting	Antibiotics	Meta. Stimulus	Time taken to competency	Shell growth (µm)	Generation time	Authors
Anaspidea								
Aplysia californica	Room	Sealed vessel	None	*Laurencia*	34 days	135 to 400	19 weeks	Kriegstein *et al.* (1974)
Phyllaplysia taylori	14.5°C	N/A	?	Direct development	Hatches after 30 days	?	?	Bridges (1975)
Aplysia dactylomela *Aplysia juliana* *Dolabella auricularia* *Stylocheilus longicauda*	24-26°C	Cetyl alcohol	Penicillin G 0.06 mg ml^{-1} Streptomycin sulphate 0.05 mg ml^{-1}	*Ulva fasciata* & *U. reticulata* *Laurencia sp.* Blue-green algae *Lyngbya majuscula*	30 days 28 days 31 days 30 days	144 to 310-315 125 to 315-330 148 to 290-300 103 to 325-340	?	Switzer-Dunlap & Hadfield (1977)
Aplysia parvula *Aplysia pulmonica*	No data 24-26°C	?	?	*Chondrococcus hornemanni* No data	? 24 days	105 to 500 128 to 330-340	?	Switzer-Dunlap (1978)
Aplysia brasiliana	21-25°C	Pipetting water	None	*Callithamnion*	30-40 days	140 to 325-375	≈ 3.5-4.5 months	Strenth & Blankenship (1978)
Aplysia brasiliana *Bursatella leachii plei*	24 ± 1°C	Sealed vessel	None	Red algae Blue-green algae: Oscillatoriaceae	34 days 19 days	111 ± 7 to 382 ±14 160 ± 4 to 286 ± 9	98-127 days 83-112 days	Paige (1986) & (1988)
Aplysia californica	20°C	Cetyl alcohol	Penicillin G 40 mg l^{-1} Streptomycin sulphate 40 mg l^{-1}	*Laurencia pacifica* *Plocamium cartilagineum*	35 days	?	?	Pawlik (1989)
Aplysia oculifera	24 ± 1°C	Cetyl alcohol	Penicillin G 0.06 mg ml^{-1} Streptomycin sulphate 0.05 mg ml^{-1} EDTA 0.25 mg ml^{-1}	*Enteromorpha intestinalis*	>60 days	102 ± 2 to 385 ±11	?	Plaut *et al.* (1995)
Akera bullata	18-20°C	0.41 µm nylon mesh floated on meniscus	Penicillin G 60 µg ml^{-1} Streptomycin sulphate 50 µg ml^{-1}	Unspecific: biofilm, phytoplankton spp., *Chondrus*, *Nemalion* or *Ulva*	Within 24 hrs	255 ± 13	?	This study
Nudibranchia								
Berghia verrucicornis	23.9 ± 1.3°C	None	None	*Aiptasia pallida* or none for direct developers	1-3 days or capsular metamorphosis	251.4 ± 7 no growth	47 days	Carroll & Kempf (1990)
Rostanga pulchra	10-15°C	Cetyl alcohol	Penicillin G 0.06 mg ml^{-1} Streptomycin sulphate 0.05 mg ml^{-1}	*Ophlitaspongia pennata*	35-40 days	148 to 300	?	Chia & Koss (1978)
Onchidoris bilamellata	15°C	Cetyl alcohol and frozen distilled H_2O	None	*Semibalanus balanoides*, *Chthamalus stellatus* & *Elminius modestus*	35 days	166 to 440	?	Todd (1981)
Eubranchus farrani	?	?	?	*Obelia geniculata*	10 hours	245 no growth	?	Todd (1981)
Tritonia diomedea	11.9 ± 1.3°C	Cetyl alcohol	Penicillin G 0.06 mg ml^{-1} Streptomycin sulphate 0.05 mg ml^{-1}	Possibly *Virgularia* sp.	34 days	144.6 to 329 or 321 depending upon diet	?	Kempf & Willows (1977)
Adalaria proxima	9-10°C	Pipetting water	None	*Electra pilosa*	1-2 days in obligatory phase	280-300 no growth	?	Thompson (1958)
Adalaria proxima	7.9°C ± 0.05 (SE)	Cetyl alcohol	Penicillin G 60 µg ml^{-1} Streptomycin sulphate 50 µg ml^{-1}	*Electra pilosa*	Can survive 15-20 days at 7.4 °C ± 0.06	279 ± 10	?	Kempf & Todd (1989)
Adalaria proxima	?	Larvae left "rafted"	None	*Electra pilosa*	24-48 hours	?	?	Lambert & Todd (1994)
Goniodoris nodosa	?	?	?	Presumably *Alcyonidium* spp.	10-13 weeks	?	?	Todd *et al.* (1998)
Onchidoris bilamellata	11°C or 7°C	?	?	*Chthamalus dalli*: conditioned sea water or living barnacles	32 days at 11 °C 60-80 days at 7.5 °C	165 to 320	?	Chia & Koss (1988)
Hermissenda crassicornis	12°C	None	Chloramphenicol 10 mg l^{-1} EDTA 0.25 mg l^{-1}	*Tubularia* but also other hydroids and anemones	41-50 days	≈120 to 250-350	?	Avila *et al.* (1997) Avila (1998)
Doridella obscura	4 to 25°C	Sealed vessel	?	*Electra crustulenta*	11 days at 22 °C or 9 days at 25 °C	110 to 190-195	26 days	Perron & Turner (1977)

Table 3.1. Caption on page 49.

Species	°C	Rafting	Antibiotics	Meta. Stimulus	Time taken to competency	Shell growth (µm)	Generation time	Authors
Nudibranchia								
*Phestilla melanobranchia**	21°C & 25-27°C	Cetyl alcohol	Penicillin 0.5-1.0 units ml^{-1} of culture medium	*Dendrophyllia elegans*	8 days	200 no growth	60 days	Harris *et al.* (1975)
Phestilla sibogae				*Porites* sp.	3-4 days	264 no growth	38 days	
Phestilla sibogae	?	?	?	*Porites* sp.	4-6 days	?	?	Hadfield & Karlson (1969)
Phestilla sibogae	?	Cetyl alcohol	Penicillin G 0.06 mg ml^{-1} Streptomycin sulphate 0.05 mg ml^{-1}	*Porites compressa*	1 day	240 no growth	?	Bonar & Hadfield (1974)
Hermissenda crassicornis	13.8 ± 1.2°C	Cetyl alcohol	5 ppm Chloramphenicol	Unspecified Hydroid	>35 days	105.9 ± 6.3 to 310.4 ± 9.8	?	Harrigan & Alkon (1978)
Cadlina laevis	10°C	None	None	None	Hatches after 50 days	?	?	Thompson (1967)
Tritonia hombergi	?	Pipetting water	?	*Alcyonium digitatum*	1-2 days	?	?	Thompson (1962)
Onchidoris muricata	10°C	?	Penicillin G 6 µg ml^{-1} Streptomycin sulphate 5 µg ml^{-1}	*Electra pilosa*	58-59 days at 10°C/47 days at 15°C	Growth to 225-235	72 days at 10 °C	Todd & Havenhand (1985)
Archidoris pseudoargus	10 or 15°C			*Halichondria panicea*	37 days at 10 °C	Growth to 340	67 days at 10 °C	
Adalaria proxima	10°C			*Electra pilosa*	4-6 days at 10 °C	273 no growth	36 days at 10 °C	
Armina tigrina	23°C	None	None	Presumably *Renilla reniformis*	<31 days	290 no growth?	Approximately 2.5 months after metamorphosis	Eyster (1981)
Tenellia adspersa	20.0-25.5°C	?	?	Capsular metamorphosis from Copenhagen	N/A	0.3 mm no growth	?	Rasmussen (1944)
				No cue for pelagic	125 hours			
Tenellia adspersa	15-25°C	None	None	Capsular metamorphosis	N/A	195 no growth	3-4 weeks capsular	Eyster (1979)
				? for pelagic	Unknown			
Tenellia adspersa	20 or 25°C	?	?	Capsular metamorphosis	7 days within the egg mass	?	?	Chester (1996)
					1-2 days as lecithotrophic veliger			
Melibe leonina	12-14°C	?	Penicillin G 60 µg ml^{-1} Streptomycin sulphate 50 µg ml^{-1} at 2-6 days intervals	No cue required	30-48 days	149 to 250	?	Bickell & Kempf (1983)
Cuthona gymnota	?	?	?	*Tubularia indivisa*	?	?	?	Swennen (1961)
Tenellia adspersa				Capsular metamorphosis	5 days if pelagic		5-20 days	
Tergipes tergipes				?	?		5 weeks	
Hypselodoris infucata	23.5-28.8°C	Cetyl alcohol	Penicillin G 60 µg ml^{-1} Streptomycin sulphate 50 µg ml^{-1}	*Dysidea* sp., *Halichondria sp.*, *Sigmodocia* sp., *Tedania* sp. & "seasoned" glass slide	16 days	149 to 306	?	Hubbard (1988)
Cuthona nana	11-13°C	None	?	No cue required	1-2 days	?	14 weeks	Rivest (1978)
Cuthona nana	11-12°C	?	?	*Hydractinia echinata*	1-2 days	?	?	Harris *et al.* (1975)
Eubranchus doriae	22-24°C	?	?	*Kirchenpaueria pinnata*	Approx. 3 days	250-300 no growth	Approximately 2 months	Tardy (1962b)
				echinulata				
Aeolidiella alderi	21°C	None	?	Capsular metamorphosis	N/A	?	3-3.5 months	Tardy (1962a)
Coryphella stimpsoni	4-8.5°C	None	None	Capsular metamorphosis	Hatch out after 52 days at 4-5 °C or after 25-34 days at 5-8.5 °C	None	?	Morse (1971)
Casella obsoleta	≈ 27.2°C	None	?	Capsular metamorphosis	Hatch out after 25 days at 27.2 °C	None	?	Gohar & Soliman (1967b)
Discodoris erythraeensis	≈ 19°C	?	?	No cue required	≈ 7 days	None	?	Gohar & Abdul-Ela (1959)
Trippa spongiosa	≈ 27°C	?	?	No cue required	≈ 30 minutes	270 no growth	?	Gohar & Soliman (1967a)

Species	°C	Rafting	Antibiotics	Meta. stimulus	Time taken to competency	Shell growth (µm)	Generation time	Authors
Dermatobranchus striatellus	24°C	?	?	Capsular metamorphosis	Hatch after 10 days at 24 °C	?	?	Hamatani (1967)
Glossodoris sibogae	27-29°C	?	50 units Penicillin G ml^{-1}	Capsular metamorphosis	Hatch after 25-28 days at 27-29 °C	?	?	Usuki (1967)
Aeolidiella mannarensis	29-31°C	?	?	No cue required	24 hours	230 no growth	?	Rao & Alagarswami (1960)
Cuthona adyarensis	22-24°C	?	?	No cue required	Few hours after hatching	370 no growth	?	Rao (1961)

Table 3.1. A review of the conditions used in veliger culture since 1958. Only the substrata that induced the best post larval development are listed for the metamorphosis stimulus. Unless otherwise stated the species of phytoplankton listed were used to rear all the species within one study. D. type, Developemental-type; S. type, Shell-type; C, cup-shaped shell. *Only *Phestilla melanobranchia* were fed phytoplankton.

types of egg masses:

Type A. These include the ribbon shaped masses common to dorids. One edge of the mass is secured to the substratum; if this edge is shorter than the free edge the ribbon becomes convoluted creating a "fluted rosette". Embryos occur throughout. The masses can be laid spirally to from a coil, or in random directions. Produced by *Onchidoris bilamellata* (L.), *O. muricata* (Müller), *Melibe leonia* (Gould), *Archidoris pseudoargus* (Rapp) etc. (Fig. 3.4A, 3.6A).

Type B. These form a long cylindrical string, which is attached to the substratum by a thin jelly sheet on one side which is capsule free. The string contains the capsules, this area can appear to be subdivided into "pinched" pockets of eggs. The overall egg string has secondary folding, which can give the mass a messy appearance. These masses are characteristic of aeolids. Produced by *Aeolidia papillosa* (L.), *Coryphella fusca* O'Donoghue, *Dendronotus iris* Cooper etc.

Type C. These form a "globular jelly bag" which is attached to the substratum by a string of jelly. Often the animal attaches the egg mass by burrowing into the sand i.e. *Acteon tornatilis* (L.).

Type D. These are formed into a "sac-like" structure. Hurst (1967) noted that the masses are not morphologically uniform. If coiled, they do not form a complete 360° turn. These masses are characteristic of very small aeolids i.e. *Eubranchus olivaceus* (O'Donoghue) (Fig. 3.8A).

The number of embryos per capsule varies depending on the species and the type of egg string produced. The numbers range from none, this is typical towards the end of the egg masses when no eggs remain within the animal, to as many as 15 eggs per capsule (Hurst, 1967). The colour of the masses varies depending on the species and their prey, although it has also been found to be dependent on age (Hurst, 1967). *Archidoris* spp. spawn masses vary from bright yellow to cream, *Rostanga pulchra* MacFarland masses are orange but fade over time, and *A. papillosa* produces masses varying from pink to white (Hurst, 1967).

3.1.2 *EMBRYOLOGICAL DEVELOPMENT*

There have been many studies following the ontogeny of nudibranch molluscs. The most extensive was written by Casteel (1904) investigating *Fiona pinnata* (Eschscholtz). Since then, only a few authors have documented the development of the embryo, and certainly

none to the extent of Casteel (1904). Over the last fifty years the only detailed studies of nudibranch embryology are Thompson (1958) on the early development of *Adalaria proxima* (Alder & Hancock); Gohar & Soliman (1963) on the embryology of *Hexabranchus sanguineus* (Rüppell & Leuckart) and Tardy (1970) on the embryology of *Aeolidiella alderi* (Cocks).

After fertilization, the egg masses are laid, and development of the embryo begins. The rate of development varies depending upon the species, and will not be addressed in this report; however the ontogenetic development is fundamentally the same between species. At the animal pole the polar bodies are formed. The first arises adjacent to the polar spindle, shortly followed by release, from the same location, of the second polar body (Casteel, 1904). The third polar body is formed from division of the first, however this division does not always occur, if not, the first polar body remains larger than the second (Casteel, 1904). The first cell division results in the formation of two equally sized cells. After separation, they become closely associated with one another, and lie so that the area of cell membrane touching becomes flattened (Casteel, 1904; Thompson, 1958). The division of these two cells occurs simultaneously in *H. sanguineus* (Gohar & Soliman, 1963), resulting in four blastomeres, one of which is slightly larger than the other three (termed the D cell). The cells meet at a common point dorsally, while ventrally there is a polar furrow which prevents cells A and C meeting (Casteel, 1904; Thompson, 1958). Following this, the segregation of the ectoblast commences (Casteel, 1904). The four blastomeres divide by dextrorotation to form four smaller micromeres, termed the first quartet, and four macromeres. This transition occurs asynchronously. Thompson (1958) commented that five, six, and seven intermediate cell stages can be observed. The second quartet arises from the division of the macromeres, which divide by laevorotation (Casteel, 1904; Thompson, 1958). These cells are much larger that those constituting the first quartet (Thompson, 1958; Gohar & Soliman, 1963). The first quartet then divides to form eight cells of equal size, called the "primary trochoblasts" or "turret cells", these do not divide again until 60 cells are present (Casteel, 1904). The third quartet is formed by dextrorotation from the division of the second quartet, which occurs simultaneously with the third division of the macromeres, this results in a 24 cell embryo. The remaining cell divisions alternate between dividing by laevorotation and dextrorotation (Thompson, 1958). For further detail consult Casteel (1904).

Gastrulation occurs by invagination followed by epiboly (Thompson, 1958; Gohar &

Soliman, 1963). The "prospective" endodermal cells move away from the medio-ventral groove to create the blastopore (Gohar & Soliman, 1963). The embryo continues to increase in size, causing the blastopore to close. During closure, the anterior end becomes covered with fine cilia, and the embryo enters the trochophore stage (Gohar & Soliman, 1963). Concurrently, the shell gland invagination appears. This forms as a pit, which everts, spreading over the posterior end of the embryo, the shell is secreted from these cells over several days. The appearance of anal cells differs between species. Gohar & Soliman (1963) noted their formation at the same time as the shell gland invagination in *H. sanguineus*; a similar situation was reported by Williams (1980) for *Hermissenda crassicornis* (Eschscholtz) and *A. papillosa*. In *A. proxima* they do not appear until after the formation of the mouth (Thompson, 1958). In *A. papillosa* and *H. crassicornis* the anal cells emerge on the left, postero-laterally, but are gradually pushed to the right as a result of the shell gland invagination (Williams, 1980). This movement of the anal cells does not occur in *A. proxima* (Thompson, 1958). These cells mark the spot of the anus, although their exact function is unknown (Thompson, 1958). Following shell gland invagination in *A. proxima*, Thompson (1958) described the edge of the gland forming a "collar" which contributed to the mantle fold; and the mouth appeared from the area formerly occupied by the blastopore (Casteel, 1904; Thompson, 1958). The rudimentary velum forms into two obvious lobes. The cilia become longer and stronger, and begin to beat synchronously (Thompson, 1958; Gohar & Soliman, 1963).

The embryo, now in the veliger stage, becomes covered with cilia, and the operculum is secreted on the posterior surface. The foot enlarges, and internally the foregut forms, along with the midgut, although initially they remain separate. The larval kidney develops from a large undifferentiated cell close to the developing midgut, and the two diverticula form from the undifferentiated visceral cells (Thompson, 1958). During internal differentiation, the mantle begins to pull away from the shell, creating a visible gap between shell and viscera (Thompson, 1958). The statocysts form within the base of the foot, and can be seen to rotate due to the movement of cilia lining the sacs. The nervous system develops, resulting in the formation of the cerebral commissure and the pedal ganglia (Thompson, 1958). Following this, the metapodial mucus glands develop, the fore- and midgut join together, and the radula sac rudiment begins to form (Thompson, 1958). The larval kidney differentiates and becomes located on the right side, and the larval retractor

muscles develop. By this point shell formation is complete. Thompson (1958) continues to describe the formation of the eye spots along with the optic ganglia in *A. proxima*. These characteristics are typical to veligers with Development-type 2; the veligers addressed in this study have a Development-type 1, embryonic development in Development-type 2 veligers will therefore not be discussed further.

Torsion occurs at different times during development in different species. Thompson (1958) stated that torsion in *A. proxima* occurs during late cleavage, therefore all structures appear in their post-torsional state. In *Tritonia hombergi* Cuvier, Thompson (1962) stated torsion was only a partial physical process, identified by the movement of the anal cells to their right position on the embryo. Tardy (1970; 1991) observed a full 180° rotation of the visceral organs for *A. alderi*. The difficulty lies in veligers with a Development-type 2; Tardy (1970) discovered that torsion in Development-type 2 veligers occurred before that in Development-type 1 veligers, and that the quantity of yolk within the former may obscure the physical process of torsion. He concluded that 180° rotation probably does occur in both *A. proxima* and *T. hombergi*, contrary to Thompson's (1958; 1962) observations. In *Phestilla*, Harris (1973) documented torsion, which involved a 180° turn of the visceral organs, occurring late in development just prior to hatching. This however, contradicts Tardy (1970) as *Phestilla* has a Development-type 2 (reviews by Tardy (1970; 1991)).

The rate of embryonic development is highly dependent upon temperature. Todd (1979) hatched *O. bilamellata* after 30 days at 4-5°C, however, at 19°C hatching occurred after eight days. The natural degradation of the egg mass by micro-organisms is thought to be important in assisting veliger liberation (Franz, 1971; Harris, 1973; 1975).

3.1.3 *SHELL TYPE*

Several authors have classified the shell type of nudibranch veligers. Thorson (1946), Thompson (1961a), and Hurst (1967) all documented the different types of shell found within opisthobranch species. Thorson (1946) divided them into three shell types:

Type A. A cup shaped shell with no whorls.

Type B. A shell with ¾ to 1 whorl only.

Type C. The "inflated" shell, characterized by its egg shape.

Thompson (1961a) reclassified these three categories into two, discarding Type A as he observed *Tritonia plebeia* Johnston on which Thorson (1946) based his Type A, having a

Type B shell. He reclassified Thorson's (1946) Type B into Shell-type 1 and Thorson's (1946) Type C into Shell-type 2. He noted that the premature hatching of *T. plebeia* resulted in veligers with deformed shells. These, Thompson (1961a) suggested, could have been the basis of Thorson's (1946) Type A. Hurst (1967) similarly commented that prematurely hatched *Dendronotus* have deformed shells that closely resemble Thorson's (1946) Type A. Some authors still acknowledge the existence of Thorson's (1946) Type A shell. Gohar & Soliman (1967b) described the shell of *Casella obsoleta* (Rüppell & Leuckart) as cup shaped which lacked whorls. Usuki (1967) identified Thorson's (1946) Type C shell in *Glossodoris sibogae* (Bergh). Both these species undergo Development-type 3, thus the shelled veliger stage is confined within the capsule. Soliman (1977) re-iterated the validity of Thorson's (1946) original shell types, and attempted to re-clarify the classification. Todd (1981) however took the debate a step further, by suggesting that Thompson's (1961a) classification should be applied only to pelagic larval stages, while Thorson's (1946) classification should be applied to all stages of development.

It was noted by Thompson (1961a) that in the Nudibranchia shell growth is minimal as the mantle fold is retracted away from the shell aperture soon after hatching. A similar comment was made by Thorson (1946), although he further suggested that for Type A and B shells the time spent within the plankton "must assumed to be very short (probably a few hours to a few days)", due to their apparent inadequacies for long term survival in the pelagic environment. However, recent literature contradicts these theories. It is now certain that for many, if not all, planktotrophic veligers of Shell-type 1, shell growth must occur followed by a period of no shell growth in order for metamorphosis to proceed (Kempf & Willows, 1977; Chia & Koss, 1978; Bickell & Chia, 1979; Hubbard, 1988). The time these veligers spend in the plankton can be substantial, lasting up to 59 days for *O. muricata* (Müller) (Todd & Havenhand, 1985). Thorson (1946) commented that Type C veligers spend a relatively short time in the plankton, this is in agreement with current literature regarding lecithotrophic and short-term planktonic Shell-type 2 veligers (Harris, 1975). With Shell-type 2 veligers, the mantle fold from which shell growth occurs is not in contact with the shell, therefore shell growth cannot occur after hatching (Tardy, 1970; Harris, 1973). The shell is large enough to accommodate the somatic growth required to reach competency, without enlargement (Todd, 1981).

As the interest in this chapter is on the free-swimming veliger stage, hereafter

Thompson's (1961a) classification shall be adopted.

3.1.4 *DEVELOPMENTAL MODES*

There are several classifications regarding development in larval invertebrates, notably those categorized by Thorson (1946). He described three types of pelagic larvae based on their need to consume plankton during their development:

PLANKTOTROPHIC LARVAE WITH A LONG PELAGIC LIFE: The larvae that belong to this group spend up to 3 months in the plankton feeding prior to settlement.

PLANKTOTROPHIC LARVAE WITH A SHORT PELAGIC LIFE: Larvae of this type spend only a short amount of time in the plankton. They have similar (or the same) morphology and size upon hatching as they do on settlement.

LECITHOTROPHIC PELAGIC LARVAE: These larvae hatch from large eggs with yolk; they do not feed upon plankton. The time spent in the plankton is variable.

Thompson (1967) discussed the developmental strategies of the Opisthobranchia, and reclassified Thorson's (1946) three larval categories to include a developmental process in which some opisthobranchs bypass the pelagic veliger stage and metamorphose within the egg capsule. Thompson (1967) classified the three developmental processes as follows:

DEVELOPMENT-TYPE 1, SPECIES WITH PLANKTOTROPHIC LARVAE: The veligers of this type are the least developed. Feeding is essential (obligate planktotrophs) if they are to metamorphose. The time spent in the plankton exceeds 3 days.

DEVELOPMENT-TYPE 2, SPECIES WITH LECITHOTROPHIC LARVAE: These veligers do not need to feed in order to undergo metamorphosis, however some are facultative planktotrophs. The mantle fold is pulled away from the shell aperture prior to hatching, therefore shell growth does not occur. The time spent in the plankton is very short, not exceeding two days. This classification covers both of Thorson's (1946) latter two categories (see above).

DEVELOPMENT-TYPE 3, SPECIES WITH DIRECT DEVELOPMENT: These larvae undergo a rudimentary veliger stage within the egg capsule and hatch out as benthic adults. However, some species are known to bypass the veliger stage altogether.

To add confusion some nudibranchs exhibit poecilogony, i.e. they produce more than one development type. In *Berghia verrucicornis* (Costa) both Development-type 2 and 3 occur within the same egg mass if the culture is not aerated. The numbers of each development

type are highly variable (Carroll & Kempf, 1990). When Chester (1996) reared *Tenellia adspersa* (Nordmann) under normal conditions 7-13% of the eggs laid in spawn masses produced capsular metamorphic juveniles (Development-type 3). However when the adults were starved this percentage dropped to zero and all masses laid produce veligers of Development-type 2. However, Eyster (1979) never observed masses containing mixed development types in *T. adspersa*. When Harris *et al.* (1975) first attempted to rear *Cuthona nana* (Alder & Hancock) they reported that the veligers hatched after 7-10 days at 13°C, and had a Development-type 1. However, two years later, they reported that the veligers hatched after 21 days at 11-12°C, and had a Development-type 2; metamorphosis was successful in the presence of *Hydractinia echinata*. They attributed this switch in development type to the poor nutritional state of the adults when they were first collected.

Many authors have discussed the developmental types of opisthobranchs, notably Todd & Doyle (1981) and Hadfield & Miller (1987). In the former study, Todd & Doyle (1981) discovered a positive relationship between egg diameter and development time in 30 species of North Atlantic nudibranchs. Thus the larger the egg diameter the longer the development time, this corresponded to those species with lecithotrophic or direct development. They also adopted the "settlement-timing hypothesis" to explain why *O. bilamellata* produces Development-type 1 veligers when it is energetically able to produce Development-type 2 veligers. It was concluded that as Development-type 1 veligers incurred the longest egg to juvenile time, the metamorphosis of the veligers would coincide with the recruitment of *Semibalanus balanoides* (L.) their prey, a year later. However, this hypothesis has been a source of interesting discussion (Grant & Williamson, 1985; Todd, 1985), especially as Chia & Koss (1988) observed juvenile *O. bilamellata* consuming detritus. Hadfield & Miller (1987) addressed the relationships between egg size and the size of the veligers on hatching, in association with their development type. In general they concluded that Development-type 1 veligers hatch from small eggs, typically < 130 μm; Development-type 2 veligers hatch from larger eggs, typically < 185 μm; and direct developers hatch from eggs between 120-380 μm. There is a positive relationship between egg size and hatching shell size, although no correlation was discovered between hatching shell size and settling shell size, or hatching shell size and larval duration (Hadfield & Miller, 1987).

3.1.5 *PROBLEMS ASSOCIATED WITH VELIGER CULTURE*

There are numerous problems associated with veliger culture, many of which can be overcome with simple and careful husbandry. Franz (1975) detailed some of the obstacles encountered and how to resolve them, including issues such as caring for the adults, phytoplankton culture, and larval development. The majority of problems arise when rearing larvae which are planktotrophic, as they must feed to become competent to metamorphose and can spend several months in the pelagic stage. Lecithotrophic veligers require less husbandry, they can metamorphose without food and only remain in the pelagic stage for a short period. The maximum time for a Development-type 2 veliger is 2 days (Thompson, 1967).

The first problem encountered with culturing involves the contamination of the egg masses. If gathered from the field, the egg mass is likely to be contaminated with protozoa, nematodes, diatoms, bacteria etc. Hurst (1967) noted that egg masses deprived of good water circulation became "unhealthy" due to infestation by copepods and protozoans. To overcome this, she suggested the egg masses should be exposed to strong water currents. Gohar & Soliman (1963) noted an increase in the numbers of veligers hatching when a continuous supply of sea water is available; without it development was impeded. Harris (1973) contradicted Hurst's (1967) reasoning; he observed that egg masses left in running sea water are continually subjected to bacteria, protozoa, nematodes, annelids and crustaceans which assist in the breakdown of the matrix, thus allowing the veligers to escape. If newly laid masses were removed immediately and placed into filtered still sea water, the veligers could not escape unless artificially assisted (Harris, 1973). He also observed *Phestilla* veligers seemingly taking the wall of the capsule into the "mouth area", thereby creating a small hole through which the veligers escaped. Morse (1971) noted that some *Coryphella stimpsoni* (Verrill) juveniles use their radular teeth to break through the capsule. This method of escape would only apply to veligers with a Development-type 3.

Todd (1981) described three main problems associated with veliger culture:

- Duration of time spent in the planktonic stage and the inevitable infections associated with it.
- High mortality of veligers.
- The necessity of a suitable algal diet to ensure successful growth and development.

The time Development-type 1 veligers spend in the plankton can vary from 9 days in *Doridella obscura* Verrill (Perron & Turner, 1977), to 59 days in *O. muricata* (Todd & Havenhand, 1985). This duration could be substantially increased when in the natural environment. Todd & Doyle (1981) suggested *O. bilamellata* could spend up to 69 days in the plankton, despite reaching competency in 32 days under artificial conditions. When culturing a species of nudibranch for the first time there is no way of knowing how long the planktonic stage will last. Despite clear correlations between egg size and larval type (Todd & Doyle, 1981; Hadfield & Miller, 1987), the relationship between egg size and development time is much more difficult to determine. Using evidence from previously published accounts of veliger culture (Table 3.1), one can say with reasonable certainty that lecithotrophic veligers (with a larger egg size) have a longer egg to larvae duration and far shorter larvae to adult duration than planktotrophic veligers. For the purposes of Development-type 1 veliger culture, preparations should be made for a minimum of one month in culture. Studies on several species have shown that veliger culture is temperature dependent with reduced planktonic periods at higher temperature e.g. *D. obscura* (Perron & Turner, 1977), *O. bilamellata* (Chia & Koss, 1988) and *O. muricata* (Todd & Havenhand, 1985). Despite this, to study larval ecology the veligers should be raised as close to ambient conditions as possible so that the development can be approximated to their natural environment.

The longer the veligers spend in culture the greater the associated mortality. There are many factors during culture that lead to death, the most significant is "rafting". On hatching the veligers are positively phototatic and or negatively geotatic, thus they have a tendency to swim upwards (Hurst, 1967; Franz, 1975; Harris, 1975). Due to the hydrophobicity of their shells they become trapped at the air-water interface, and will die if not dislodged. Cetyl alcohol flakes were used by Hurst (1967) to reduce the surface tension thereby minimising larval mortality. Harris (1970) (cited in Franz (1975)) also found it effective when rearing *P. melanobranchia.* Several authors have adopted other techniques e.g. utilizing sealed vessels (Kriegstein *et al.*, 1974; Perron & Turner, 1977; Paige, 1986), frozen distilled water (Todd, 1981), or the simple technique of pipetting water onto the trapped veligers to knock them free (Thompson, 1958; 1962).

Bacterial and protozoan infections are another cause of larval mortality. Many authors have used the antibiotics Penicillin G and Streptomycin sulphate in accordance with

Switzer-Dunlap & Hadfield's (1977) methods when rearing aplysiids. Other chemicals used include Terramycine (Hadfield, 1963), Chloramphenicol (Harrigan & Alkon, 1978; Avila *et al.*, 1997) and EDTA (Avila *et al.*, 1997). Some have eliminated the use of chemicals altogether, employing other methods including increased water and or vessel changes (Carroll & Kempf, 1990), or increased culture temperature thereby shortening the veliger phase (Perron & Turner, 1977; Todd, 1981; Todd & Havenhand, 1985; Chia & Koss, 1988). Lambert & Todd (1994) warned against the use of antibiotics if investigating the stimulus of metamorphosis as many compounds have been known to induce metamorphosis artificially (review by Hadfield (1978)). Obviously to obtain unimpaired and reliable results the use of chemicals in culture should be minimized or if practicable avoided altogether as it may affect veliger development and or behaviour.

When changing the culture water it is necessary to employ a technique which minimises larval mortality without exposing the veligers to the air. Several different methods have been employed but by far the most frequent is one described by Switzer-Dunlap & Hadfield (1981) (Fig. 3.1). After concentrating the veligers within a filter they are then transferred into clean culture vessels. Any veligers which have become trapped at the air-surface interface are knocked down with water from a pipette. For cultures using larger volumes, Todd (1981) used a siphon with a fine mesh to cover one end. The mesh permitted removal of dead larvae, algae, and faeces from the bottom of the vessels whilst leaving healthy veligers behind. The medium was reduced to approximately 10% of the original volume which was then topped up with clean filtered sea water.

Different species of veliger require different dietary regimes; many authors have failed to rear veligers through metamorphosis due to inadequate food quality. Harris (1970) cited in Franz (1975), found *Phaeodactylum* was nutritionally better than other phytoplankton. However, the *Phaeodactylum* cells settled, thus to re-suspend it the vessels were shaken which was found to be detrimental to *P. melanobranchia* veligers. Todd (1981) advised against the use of *Phaeodactylum* and other diatoms as they contain indigestible organics, and have a tendency to settle. Most authors who have reared veligers successfully used one or both of *Isochrysis galbana* and *Pavlova* (*Monochrysis*) *lutheri* often in combination with other species (Table 3.1). To add to the problem, some veligers when fed on inadequate diets may appear to develop normally, but will ultimately fail to metamorphose. When attempting to

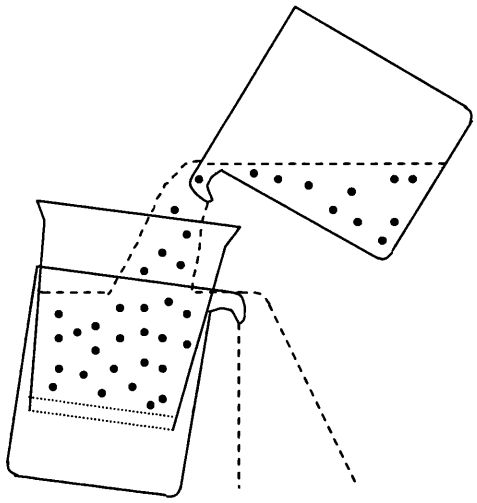

Figure 3.1. The filtering method employed by Switzer-Dunlap & Hadfield (1981). A modified plastic beaker with a 41 or 73 μm mesh replacing the bottom was used as a filter. It was placed into a larger beaker containing fresh sea water. The veligers were poured into the filter where they were retained. The water was allowed to overflow from the bottom beaker, thus allowing the old culture water to escape. This method concentrated the veligers and allowed them to be washed using fresh sea water. The final volume was then reduced in the filter and the veligers transferred to new vessels.

rear *O. bilamellata* using *Monochrysis*, Hadfield (1963) noted shell growth but the veligers failed to metamorphose, the veligers survived for up to 32 days. Todd (1977) cited in Todd (1981) had similar results with *O. bilamellata*. Growth was observed when fed with *Pavlova* and the combination of *Pavlova* and *Phaeodactylum*, but the veligers never attained competency. When *T. diomedea* Bergh veligers were fed monocultures of *Dunaliella*, *Isochrysis* and *Monochrysis* all failed to reach competency, despite the latter resulting in propodial development (Kempf & Willows, 1977). The type of phytoplankton that results in a successful outcome for one species is unlikely to work for another. However, development and metamorphosis is more likely with mixtures of phytoplankton, and this unfortunately adds to the intensity of labour during veliger culture.

3.1.6 *STAGES OF DEVELOPMENT*

After hatching veligers exhibiting Development-type 1 (Thompson, 1967) undergo significant morphological changes prior to become competent to metamorphose. These changes were described in detail by Bickell & Chia (1979) when rearing *Doridella steinbergae* (Lance). They identified four stages:

Stage 1. During this stage the mantle fold remained in contact with the shell aperture. Shell growth began, and as soon as feeding commenced the left digestive diverticulum distended.

Stage 2. Eye spots formed and the mantle fold began to pull away from the shell aperture. The shell size increased from 142 μm to 168 μm. At this point shell growth ceased but the left digestive diverticulum continued to distend. The larval heart began to beat. This stage was attained 10-12 days post-hatching.

Stage 3. The metapodium started to inflate to form the propodium. The mantle fold began to undergo hypertrophy, the radular sac rudiment evaginated, and the adult kidney rudiment appeared. This stage was attained 20-22 days post-hatching.

Stage 4. The propodium became fully inflated, the radular teeth appeared and the oral lip glands formed. Gland cells appeared in the inner epithelium of the mantle fold. At this point the larvae were able to metamorphose. This stage was attained 23-24 days post-hatching.

It took 33-34 days from oviposition for *D. steinbergae* to reach competency. Bickell & Chia (1979) noted that the veligers ceased shell growth at 168 μm, this was attributed to the precocious retraction of the mantle fold, which they concluded to be necessary due to the relative short duration of the larval phase. Perron & Turner (1977) observed a similar development process in *D. obscura*, which attained a shell size of 190-195 μm prior to metamorphosis. *D. obscura* spent only 9 days as a veliger when reared at 25°C (Perron & Turner, 1977). This is exceptionally short for a Development-type 1 veliger. The small shell length attained by *D. steinbergae* and *D. obscura* prior to metamorphosis is unusual; nudibranchs with a Development-type 1 and a Shell-type 1 can reach up to 440 μm in shell length (Todd, 1981), however 250-300 μm is more typical. The majority develop eye spots before the retraction of the mantle fold, especially in species attaining a large maximum shell length (Kempf & Willows, 1977; Kriegstein, 1977; Chia & Koss, 1978; Bickell & Kempf, 1983).

Bickell & Kempf (1983) identified three stages in the larval development of *M. leonina* (Gould): the hatching stage; the eye spot-mantle retraction stage attained 16-20 days post-hatching; and the metamorphic competence stage attained 30-48 days post-hatching. At the mantle retraction stage the veliger shell ceased to grow after reaching a size of approximately 250 μm, shortly after this the larval heart began to beat. Despite minor

discrepancies between species, the pattern of veliger development described by Bickell & Chia (1979) for *D. steinbergae* is typical for all, although the exact timing of the mantle fold retraction and therefore shell growth cessation does vary from species to species.

Kriegstein (1977) identified six stages in the larval growth of *Aplysia californica* Cooper which has veligers with a Shell-type 1 (Thompson, 1961a) and a Development-type 1 (Thompson, 1967). Stage 1 was attained immediately on hatching before any development had occurred. Stage 2 included the development of eye spots and the enlargement of the velar lobes. Shell growth began to occur and the shell started to coil dextrally. Stage 3 was characterized by the development of the larval heart. At Stage 4 shell growth had ceased, and the shell measured 400 μm in length. At Stage 5 the propodium developed and the mantle fold began to retract away from the shell aperture. At Stage 6 the veligers were competent to metamorphose. This stage was characterised by the development of red spots. This sequence of development for *A. californica* veligers is similar to that described for the Nudibranchia, except Stage 6.

Switzer-Dunlap & Hadfield (1977) and Switzer-Dunlap (1978) noted that aplysiid veligers grow to a "species-specific size" prior to the development of other features necessary for metamorphosis. This has not been recorded for the Nudibranchia, however it does appear that in order to attain metamorphic competence they must undergo a period of no shell growth which is only achieved once they have attained a "maximum" size and this may well be species specific.

There are a few species that do not follow the typical pattern of veliger development. Thiriot-Quievreux (1977) studied the development of *Aegires punctilucens* (D'Orbigny). She divided the veliger stage into two, based on the presence or the absence of the shell. Unusually, the veligers possessed velar lobes which measured 200-300 μm, consequently they could not be retracted into the shell, instead "...il replie les lobes l'un sur l'autre comme des ailes de papillon". During the first stage, the mantle grew rapidly around the shell aperture, the shell was then cast, and the mantle continued to grow. Unusually the veligers remained planktonic following shell loss. The velar lobes grew to a size of 350-500 μm. The mantle extended to cover the whole body and papillae and spicules developed. When the veligers were exposed to *Ulva* or *Posidonia* settlement was stimulated (Thiriot-Quievreux, 1977). Gohar & Soliman (1967a) described a similar development for *Trippa spongiosa* (Kelaart) veligers. Within 30 minutes of hatching the shell was cast, followed

shortly after by the loss of the operculum. Gohar & Soliman (1967a) observed the veligers swimming following shell loss. During metamorphosis, the velum was absorbed and the veliger assumed the adult form.

The process of metamorphosis is not discussed here as none of the veligers reared in this study reached the level of development required for metamorphosis (reviews by Tardy (1970), Bonar & Hadfield (1974), Bonar (1978), Chia & Koss (1978), Bickell & Kempf (1983)).

3.1.7 *AIMS OF STUDY*

The veliger culture of *O. bilamellata*, *Cuthona gymnota* (Couthouy), *Palio nothus* (Johnston), *Ancula gibbosa* (Risso), and *Thecacera pennigera* (Montagu) was attempted under laboratory conditions, with the aim of investigating the morphological changes that occur during metamorphosis, and to determine the possible cues stimulating settlement. Unfortunately, metamorphosis was never reached in any of the five species, despite significant morphological development in *T. pennigera* and *O. bilamellata.*

3.2 *MATERIALS AND METHODS*

Onchidoris bilamellata, *Thecacera pennigera*, *Ancula gibbosa*, *Palio nothus* and *Cuthona gymnota* adults and spawn were collected from Mumbles pier, Swansea (003°58'04"W 051°34'02"N) from September 2001 to September 2003. Sampling occurred on every spring tide, weather permitting. The adults and spawn were brought back to the laboratory using a stainless steel vacuum flask. This prevented the adults suffering heat stress which during the summer months was often a source of significant mortality, particularly in *T. pennigera.*

The adults were kept in covered plastic holding tanks (30×20×8 cm), an inverted perforated plastic Pasteur pipette was inserted through the lid which was then connected to an air line. Food was added to the tanks and changed when necessary. Towards the end of 2002, the adults began to suffer severe stress when kept in aquarium sea water. The aquarium sea water was pumped from Swansea Bay into tanks located beneath the University aquarium (henceforth referred to as aquarium sea water). This water was found to be high in nitrates, nitrites and phosphates, and the tanks were only infrequently renewed. Thus sea water collected from White Oyster Ledge (003°59'05"W 051°31'02"N) aboard R.V. *Noctiluca* was used (henceforth referred to as WOL sea water). It was stored in 25 litre carboys in a dark room at room temperature to reduce phytoplankton growth. The

sea water in the holding tanks was changed every third day. During the water change, the tanks were emptied of their contents, and washed thoroughly using a scourer and aquarium sea water. The tanks were then dried and filled with fresh unfiltered (aquarium and then WOL) sea water.

3.2.1 *SPAWN MAINTENANCE*

Spawn was typically laid on the side of the holding tank; it was carefully removed using a scalpel. The spawn was observed for debris under an Olympus S40 binocular microscope, and any debris present was removed using fine forceps. The spawn was then transferred to autoclaved 100 ml Pyrex beakers; 50 ml of filtered WOL sea water was added. The WOL sea water was filtered using a Sartorius™ pressure filter composed of a 293 mm sanitary flange holder connected to a 40 litre pressure tank. Cellulose acetate membrane filters (catalogue number: 11106-293) were used with a pore size 0.45 μm. Antibiotics were added following Switzer-Dunlap & Hadfield's (1981) methods for *Aplysia* spp. culture. 0.6 g Penicillin G (Sigma™ P-7794) and 0.5 g Streptomycin sulphate (Sigma™ S-9137) were added to 100 ml of 0.45 μm filtered WOL sea water. This was then stored at -20°C in 1 ml aliquots. Prior to use, the aliquots were removed from the freezer and defrosted at room temperature. 0.5 ml of the antibiotic solution was added to each beaker to produce a final concentration of 60 μg ml^{-1} Penicillin G and 50 μg ml^{-1} Streptomycin sulphate. A Millipore™ nylon mesh (41 μm pore size, Ø 47 mm, catalogue number: NY4104700) was floated on the top to prevent the newly hatched veligers from rafting. This was found to be more effective than the use of cetyl alcohol flakes. Parafilm™ was then used to cover the beakers to reduce evaporation. The beakers were left in a constant temperature room at ambient sea water temperature (6-15°C), with a L:D cycle of 12:12 hours. The WOL sea water was changed every alternate day.

3.2.2 *VELIGER CULTURE*

On hatching, the veligers were collected using a modified filter (Fig. 3.1). A 50 ml syringe (Sartorius™, catalogue number 16647) was sawn in half and the edges smoothed using wet and dry abrasive paper (P1000C). A 41 μm Millipore™ nylon net filter (Ø 47 mm, catalogue number: NY8004700) was glued to the end using Loctite Super Glue Gel™. Prior to use, the filter was washed thoroughly with distilled water to ensure that possible contamination of the glue residues into the culture were minimal. The veligers were poured

into the filter, which was held in a 100 ml beaker full of aquarium sea water. This ensured the veligers stayed immersed and prevented rafting. The veligers were washed using WOL unfiltered sea water. The filter (in the beaker) containing the larvae was placed under an Olympus S40 binocular microscope. The healthy veligers (identified as those actively swimming with a complete shell) were transferred into the culture vessels using a Pasteur pipette.

The culture vessels consisted of 500 ml autoclaved Erlenmeyer flasks. Each flask was filled with WOL 0.45 μm filtered sea water. Four species of phytoplankton were added in equal proportions: *Rhinomonas reticulata* var. *reticulata* (initially *Rhodomonas baltica* was used), *Isochrysis galbana*, *Chaetoceros calcitrans* and *Tetraselmis* sp. See section 3.2.3 for methods used in phytoplankton culture. The final concentration of phytoplankton in the culture vessels was approximately 100 cells μl^{-1}, this was measured using a haemocytometer. Into these flasks approximately 500 larvae were added, to ensure the recommended culture concentration of 1 larva ml^{-1} was achieved (Switzer-Dunlap & Hadfield, 1977; Switzer-Dunlap & Hadfield, 1981). The final volume within the Erlenmeyer flasks was 500 ml. Penicillin G and Streptomycin Sulphate were added to the vessels to give a final concentration of 60 $\mu g\ ml^{-1}$ and 50 $\mu g\ ml^{-1}$ respectively. 1-hexadecanol (Sigma™, catalogue number C-7882) was sprinkled onto the surface of the culture to prevent the "rafting" of larvae (Hurst (1967); Harris (1970) cited in Franz (1975)). Frozen distilled water suggested by Todd (1981) resulted in an unpleasant scum on the surface of the culture vessels and provided only a temporary solution to rafting. The flasks were plugged using a foam bung, and were placed into a CT room at ambient sea water temperature with L:D cycle of 12:12. The culture vessels were changed every 4-5 days.

The veligers were filtered using the same technique used to collect them after hatching. The filter was placed into a beaker containing aquarium sea water and the veliger culture was carefully poured into the filter. The larvae, concentrated in the filter, were then washed using WOL 0.45 μm filtered sea water. They were transferred into an autoclaved flask containing WOL filtered sea water using a Pasteur pipette, and each of the four species of phytoplankton was added. Antibiotics were added at each water change. 1-hexadecanol remained on the surface of the culture medium throughout. Due to larval mortality the numbers of veligers at each water change reduced, thus after two to three weeks of culture the vessels were reduced to 250 ml volume.

Photographs were taken of the spawn and veligers when the sea water was replaced. Small sections of the spawn mass were sliced off using a scalpel and placed onto a dimpled slide. The larvae were removed from the filter using a Pasteur pipette and placed onto a glass slide. A central cover slip was supported by cover glasses at both ends of the glass slide to prevent crushing. They were observed under an Olympus BH2 binocular microscope. Photographs were taken with a JVC TK1270 digital camera using Image-Pro Plus version 4.1 for Windows 95/NT/98.

3.2.3 *PHYTOPLANKTON CULTURE*

Four species of phytoplankton: *Rhinomonas reticulata* var. *reticulata* (*Rhodomonas baltica* was used in the first veliger culture), *Tetraselmis* sp., *Chaetoceros calcitrans*, and *Isochrysis galbana*; were cultured and nourished using f/2 nutrients (Guillard & Ryther, 1962; Guillard, 1975). Todd (1981) used combinations of the four phytoplankton species in the veliger culture of *O. bilamellata* which resulted in successful metamorphosis.

The phytoplankton species were cultured in 500 ml Erlenmeyer flasks using WOL sea water. The sea water was filtered using Whatman™ glass microfibre filters (GF/C pore size 1.2 μm) under vacuum, and 300 ml was added to each 500 ml Erlenmeyer flask. A foam bung was inserted into the mouth of the flask, which was covered with foil and sealed using autoclave tape. The flasks were autoclaved for 20 minutes at 120°C and 15 lb. The flasks were removed at 100°C and placed immediately on ice to reduce precipitation, then they were allowed to cool overnight.

The flasks were supplemented with the f/2 media (Appendix pg. 234) within a laminar flow cabinet. A 5 ml plastic syringe was fitted with a sterile Satorius™ Minisart High-Flow filter (0.2 μm pore size, catalogue number 16532). The filter was dampened prior to use by passing 1 ml of f/2 through which was then discarded. After removing the bung from the flask containing the autoclaved WOL sea water, the lip of the vessel was flamed. The appropriate amount of the f/2 was added (5 ml of f/2 for every 1 litre of WOL sea water). The lip of the vessel was flamed again and the foam bung re-inserted. The filter attached to the syringe was never used more than three times. The silicate supplement was added separately to the flasks containing *Chaetoceros* only. The flasks were then thoroughly mixed. Approximately 25 ml of one species of phytoplankton was added to each flask using autoclaved glass Pasture pipettes. Initially sub-cultures from the cell stock at the University

of Wales Swansea were used to inoculate the culture vessels (except *Rhinomonas* which was obtained from CCAP; strain number CCAP 995/2). After the initial inoculation, separate sub-cultures of each of the species were created in 100 ml Erlenmeyer flasks using 50 ml of WOL filtered sea water. Exactly the same method described above was used to prepare the sub-cultures. These were placed into a different constant temperature room to the main cultures, and were used to inoculate future cultures as required. The sub-cultures were renewed after four weeks, and the main stock was renewed every three to four weeks or more frequently if required. The flasks were kept in a constant temperature room at 18-20°C, with a L:D cycle of 12:12.

3.3 *RESULTS*

Veliger culture was not successful to metamorphosis for any of the five species of nudibranch studied. However, the veligers of *Onchidoris bilamellata* and *Thecacera pennigera* exhibited shell growth and significant somatic development. *O. bilamellata* and *Palio nothus* were studied from oviposition, and the major developmental events were documented. *Cuthona gymnota* and their egg masses were found on *Tubularia* sp. at the time of veliger culture, therefore they too were investigated. Little success was had with both *C. gymnota* and *Ancula gibbosa* (Appendix pg. 234).

3.3.1 ONCHIDORIS BILAMELLATA

O. bilamellata was studied through the embryonic phase, hatching, and partial veliger development.

O. bilamellata lays an egg mass of Type A (Hurst, 1967). The number of eggs per capsule varied between one and two (Fig. 3.2A). Empty capsules were common towards the end of the string (Fig. 3.2B). At the one cell stage, two polar bodies were often visible at the animal pole (Fig. 3.2C). The first division resulted in two cells of equal size which settled alongside one other (Fig. 3.2D). These divided equally to form four indistinguishable cells (Fig. 3.2E). The third division resulted in the formation of the four micromeres which were positioned at the animal pole. Following this, the second quartet cleaved resulting in the formation of a twelve cell embryo (Fig. 3.2F). Cell division continued as normal according to Thompson (1958) & Casteel (1904). After five days gastrulation began. At this point the blastopore formed which lead into the sagittal cleft (Fig. 3.2G). At day nine the early trochophore stage was reached. The blastopore closed and the eversion of the shell gland

began. By this point the apical end of the veliger had developed cilia (Fig. 3.2H), and within the capsule the veliger started to rotate. The early veliger stage was reached after 13 days. The two velar lobes had developed and the shell began to form (Fig. 3.2I). After 17 days, the mid veliger had an obvious metapodium and the shell gland had retracted to form the mantle fold (Fig. 3.2J). Hatching occurred 22-28 days after oviposition at a culture temperature of 6-9°C.

On hatching the veliger has a Shell-type 1 (Thompson, 1961a) which is 165.7 ± 9.5 μm in length. All shell lengths are given as the mean ± the standard deviation. They undergo a Development-type 1 (Thompson, 1967) (Fig. 3.1K). After hatching the veligers commenced feeding, and after three days lipids were observed within the left digestive diverticulum (Fig. 3.2L, M). Shell growth was initially slow, but after ten days growth striations were clearly visible on the shell (Fig. 3.2N). After 26 days the veligers had developed eye spots and the mantle fold had begun to pull away from the shell aperture (Fig. 3.2P). The maximum shell length obtained was 260.9 ± 0.2 μm (Fig. 3.3). Unfortunately, due to larval mortality only a few individuals reached this stage of development, and none developed further.

3.3.2 PALIO NOTHUS

Palio nothus was studied through embryogenesis into the veliger stage; unfortunately the veligers failed to exhibit any further development beyond an increase in shell size.

P. nothus lays spawn masses of Type A (Hurst, 1967). Each capsule contains one embryo (Fig. 3.4A, B). The process of embryonic development follows a similar pattern to that of *O. bilamellata*, except that the time taken to hatch is shorter when reared at the same temperature. Within 24 hours of oviposition, the zygote had cleaved to form two blastomeres (Fig. 3.4C). Polar bodies were often observed and varied between two and three. Within 48 hours the two cells had divided to form four blastomeres of equal size (Fig. 3.4D). The four micromeres cleaved to form the first quartet, shortly followed by the second division to form the second quartet (Fig. 3.4E). After four days, gastrulation had occurred by the formation of the blastopore (Fig. 3.4F). The developing veliger then passed through the trochophore stage to the veliger stage (Fig. 3.4G). Hatching occurred 11-14 days after oviposition when reared at 6-9°C.

P. nothus veligers have a Shell-type 1 (Thompson, 1961a) with a length of 126.3 ± 5.8

μm on hatching. They have a Development-type 1 (Thompson, 1967) (Fig. 3.4H, I). Feeding starts immediately after hatching. The digestive diverticulum became pigmented due to the ingestion of phytoplankton, and lipid droplets were clearly seen (Fig. 3.4J). After 28 days following considerable shell growth, there were no other signs of development beyond that achieved on hatching (Fig. 3.4K, L). The maximum shell length reached was 176.3 ± 11.2 μm (Fig. 3.5).

3.3.3 ANCULA GIBBOSA

Ancula gibbosa gathered from the field failed to produce viable spawn when brought back to the laboratory. However, spawn gathered from the field with the adults was fertilized and therefore the "wild" spawn was cultured. As the time of spawning could not be accurately determined, ontogeny was not studied.

A. gibbosa lays a spawn mass of Type A (Hurst, 1967). Each capsule contains one embryo (Fig. 3.6A, B). The veligers have Shell-type 1 (Thompson, 1961a) which measured 105.6 ± 2.5 μm in length on hatching, and a Development-type 1 (Thompson, 1967) (Fig. 3.6C, D). Feeding commenced immediately after hatching, and after five days the veligers had a pigmented digestive diverticulum (Fig. 3.6E). After ten days some larvae were observed to have their visceral mass pulled slightly away from the shell wall (Fig. 3.6F). None of the veligers survived beyond 13 days in culture. Many died after becoming caught up in debris within the culture (Fig. 3.6G). At no point during culture were eye spots, a larval heart or a propodium developed by any of the veligers. There were large numbers of deformed veligers, many lacked shells but retained the operculum, and were still able to feed (Fig. 3.6H). During development shell growth was negligible; the maximum shell length attained was 125.8 ± 7.8 μm after 8 days (Fig. 3.7).

3.3.4 CUTHONA GYMNOTA

Cuthona gymnota lays spawn masses of Type D (Hurst, 1967) (Fig. 3.8A, B), they contained only one embryo per capsule. Due to the small number of veligers available per egg mass, and high mortality rates development through to metamorphic competency was not achieved.

On hatching, *C. gymnota* veligers possesses a Shell-type 2 (Thompson, 1961a) which measures 246.5 ± 13.2 μm in length, and a Development-type 1 (Thompson, 1967) (Fig.

3.8C). Their internal organisation was similar to that described by Hurst (1967) for Shell-type 2 veligers, except that on hatching they possess eye spots (Fig. 3.8D, E). Feeding commenced immediately after hatching, noted due to the pink tinge within the stomach (Fig. 3.8F). During culture the digestive diverticulum became pigmented, and distended to fill most of the remaining shell space (Fig. 3.8G). By day nine the veligers had developed no further than on hatching (Fig. 3.8H). There was only a slight increase in shell length (probably due to the smaller number of veligers reducing the accuracy of measurement rather than actual shell growth), after 9 days the veligers measured 261.4 ± 7 μm (Fig. 3.7). None of the veligers survived beyond day nine.

3.3.5 THECACERA PENNIGERA

None of the spawn laid in the lab developed beyond the 32 cell stage. Consequently all of the veligers reared in this study were hatched from "wild" spawn gathered from the field on *Bugula plumosa* (Pallas). Initial attempts were made to rear the veligers at 12-15°C as this corresponded to ambient sea temperature at the time of culture. Development at this temperature was slow and the mortality rate was high, as a result the temperature was increased to 20°C in the second attempt; also *Rhinomonas reticulata* var. *reticulata* was used instead of *Rhodomonas baltica.*

T. pennigera lays spawn masses of Type A (Hurst, 1967) (Fig. 3.9A), and the capsules contain only one embryo (Fig. 3.9B). Figures 3.9C and D show early cell cleavage, resulting in the formation of four and eight cells respectively. Cell division is equal and cannot be distinguished at this early stage.

On hatching the veligers possess a Shell-type 1 (Thompson, 1961a) which measures 124.6 ± 11.1 μm in length, and have a Development-type 1 (Thompson, 1967) (Fig. 3.9E). Immediately after hatching the veligers commenced feeding. When reared at 12-15°C, by day 14 the left diverticulum had distended significantly due to food ingested, and shell length had increased (Fig. 3.9F-H). Eye spots appeared after 22 days at a shell length of 177.2 ± 8.5 μm (Fig. 3.9I, J).

Culture at 20°C increased the rate of veliger development substantially. *T. pennigera* commenced feeding on as the alimentary canal acquired a pink hue (Fig. 3.9K). After ten days in culture eye spots had developed, the larval heart had started beating, and the propodium had begun to swell (Fig. 3.9L). The mantle fold had retracted from the shell

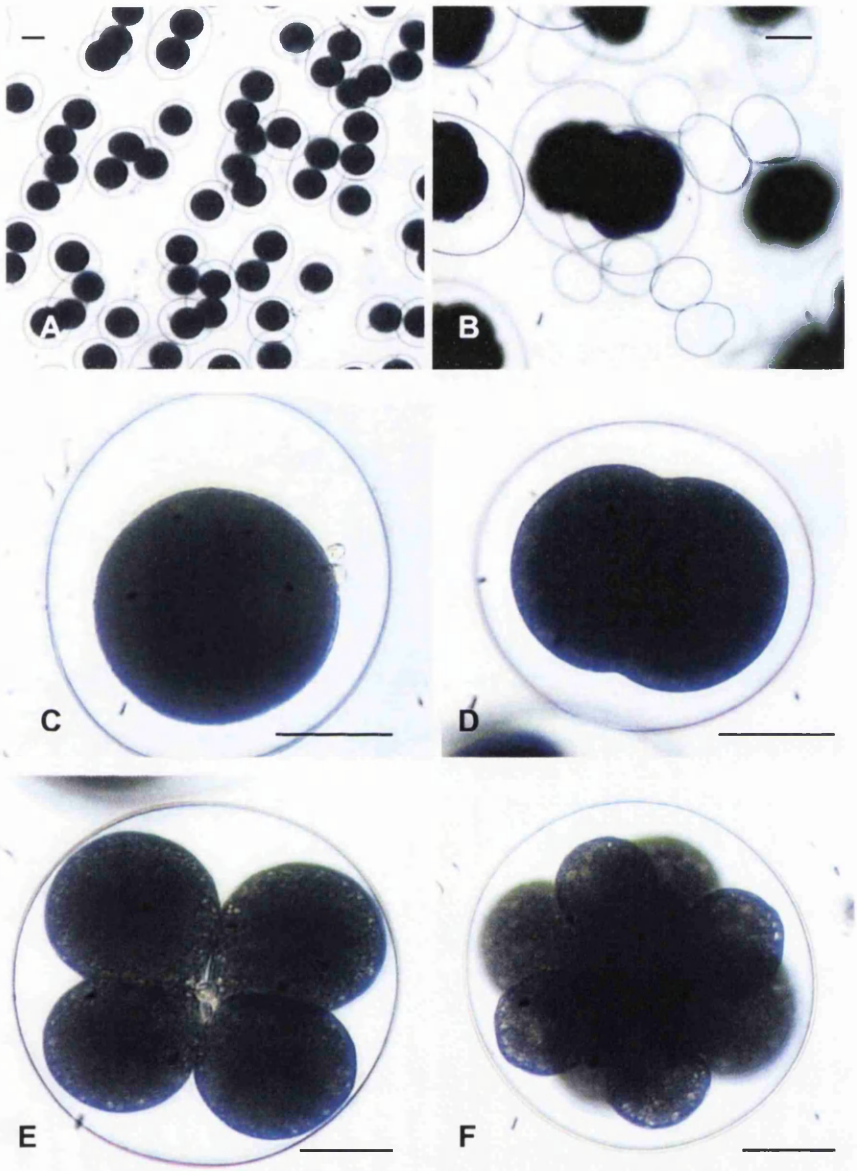

Figure 3.2. *Onchidoris bilamellata*. **A**. Spawn, showing the capsules which contain the zygotes. **B**. Empty capsules. **C**. One cell stage with two polar bodies. **D**. Two cell stage. **E**. Early four cell stage. **F**. The division of the second quartet, viewed from the vegetal pole. Scale bars: **A-F** = 50 μm. Abbreviations of labels given on page 82.

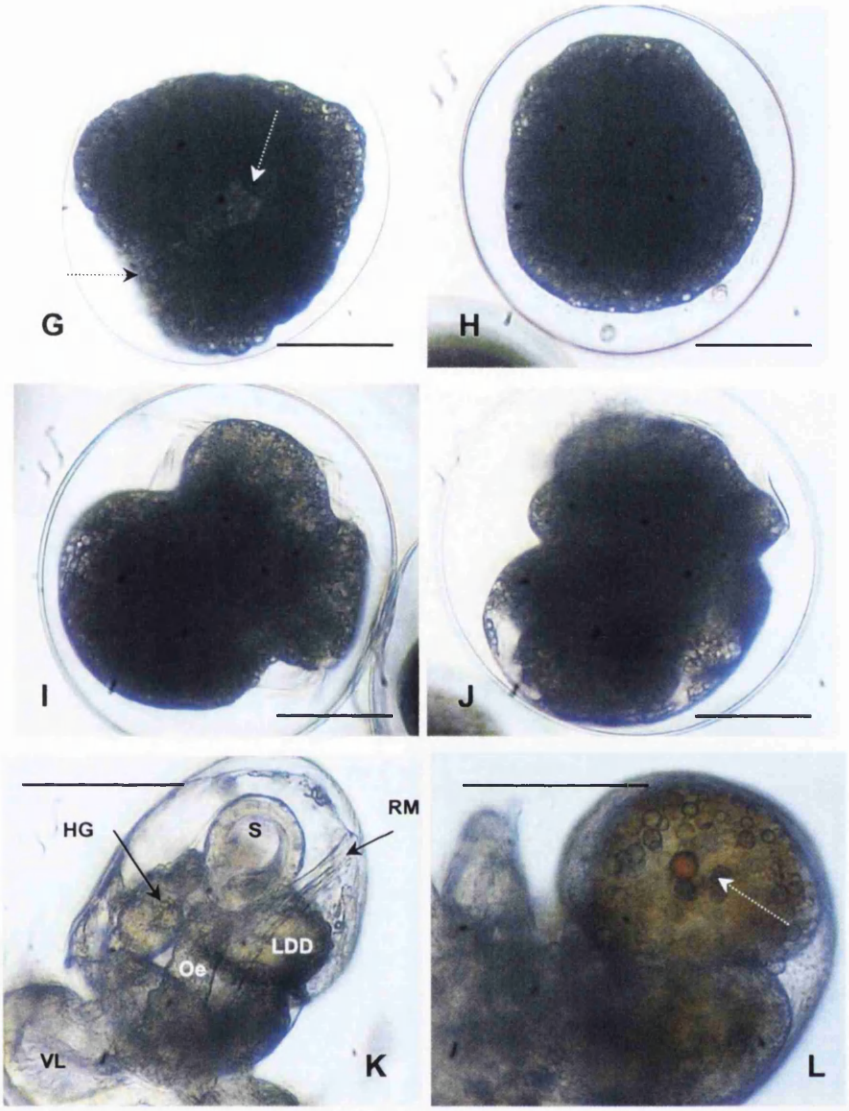

Figure 3.2. *Onchidoris bilamellata*. **G.** Gastrulae at day five, showing the blastopore (dashed arrow), which leads to the sagittal cleft (white arrow). **H.** Early trochophore prior to the shell gland spreading. **I.** Early veliger, with shell in formation. **J.** Mid veliger. **K.** Hatched planktotrophic veliger with a Shell-type 1. **L.** A day three veliger with lipids in the left digestive diverticulum (dashed arrow). Scale bars: **G-L** = 50 μm.

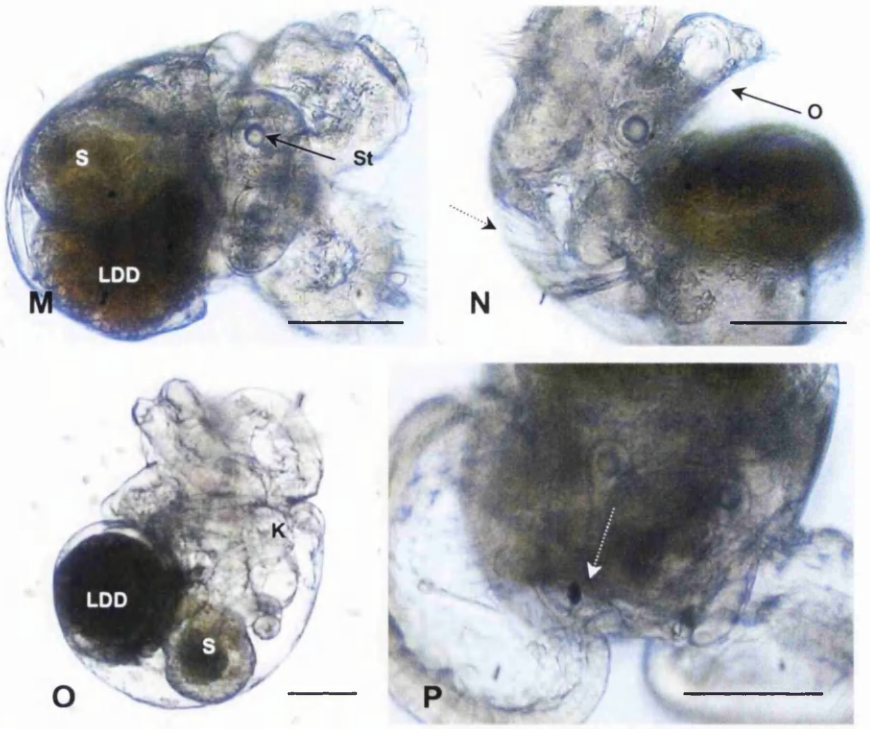

Figure 3.2. *Onchidoris bilamellata*. **M.** Day seven veliger. **N.** Day ten veliger growth striations on the shell (dashed arrow). **O.** Day 18 veliger. **P.** Day 26 veliger with eye spots (dashed arrow). Scale bars: **M-P** = 50 μm.

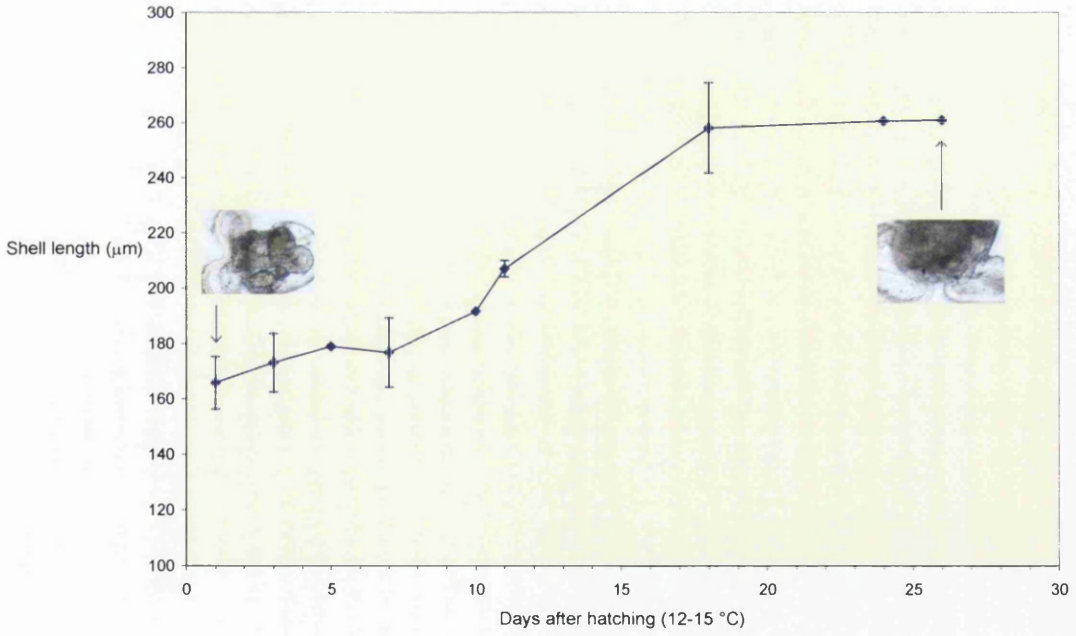

Figure 3.3. Shell growth of *Onchidoris bilamellata* with indications of further development due to the presence of eye spots. Data values are the mean with standard deviation error bars (n=105).

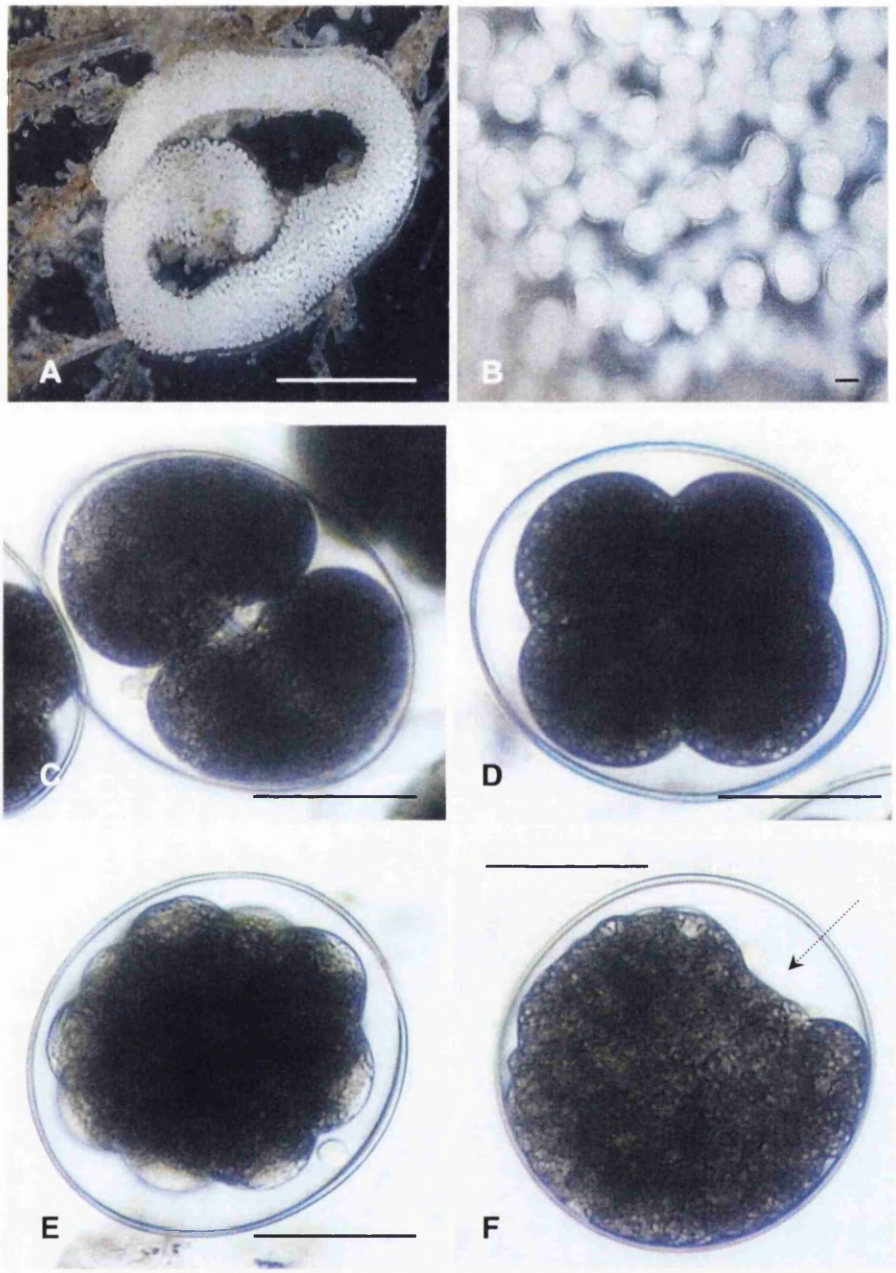

Figure 3.4. *Palio nothus*. **A**. Spawn on *Bowerbankia* sp. **B**. Enlarged view of the same spawn mass. **C**. The transition from the two cell stage to four. **D**. Four cell stage. **E**. Division of the second quartet. **F**. Gastrulation with the formation of the blastopore, dashed arrow. Scale bars: **A** = 10 mm; **B-F** = 50 μm.

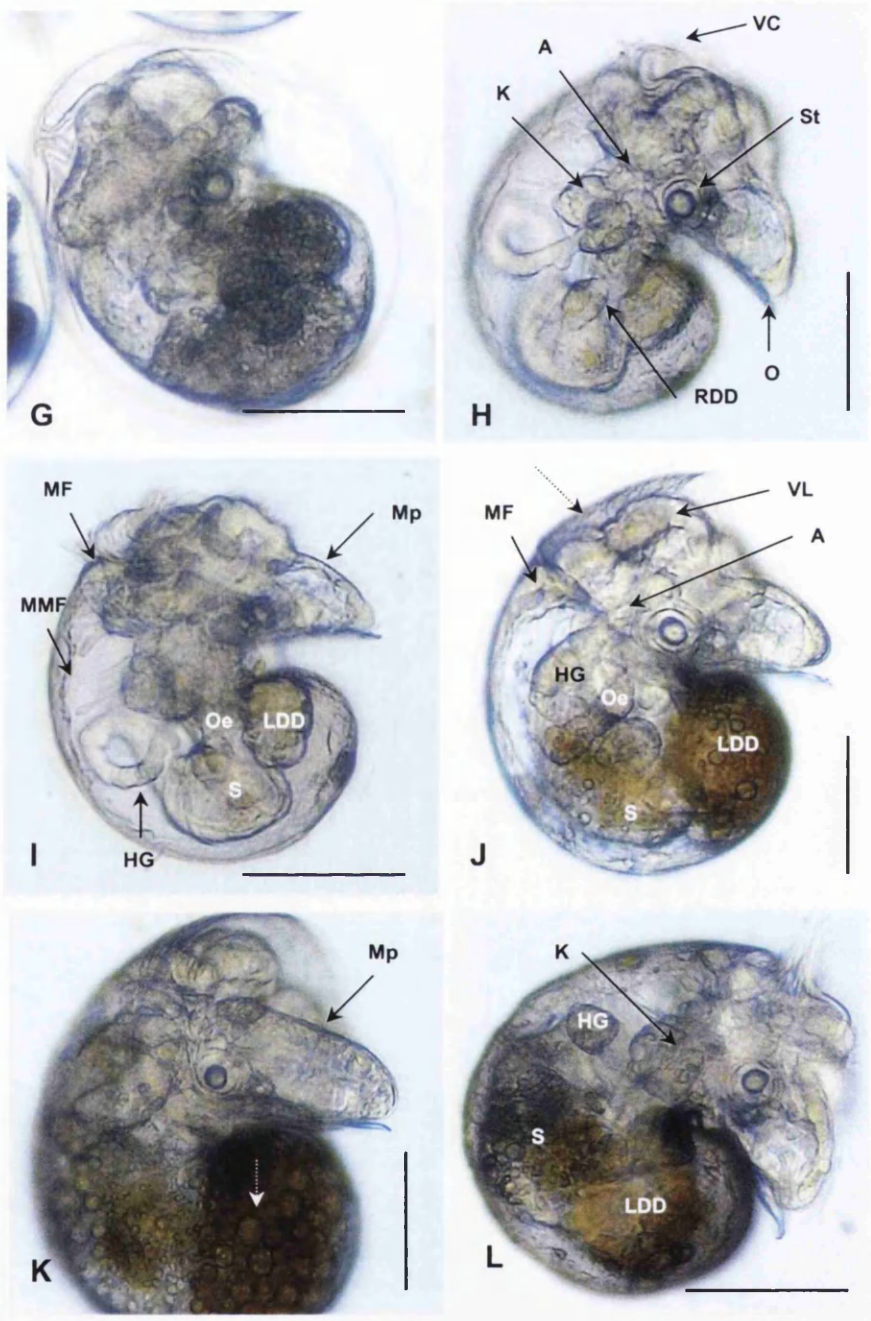

Figure 3.4. *Palio nothus*. **G.** Late veliger. **H, I.** Hatched planktotrophic veliger with a Shell-type 1. **J.** Day ten veliger, shell growth can be identified as striations on the shell (dashed arrow). **K.** Day 14 veliger, note the uninflated metapodium. **L.** Day 28 veliger showing no obvious further development. Scale bars: **G-L** = 50 μm.

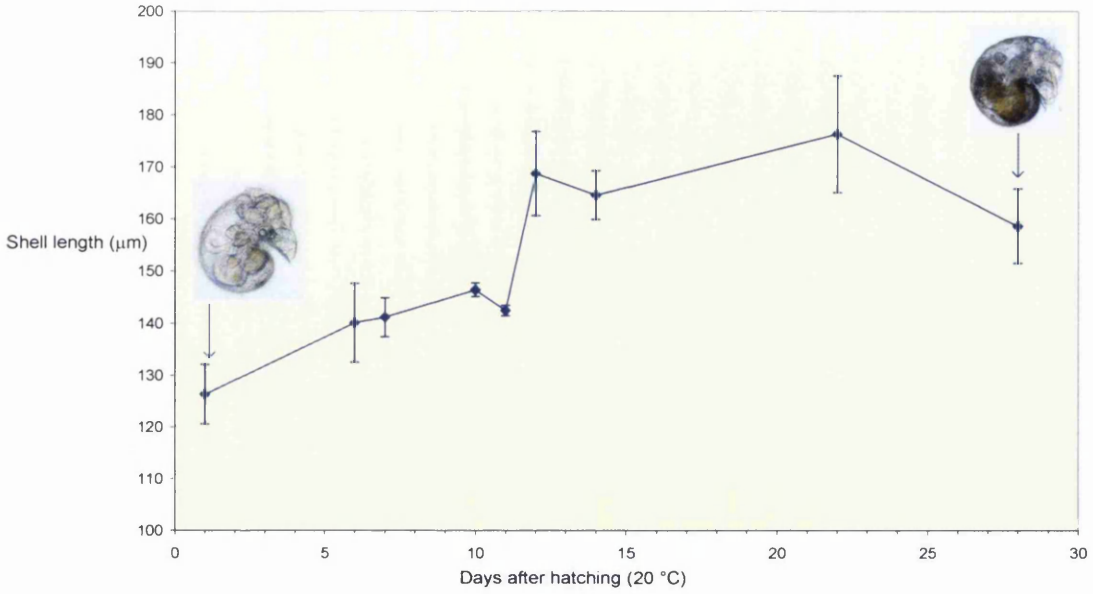

Figure 3.5. Shell growth of *Palio nothus* at 20°C. Despite exhibiting shell growth they failed to develop any further than upon hatching. Data values are the mean with standard deviation error bars (n=86).

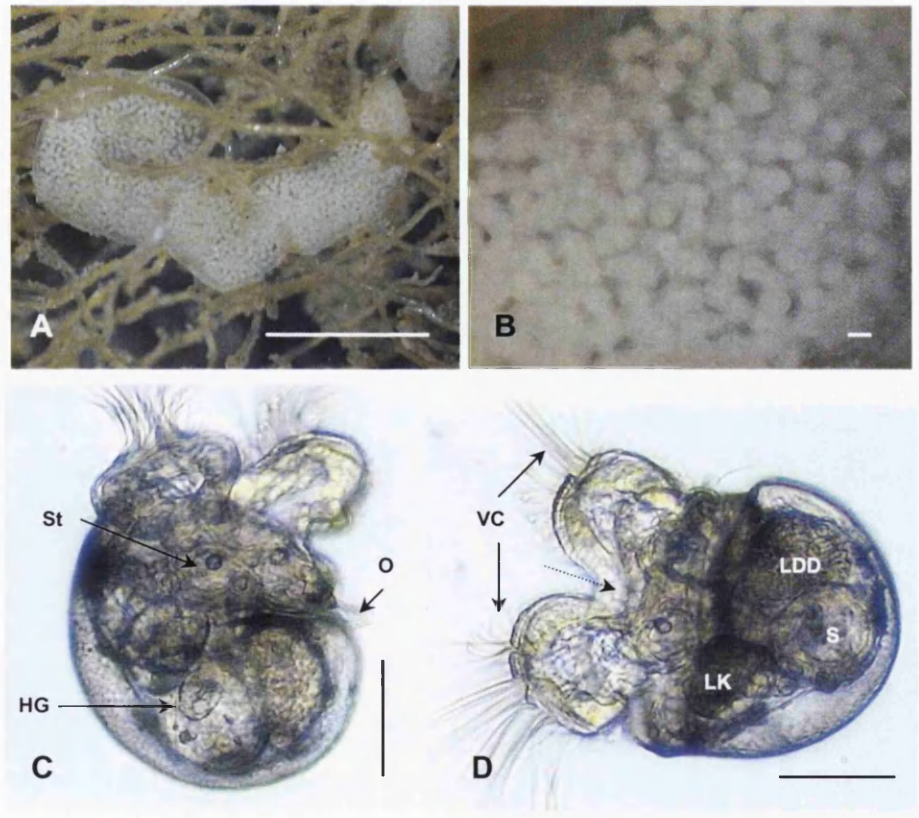

Figure 3.6. *Ancula gibbosa*. **A.** Spawn mass. **B.** Enlarged view of the same mass. **C.** Newly hatched veliger. **D.** Newly hatched veliger showing mouth (dashed arrow). Scale bars: **A** = 10 mm; **B** = 100 µm; **C-D** = 50 µm.

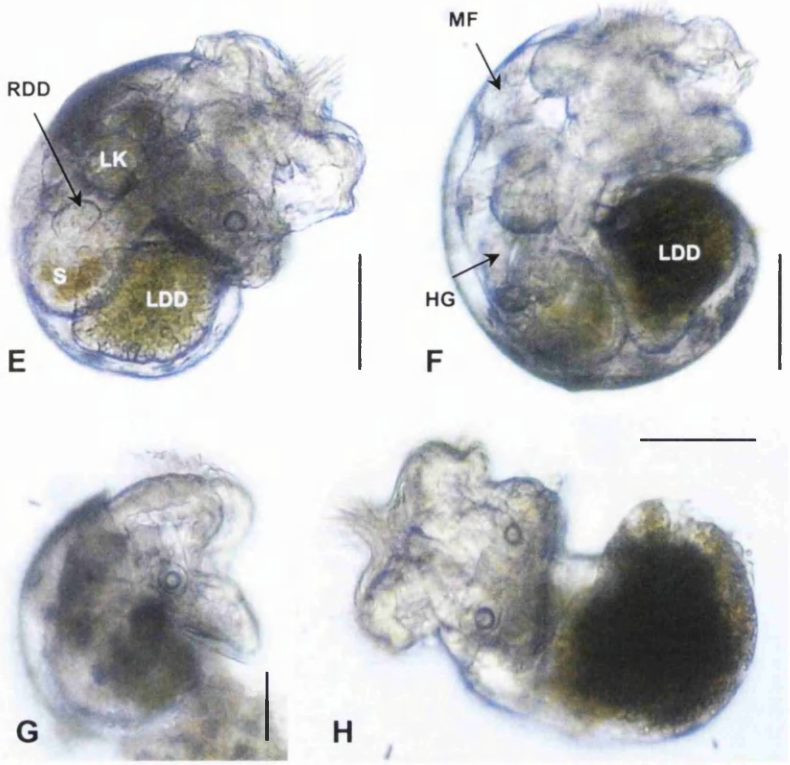

Figure 3.6. *Ancula gibbosa*. **E**. Day five veliger. **F**. Day ten veliger. **G**. Day 13 veliger caught up in debris. **H**. Deformed veliger with no shell, showing evidence of feeding due to the pigmented diverticulum. Scale bars: **E-H** = 50 μm.

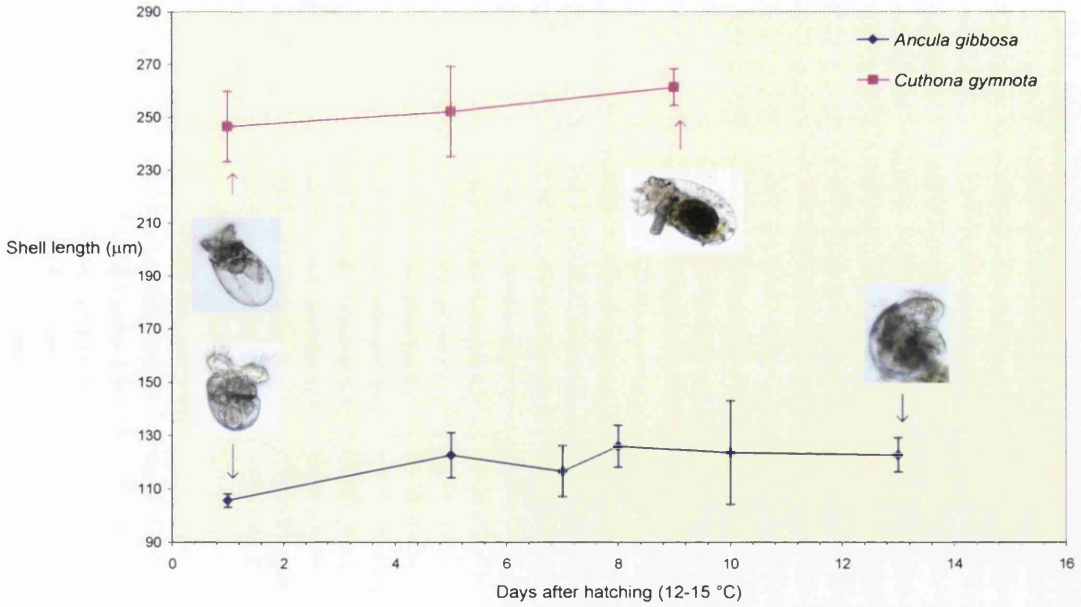

Figure 3.7. Shell growth of *Ancula gibbosa* (n=142) and *Cuthona gymnota* (n=36). Neither of the two species showed any sign of development. Data values are the mean with standard deviation error bars.

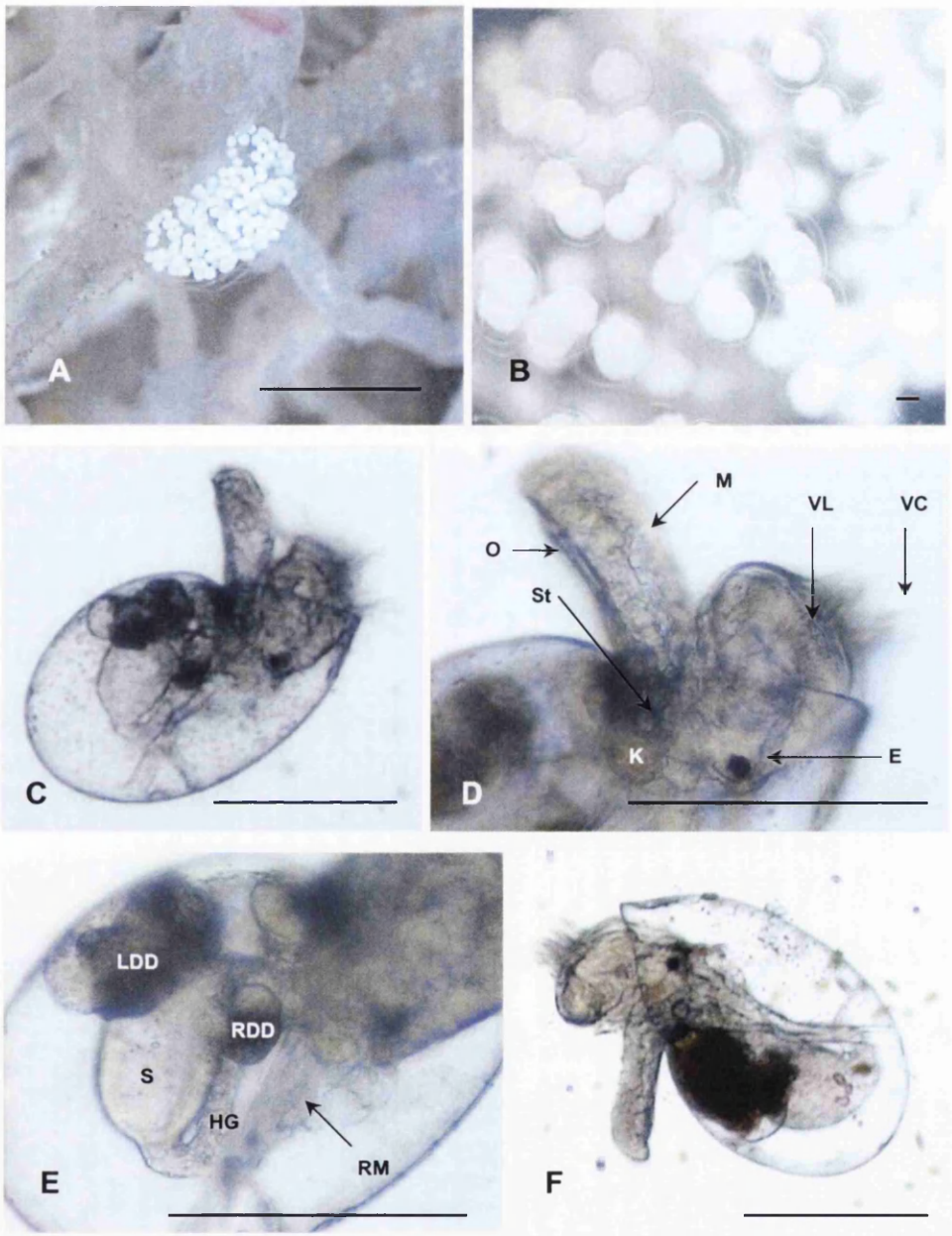

Figure 3.8. *Cuthona gymnota*. **A.** Spawn mass. **B.** Enlarged view of the same mass showing embryos. **C.** Newly hatched planktotrophic veliger. **D.** Detailed view showing the apical end. **E.** Detailed view showing the shell end. **F.** Day five veliger. Scale bars: **A** = 5 mm; **B-F** = 100 μm.

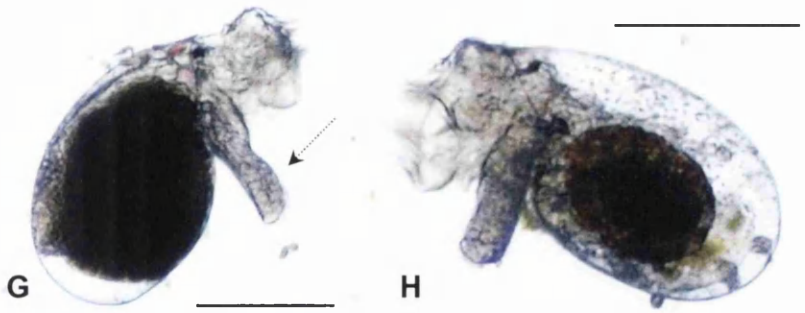

Figure 3.8. *Cuthona gymnota*. **G.** Veliger with a grossly distended digestive diverticulum, note the uninflated metapodium (dashed arrow). **H.** Day nine veliger. Scale bars: **G-H** =100 μm.

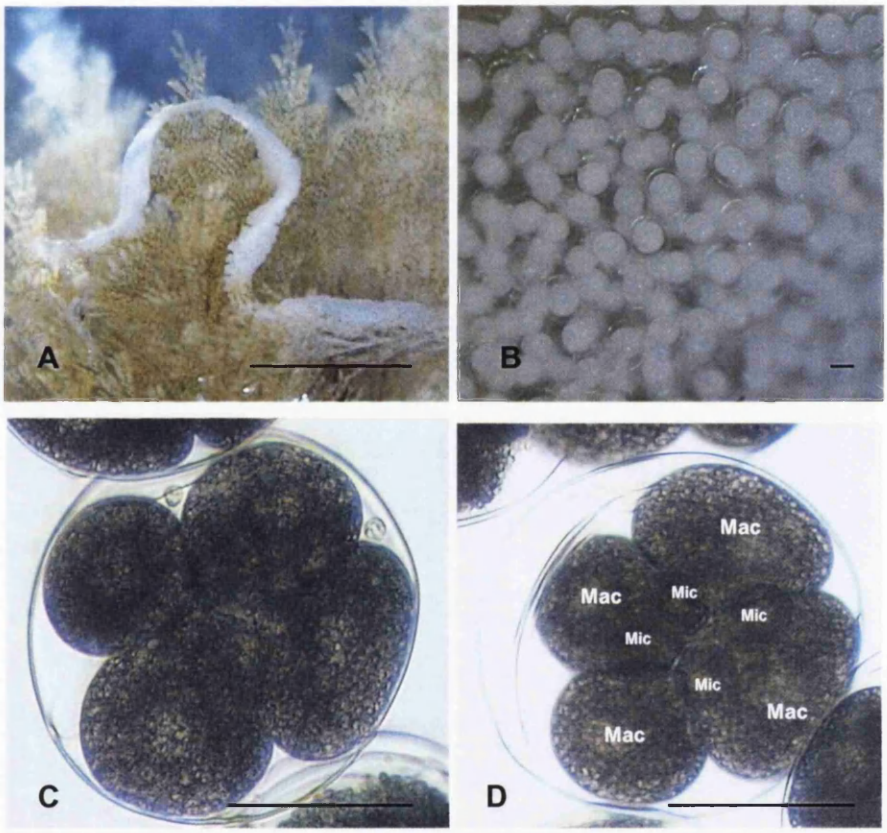

Figure 3.9. *Thecacera pennigera*. **A.** Spawn mass on *Bugula plumosa*. **B.** Enlarged view of the same mass. **C.** The four cell stage. **D.** Eight cell stage viewed from the animal pole. Scale bars: **A** = 10 mm; **B** = 100 μm, **C-D** = 50 μm.

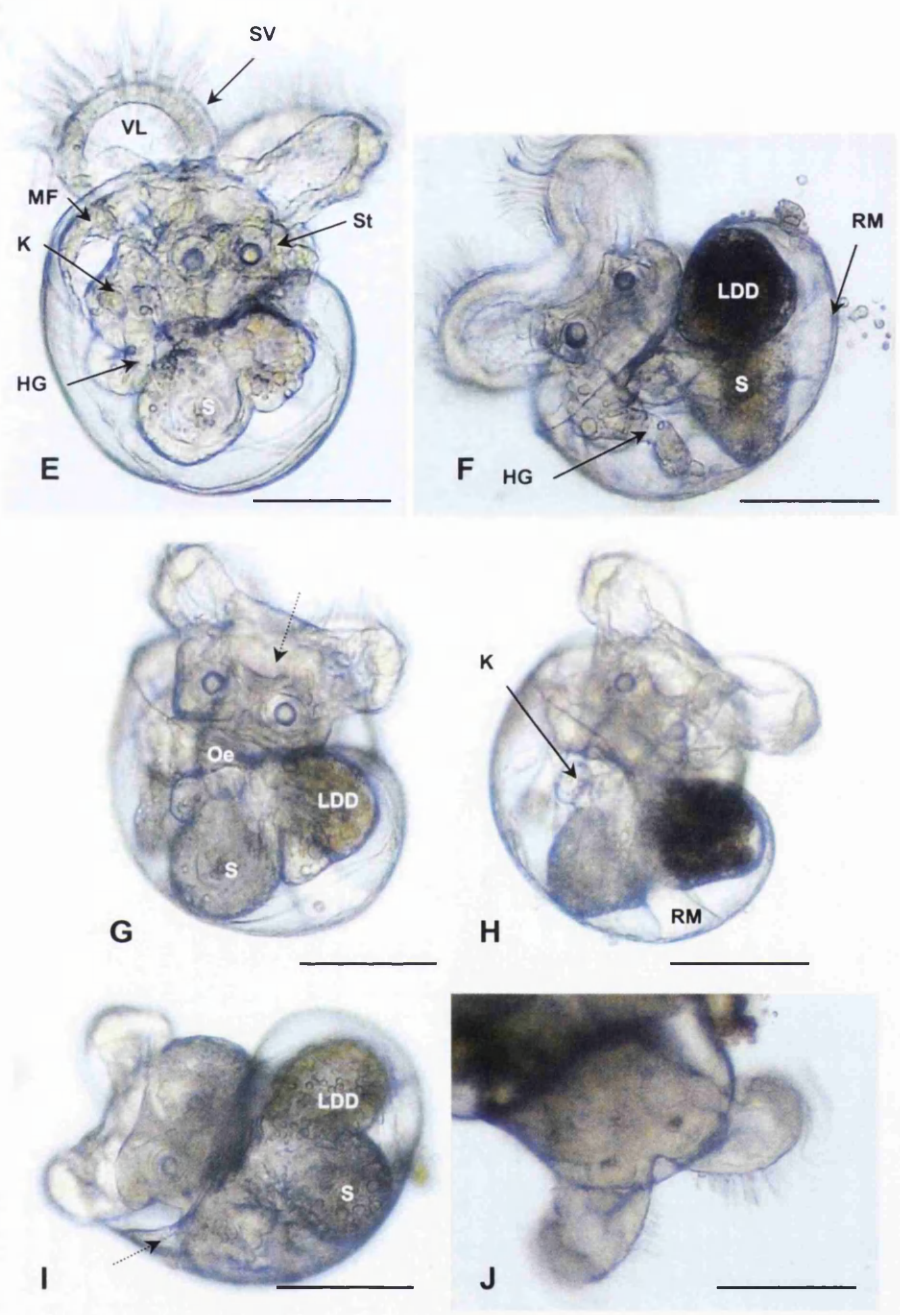

Figure 3.9. *Thecacera pennigera*. **E**. Newly hatched veliger. **F**. Day seven veliger. **G**. Day 14 veliger, showing mouth (dashed arrow). **H**. Day 14 veliger. **I**. Day 22 veliger with shell growth, dashed arrow. **J**. Enlarged view of the same veliger showing eye spots. All reared at 12-15°C. Scale bars: **E-J** = 50 μm.

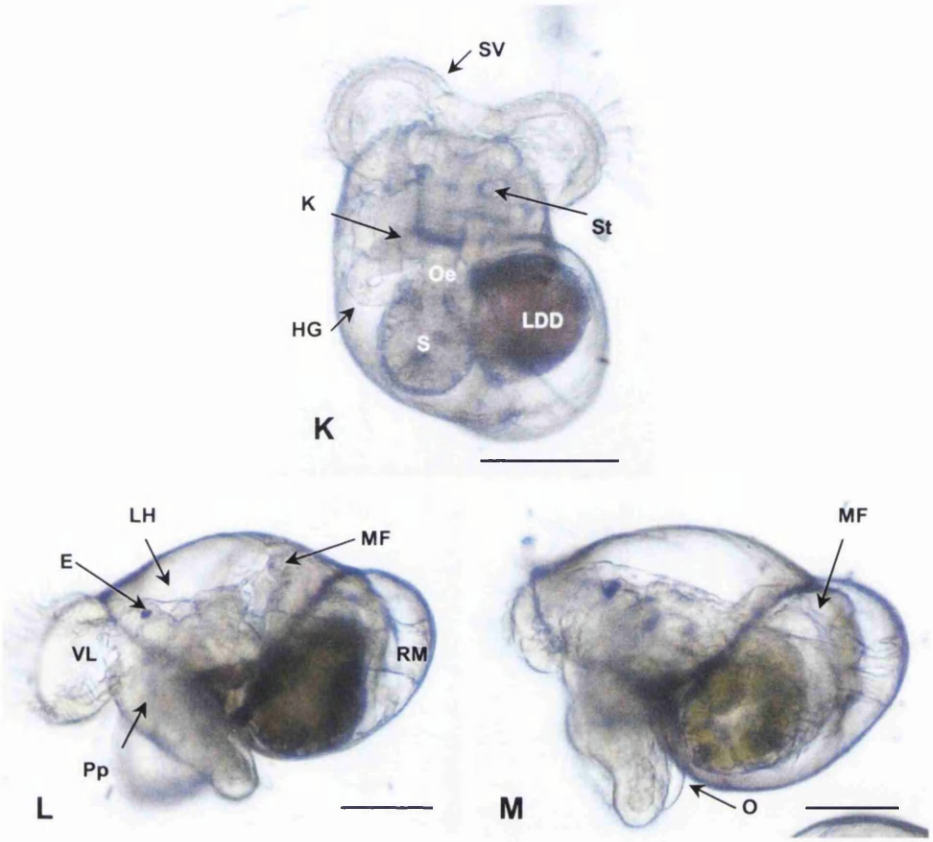

Figure 3.9. *Thecacera pennigera*. **K**. Day three veliger reared at 20°C using *Rhinomonas*. **L**. Day ten veliger reared at 20°C. **M**. Day 16 veliger showing complete retraction of the mantle fold, reared at 20°C. Scale bars: **K-M** = 50 μm.

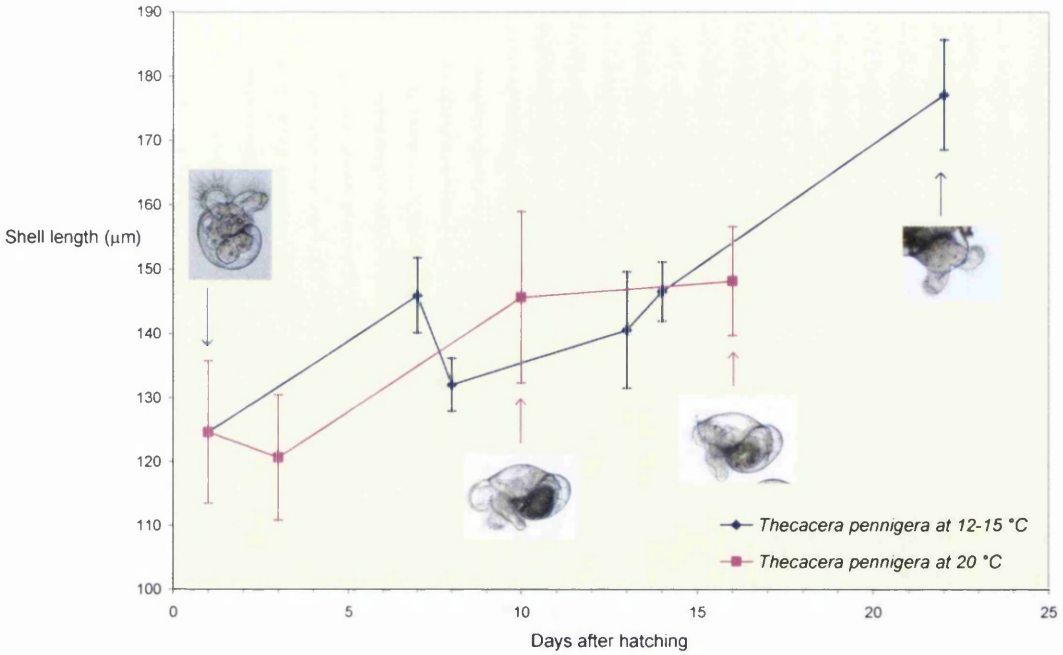

Figure 3.10. Shell growth of *Thecacera pennigera* at different culture conditions, showing significant larval development at 20°C but reduced shell growth (n=124 at 12-15°C, n=162 at 20°C). Data values are the mean with standard deviation error bars.

Abbreviations to figures:

A	anus
E	eye
HG	hind gut
K	larval kidney
LDD	left digestive diverticulum
LH	larval heart
Mac	macromeres
Mic	micromeres
MMF	mantle muscle fibres
Mp	metapodium
O	operculum
Oe	oesophagus
Pp	propodium
RDD	right digestive diverticulum
RM	retractor muscle
S	stomach
St	statocyst
SV	subvelum
VC	velar cilia
VL	velar lobes

aperture indicating a cessation of shell growth. At day 16 the propodium had almost fully developed (Fig. 3.9M). The maximum shell size attained was 148.2 ± 8.5 μm (Fig. 3.10). At this stage in their development the veligers were transferred into finger bowls containing *B. plumosa* to see whether metamorphosis could be triggered. Unfortunately the quality of the available *B. plumosa* was extremely poor, and metamorphosis failed to occur.

3.4 *DISCUSSION*

Veliger culture was not successful through to metamorphosis in any of the species studied. The greatest success achieved was with *Thecacera pennigera* where competency was almost reached. Initially the culture of *Onchidoris bilamellata* was undertaken to develop and improve my techniques. However, as I had considerably more success using *O. bilamellata* than some of the other species attempted, my findings are included in this chapter.

3.4.1 *EMBRYONIC DEVELOPMENT*

O. bilamellata produces Type A spawn containing capsules that typically contained one to two embryos (Fig. 3.2), this corresponds to Hurst's (1967) observations. Towards the end of the egg mass the capsules were frequently observed empty, due to the lack of available eggs (Hurst, 1967). Embryonic development progressed at a similar rate to that documented by Todd (1979) when he reared *O. bilamellata* at 7°C. The embryonic development is similar to that described by Casteel (1904) and Thompson (1958) for *Fiona pinnata* and *Adalaria proxima* respectively (except for the later stages in *A. proxima* which differ due to its different development type). In this study only the major developmental processes were recorded. The first cell cleavage in *O. bilamellata* occurred in the first day of spawning, resulting in two cells of equal size. After a period of time they laid to rest alongside one another with their cell surfaces meeting over a large area (Fig. 3.2D). Two or three polar bodies were observed, which were located at the animal pole. The second cleavage occurred two days after oviposition, resulting in four cells of equal size. Gastrulation occurred five days after oviposition; at this point the embryo adopted the characteristic heart-shape. The formation of the blastopore occurs by epiboly (Casteel, 1904; Thompson, 1958). The blastopore formed the sagittal cleft (Fig. 3.2G); the pore closed and the embryo entered the trochopore stage. At this stage in development the shell gland formed as a small pit bordered by large cells, which then evaginated and spread out over the base of the embryo to secrete the shell. After nine days small cilia were observed at

the apical end of the embryo (at the head vesicle), this region later developed into the velar lobes (Fig. 3.2H). After 13 days the embryo had assumed the characteristic veliger shape and had entered the veliger stage. The rudimentary velar lobes were observed and the cilia had developed longer and stronger (Fig. 3.2I). At this point the veliger was observed to rotate within its capsule. During the veliger phase the main features begin to develop (although not identified in this study), including the formation of the stomatodæum which develops from the location of the blastopore, the continuation of secretion and shaping of the shell by the shell-gland spreading over the posterior end and then retracting, the development of the foot from the ventral prominence (which also supports the cells that secrete the operculum), and the differentiation of cells to form the internal organs (Casteel, 1904; Thompson, 1958). After 22-28 days at 6-9°C the veligers hatched. The hatching time closely correspond with Todd's (1979) culture of *O. bilamellata* at 7°C, in which the veligers hatched after 22 days.

Cuthona gymnota lays egg masses of Type D (Hurst, 1967). The veligers posses a Shell-type 2 which measures 246.5 ± 13.2 μm in length, and have a Development-type 1. On hatching they are slightly more advanced in their development than most planktotrophic veligers as they possess eye spots. A similar morphology was observed in *Cratena albocrusta* MacFarland by Hurst (1967) and possibly *Catriona ornata* (Baba) (Hamatani, 1960b). Some veligers with a Development-type 1 and a Shell-type 2 do not possess eyes, *Precuthona peachi* (Alder & Hancock) (Christensen, 1977), *Dendronotus iris* (Hurst, 1967), *Eubranchus olivaceus* (Hurst, 1967). However in most reported cases of Shell-type 2 veligers with a Development-type one, the presence or absence of eyes has not been recorded: *Eubranchus exiguus* (Alder & Hancock) (Hadfield, 1963), *F. pinnata* (Holleman, 1972), *Cuthona nana* (Harris *et al.*, 1975) and *Phestilla melanobranchia* (Harris, 1975).

Ancula gibbosa, *Palio nothus* and *T. pennigera* lay spawn masses of Type A (Hurst, 1967), which typically contain only one embryo per capsule. Embryological development of *A. gibbosa* followed a similar pattern to that described for *O. bilamellata* (see above). The spawn used within this study for *P. nothus* and *T. pennigera* was gathered from the field, termed "wild", therefore information on the embryonic period was not obtained. *A. gibbosa*, *P. nothus* and *T. pennigera* veligers possess a Shell-type 1 (Thompson, 1961a) and have a Development-type 1 (Thompson, 1967). They hatch with a shell length of 105.6 ± 2.5 μm, 126.3 ± 5.8 μm and 124.6 ± 11.1 μm respectively. Development-type 1 Shell-type 1 veligers

vary in size: 95-110 μm for *Doridella obscura* (Perron & Turner, 1977), 148 μm for *Rostanga pulchra* (Chia & Koss, 1978), 149 μm for *Melibe leonina* (Bickell & Kempf, 1983) etc. The hatching shell size of *O. bilamellata*, *A. gibbosa*, *P. nothus* and *T. pennigera* veligers was not unusual for planktotrophic species with a Shell-type 1.

The veligers of *P. nothus* hatched out after 11-14 days at 6-9°C. This embryological duration is considerably shorter than that of *O. bilamellata*, however *O. bilamellata* veligers were larger on hatching and this is probably attributable to the increased development time. Embryological duration is species dependant and can vary from 4 days for *D. obscura* at 25°C (Perron & Turner, 1977), 7.5 to 8 days for *D. steinbergae* at 12-15°C (Bickell & Chia, 1979), 10 days for *M. leonina* at 12-14°C (Bickell & Kempf, 1983), to 30.3 days for *A. proxima* at 10°C (Todd & Havenhand, 1985). The time taken for both *O. bilamellata* and *A. gibbosa* to hatch is not unusual for Development-type 1 species.

3.4.2 *FAILURE OF ONTOGENETIC DEVELOPMENT*

During this study only *Ancula gibbosa* veligers failed to exhibit larval development and shell growth. The veligers hatched with a mean shell length of 105.6 ± 2.5 μm and by day 13 had only increased to 122.5 ± 6.4 μm. The veligers seemed to display the effects of starvation (Kempf & Willows, 1977), except for the pigmentation of the digestive diverticulum. The lack of definitive shell growth or visceral development is probably due to an unsatisfactory algal diet. *Aeolidiella glauca* (Alder & Hancock) and *O. bilamellata* failed to exhibit shell growth when starved (Hadfield, 1963). Kempf & Willows (1977) had similar results with *Tritonia diomedea*. When not fed the veligers exhibited "negligible shell growth" but despite starvation those reared with antibiotics remained alive for 24 days, whereas those reared without antibiotics survived only ten days. The high incidence of deformity seen in this study could have been indicative of poor spawn quality, this might (even with a satisfactory diet) have affected veliger development. The larvae only survived until day 13. Due to the lack of shell growth no further attempts were made to rear *A. gibbosa* veligers.

3.4.3 *SPECIES EXHIBITING ONLY SHELL GROWTH*

Exactly the same culture techniques were used for *P. nothus* as for *O. bilamellata*. However, no ontogenetic development was observed during the 28 days in culture, except for an increase in shell length from 126.3 ± 5.8 μm to 176.3 ± 11.2 μm after 22 days, and then a decrease to 159 ± 7.1 μm. This reduction was probably due to the mortality of the

larger veligers in the culture, hence the survivors appeared to show a decrease in shell size. There are two possible reasons for the lack of development in *P. nothus*. Firstly, the phytoplankton diet may have been nutritionally inadequate for the veligers: even if they had survived longer than 28 days it is extremely unlikely they would have developed to competency. Shell growth with no visceral development has been recorded by several authors who attempted to rear veligers (Hadfield, 1963; Kempf & Willows, 1977; Perron & Turner, 1977; Todd, 1981). Kempf & Willows (1977) fed *T. diomedea* on monocultures of *Dunaliella*. The veligers grew from 144.6 to 165 μm over 8 days; after 28 days they measured approximately 172 μm (details obtained from an enlarged fig. 2 from Kempf & Willows (1977)), thereafter they (presumably) died. A similar situation developed when Hadfield (1963) attempted to rear *A. glauca* and *O. bilamellata* on moncultures of *Isochrysis*; in both attempts the veligers survived slightly longer than *T. diomedea*, 36 and 32 days respectively. Secondly, the larvae may have an extended planktonic phase when cultured at 6-9°C. Thus had larval mortality been lower, they may have survived long enough for development to have progressed. It seems unlikely that the first suggestion is the likely explanation for the complete lack of veliger development. The veligers were fed four different species of phytoplankton in equal quantities; all of these species whether fed in combination or as dicultures, have promoted veliger development and consequently metamorphosis in many opisthobranch species (Table 3.1). Todd (1981) and Chia & Koss (1988) had metamorphic success with *O. bilamellata* when fed *Rhodomonas*, *Isochrysis*, *Pavlova* and *Tetraselmis*; and *Dunaliella*, *Isochrysis*, *Pavlova*, *Rhodomonas* and *Phaeodactylum* respectively. Harrigan & Alkon (1978) reared *Hermissenda crassicornis* veligers to metamorphosis using *Isochrysis*, *Monochrysis* and *Chroococcus*. If the temperature of cultures had been increased, development would have almost certainly progressed faster and possibly further. Unfortunately the high rate of mortality combined with the long duration spent in the planktonic stage resulted in no larvae with which to continue the culture. The adults were not found again in the field therefore veliger culture could not be attempted at higher temperatures.

3.4.4 *SHELL-TYPE 2 DEVELOPMENT*

Cuthona gymnota spawn was gathered from *Tubularia* sp. collected from Mumbles pier. Only two spawn masses were collected, both yielded healthy veligers, and an attempt therefore was made to rear them. Data on their embryological development were not

obtained. *C. gymnota* was the only species reared in this study to possess a Shell-type 2 (Thompson, 1961a). On hatching the veligers have a mean shell length of 246.5 ± 13.2 μm, possessed eye spots and were morphologically similar to those of *C. albocrusta* described by Hurst (1967). The mantle fold was not present, therefore shell growth during the planktonic stage was not possible (Fig. 3.8D, E). Somatic growth during the veliger phase would have occupied the space available within the shell (Todd, 1981), had it occurred. The veligers have a Development-type 1 (Thompson, 1967), and fed readily after hatching. As eye spots were present at hatching, the only obvious indication of competency would have been the presence of a larval heart and an inflated propodium, although neither was observed during culture. The majority of Shell-type 2 opisthobranchs reared through metamorphosis also have a Development-type 2: *Akera bullata* (Müller) (this study), *Cuthona adyarensis* Rao (Rao, 1961), *C. nana* (Alder & Hancock) (Harris *et al.*, 1975; Rivest, 1978), *Eubranchus farrani* (A. & H.) (Todd, 1981), *E. doriae* (Trichese) (Tardy, 1962b), *Tenellia adspersa* (Nordmann) (Rasmussen, 1944; Eyster, 1979; Chester, 1996); and *Phestilla sibogae* Bergh (Bonar & Hadfield, 1974). To date the only nudibranch which has a Shell-type 2 and a Development-type 1 to have been taken through metamorphosis is *P. melanobranchia* (Harris, 1975). Other Shell-type 2 Development-type 1 veligers include: *Precuthona peachi* (Christensen, 1977), and possibly *C. albocrusta* and *D. iris*, from Hurst's (1967) descriptions, possibly *Catriona bicolor* (Bergh) and *C. ornata* (Baba) from Hamatani's (1960a; 1960b respectively) descriptions and *F. pinnata* from Holleman's (1972) description. The time these veligers spend in the plankton is unknown as they have never been reared with the intention of studying metamorphosis. *P. melanobranchia* veligers undergo metamorphosis eight days after hatching (Harris, 1975). After nine days in culture, *C. gymnota* showed no signs of development, despite the left digestive diverticulum becoming substantially distended (Fig. 3.8G). Due to the small initial number of veligers, and high larval mortality none of the veligers survived beyond nine days. With a greater number of hatched veligers development may have continued beyond 9 days, as feeding and behaviour appeared normal.

3.4.5 *VARIABLE SHELL LENGTH*

The veligers of *O. bilamellata* hatched with a mean shell length of 165.7 ± 9.5 μm. This corresponded to measurements recorded by Chia & Koss (1988) and Todd (1981), which measured 165 μm and 166 μm respectively. During culture the veligers grew to a shell

length of 260.9 ± 0.2 μm, which falls short of the size reached for metamorphosis recorded by Todd (1981) and Chia & Koss (1988). Todd (1981) documented a maximum shell length of 440 μm prior to metamorphosis, whereas Chia & Koss (1988) noted that the veligers grew to "about" 320 μm. This discrepancy is too large to be attributed to measurement inaccuracies. Perhaps the higher culture temperature used by Todd (1981) of 15°C, compared to the 4-11°C used by Chia & Koss (1988), promoted increased shell growth. Or, perhaps the algal diet Todd (1981) used was nutritionally superior to that used by Chia & Koss (1988), if that was the case their diet did not prevent the veligers in Chia & Koss' (1988) study reaching the required level of development for metamorphosis. Another possibility is that there could be an inherent genetic size difference between the population at Menai Bridge, North Wales, UK, and Argyll Lagoon, San Juan Island, Washington, USA. For aplysiids, Switzer-Dunlap & Hadfield (1977) stated that a species specific size must be reached in order for the veliger to proceed further in its development towards competency. If however, the measurements of Todd (1981) and Chia & Koss (1988) are correct it seems unlikely that Switzer-Dunlap & Hadfield's (1977) theory can be applied to *O. bilamellata*. In this study, eye spots developed at 26 days which corresponded to Todd's (1981) culture, although it would be interesting to know the size of the veligers, on attaining this stage in his study, to assess possible size differences between the different populations.

When rearing *T. pennigera*, after ten days at 20°C the veligers had developed eye spots, a larval heart, and the mantle fold had retracted away from the shell aperture indicating the cessation of shell growth. The mean shell length was 145.7 ± 13.3 μm, which was 31.5 μm smaller than when reared at 12-15°C. Genetic differences are unlikely to cause this difference in shell size as the two cohorts would have been genetically similar as a result of their long lived planktotrophic veliger stage (see Chapter 4). Four possible explanations may be provided: (1) the shell size which *T. pennigera* reaches prior to mantle retraction could be variable and not strictly adhered to as in the Anaspidea (see above). (2) The increased temperature at which one cohort was reared resulted in the precocious retraction of the mantle fold. (3) the hatching shell size may have been genetically different between the two populations; therefore, although the maximum shell length may have been different, the actual increase in shell length may have been the same. (4) The pattern of veliger development for those raised at 20°C may not have been normal, one indication of this being the reduced shell size. The reduced shell size may have also resulted in them not

being able to metamorphose. From observations made during the development of veligers up until day 16, it is impossible to conclude if at either temperature they would have ever reached competency. It is therefore not known if what was observed was the normal pattern of development. The veligers cultured at 20°C were placed in finger bowls with *Bugula plumosa* at day 16 to try to stimulate settlement. However, the condition of the bryozoan was poor, and the quality may not have been sufficient to induce metamorphosis. Precocious retraction of the mantle fold has been observed in *Doridella steinbergae* (Bickell & Chia, 1979). The veligers of this species have a shell length of 142 μm upon hatching and grew to a maximum of 168 μm. *T. pennigera* may have a similar pattern of development. However, until *T. pennigera* can be reared through metamorphosis and beyond, the developmental processes that have been observed in this study cannot be assumed as typical.

3.4.6 *LARVAL MORTALITY*

There was a high incidence of deformed veligers which hatched from the *A. gibbosa* spawn masses, the majority lacked shells (but retained their operculum), and continued to feed and swim as normal (Fig. 3.6H). In no other species studied was the incidence of deformity as high. There was also a high mortality associated with *A. gibbosa* veligers adhering to flocculated particles within the culture medium. A major problem encountered during veliger culture was poor sea water quality. Sea water held within the aquarium was pumped on a monthly basis (weather and construction work permitting) from Swansea Bay into storage tanks beneath the floor. The sea water was found to contain high concentrations of nitrates, nitrites, and phosphates (testing was carried out using a crude aquarium test). This resulted in the formation of large precipitates during autoclaving, as a result the water could not be used for phytoplankton culture. Instead sea water from White Oyster Ledge (west of Swansea Bay) was collected in 25 litre carboys aboard the R.V. *Noctiluca*, and was stored in a dark room at room temperature. This sea water still formed small crystals even when measures were applied to reduce precipitation on autoclaving. When this water was used in phytoplankton culture, the algal cells flocculated onto the crystals and sank. *Chaetoceros* and *Tetraselmis* were particularly prone to flocculation and settlement. Despite best efforts, a small minority of these crystals were transferred into the veliger culture. Mortality associated with flocculation tended only to affect the older

cultures when the larvae changed their swimming behaviour and spent more time towards the bottom of the vessels. However the *A. gibbosa* veligers were more prone to becoming caught in the precipitates than any other species reared in this study.

Spawn laid by *T. pennigera* within the laboratory appeared normal until the 32 cell stage after which the cells became abnormal; subsequent cell divisions produced cells of various sizes and the embryos failed to undergo normal gastrulation. Adult *T. pennigera* were particularly difficult to maintain within the laboratory. *B. plumosa* gathered from the field did not survive long after collection, and the adults would not feed on dead or dying *B. plumosa.* It is likely that that the spawn laid in the laboratory by *T. pennigera* was a stress response. At the start of the season (end of July), care was taken to ensure they did not warm up during transportation by transferring them into a vacuum flask whilst travelling to the laboratory. The adults were also particularly sensitive to sea water quality. Initially, they were kept in aquarium water, but as the quality declined, the adults would die within 3 days of introduction. Excessive mucus production was the first indication of stress, and similar to Marcus' (1957) description "they react to unfavourable conditions...by decreasing in size". The stressed adults would spend the majority of their time contracted, this was followed by a loss of colour, and finally death. As soon as the problem was identified sea water gathered from White Oyster Ledge was used.

3.5 *SUMMARY*

Metamorphosis was unsuccessful in all five of the nudibranch species reared in this study. *A. gibbosa* failed to exhibit shell growth and/or organ development after 13 days in culture. There was a high mortality as a result of the veligers becoming trapped in debris within the culture. Poor spawn quality, as indicated by the high number of deformed veligers, high mortality, and a poor algal diet were all implemented as possible reasons for the lack of development in *A. gibbosa.* After 28 days at 6-9°C *P. nothus* had exhibited substantial shell growth but had failed to exhibit visceral development. Poor algal diet, a low culture temperature, coupled with a prolonged planktonic stage were implemented as reasons for the failure in visceral development in *P. nothus. C. gymnota* hatches with a Shell-type 2 (Thompson, 1961a), and has a Development-type 1 (Thompson, 1967). Due to the small number of veligers hatching per egg mass, the initial number of veligers used in culture was small, this coupled with high mortality, resulted in a failure to culture the

veligers beyond 9 days. As for other Shell-type 2 veligers, no shell growth was recorded for *C. gymnota*. Despite this, the veligers acquired a highly distended left digestive diverticulum on feeding. It was concluded that the veligers have an extended planktonic stage unlike other Shell-type 2 veligers i.e. *P. melanobranchia* (Harris, 1975). Both *T. pennigera* and *O. bilamellata* exhibited shell and visceral development. After 26 days, when cultured at 6-9°C, eye spots had developed in *O. bilamellata*, and the shell had increased in length by 95.2 μm. Culture failed beyond this stage in development due to high mortality. When initially reared at 12-15°C, *T. pennigera* veligers reached a maximum shell size of 177.2 ± 8.5 μm. After 22 days they had developed eye spots. When the culture temperature was increased to 20°C, at 10 days the veligers had developed eyes, a larval heart, an inflated propodium, and the mantle fold had begun to retract. After 16 days in culture the veligers were tested to see if they were metamorphically competent; however, metamorphosis failed. Poor quality *B. plumosa* was attributed for the failure of metamorphosis. At the higher culture temperature the maximum shell length of *T. pennigera* after developing eye spots, was smaller than the shell size when reared at the lower culture temperature. Precocious retraction of the mantle fold, a variable maximum shell size, and abnormal veliger development were attributed to the differences in shell size at the different culture temperatures.

Chapter 4

Chapter 4

Population genetics of *Thecacera pennigera* (Montagu)

4.1 *INTRODUCTION*

There have been extensive studies on the phylogeny of the Opisthobranchia, many of which used cladistics (i.e. morphological and histological traits along with outgroup comparisons) to reconstruct phylogeny (Wollscheid & Wägele, 1999; Wägele & Willan, 2000; Klussmann-Kolb, 2003 etc). Morphological and histological traits are often incorporated with genetic analysis to clarify systematics (Thollesson, 1999, 2000; Wollscheid-Lengeling, Boore, Brown & Wägele, 2001; Valdés, 2002; Grande, Templado, Cervera & Zardoya, 2004; Pola, Cervera & Gosliner, 2005; Vonnemann, Schrödl, Klussmann-Kolb & Wägele, 2005). Despite this, only a few authors have investigated the population genetics of the Nudibranchia (Todd, Havenhand & Thorpe, 1988; Todd, Lambert & Thorpe, 1998; Lambert, Todd & Thorpe, 2003).

The recruitment of pelagic larvae into the benthic stage is of paramount importance for the continuation of a population in many marine invertebrates. The duration of the planktonic larval phase (and consequently larval dispersal) is likely to have a significant influence on gene flow within and between populations; this will ultimately govern the genetic differentiation expressed within a population (review by Todd (1998)). There are however, other factors; e.g. oceanographic barriers, which are known to influence larval dispersal. Populations of *Mytilus galloprovincialis* Lmk located around the Atlantic and Mediterranean exhibit two distinct clades, these were attributed to dispersal events occurring in the Pleistocene and to more recent contact between the populations with limited gene flow (Quesada, Beynon & Skibinski, 1995). The distribution of *Brachidontes exustus* around the Florida peninsula exhibits two distinct clades located on the east and west coast (Lee & Ó Foighil, 2004). Lee & Ó Foighil (2004) attributed this separation to an event in the Pliocene. The extent of the differentiation reaches a climax in the Florida populations, in which they identified four cryptic species of *B. exustus*.

An extended planktonic stage will inevitably result in increased larval mortality and an increased likelihood of dispersal away from possible suitable habitats. These factors reduce larval density, and hence lower the probability of the larvae settling in uncolonised habitats in numbers sufficient to ensure recruitment for the following generation (Johannesson, 1988). Fluctuations in fecundity within the parent population will also affect the numbers of recruiting larvae (Scheltema, 1971). Johannesson (1988) hypothesized that there is a maximum distance from a parent population that planktonic larvae can settle in order to establish a population. Further away from the parent population, the larvae settle, but in too few numbers (due to post-settlement mortality) to establish a population.

Genetic information gathered from populations provides a greater understanding of larval dispersal, vicariance events, gene flow, and the importance of recruitment in regulating nudibranch population dynamics. Despite this, the extent of larval dispersal and the impact on gene flow between nudibranch populations is only now being investigated.

4.1.1 *LIFE HISTORY OF THE OPISTHOBRANCHIA*

The majority of the Nudibranchia are annual semelparous species. The veliger metamorphoses into the benthic "slug" form, and the resulting juvenile undergoes a period of rapid growth until mature. Hermaphroditic adults copulate reciprocally with other individuals, produce several spawn masses, and die. Benthic populations of mature adults are only present for a few months of the year. The majority of these species have teleplanic or actaeplanic larvae e.g. *Onchidoris bilamellata* (Todd, 1981); the longer the larval phase the greater risk of predation, and the greater the likelihood of becoming transported via currents away from suitable habitats. Typically the adults are specialist feeders; hence their life cycle is closely associated with that of their prey (Todd & Doyle, 1981). It has been hypothesized that this dependency of many species on one or two particular prey items and the seasonality of their prey compel many to produce teleplanic larvae (Todd & Doyle, 1981). This has however, proved controversial (Grant & Williamson, 1985; Todd, 1985). Nudibranchs with Development-types 1 or 2 (Thompson, 1967) possess a planktonic veliger stage which can spend a minimum of several hours (pelagic lecithotrophs) for *Adalaria proxima* (Alder & Hancock) (Kempf & Todd, 1989), to several weeks or even months (pelagic planktotrophs) for *Onchidoris bilamellata* (L.) and *O. muricata* (Müller) (Todd, 1981; Todd & Havenhand, 1985 respectively) within the plankton. There have been several suggested reasons as to why many species produce planktonic larvae (Havenhand, 1995).

Experiments performed by Kempf (1981) demonstrated the potential for teleplanic dispersal within *Aplysia juliana* Quoy & Gaimard veligers. They were maintained in culture for 316 days, during which time they maintained the same tissue and shell mass. When tested for metamorphic competence, the percentage of those successfully undergoing metamorphosis varied from 0-60%; this was found to be independent of larval age and the failures were instead attributed to inadequate culture conditions (Kempf, 1981). Pechenik (1990) commented on the validity of Kempf's (1981) study. She suggested that the veligers which apparently had the ability to delay metamorphosis may have taken longer to reach competency, hence the length of time they are able to delay metamorphosis may not be as long as Kempf (1981) had originally concluded. Inferences can be made regarding the extent of larval dispersal by investigating the population genetics within a species; what has been typically assumed is the inevitability of teleplanic larvae to undergo extensive dispersal resulting in genetic homogeneity of populations, and non-pelagic "direct developing" larvae to give rise to heterogeneous populations; however this is not always the case (Palumbi, 1995).

After metamorphosis, adult nudibranchs have very limited dispersal capabilities (Todd *et al.*, 1998). Exceptions to this include a few pelagic species e.g. *Glaucus atlanticus*, and the potential for the adults to undergo "rafting", whereby adults and/or spawn are transported from one population to successfully colonise and interbreed with another population. In order for "rafting" to occur, adults (and/or spawn) would have to be dislodged (with or without their substratum), carried via tidal or wind influences to a suitable habitat with the required prey, copulate with others (if not having done so already), spawn and have successful recruitment, all within a very short period of time. As well as these factors, there would have to be significant numbers of veligers spawned from the rafted adults to overcome the pelagic phase and post-settlement mortality (Johannesson, 1988). The main advantage of "rafting" is that there is no limit on the dispersal distance (Johannesson, 1988). However, due to the short adult life span of most nudibranchs, Johannesson's (1988) rule can not be applied to these species.

In many species of nudibranchs there is no generation overlap; senescence occurs uniformly over the whole population and recruitment is limited to one cohort per year. This allows for accurate results to be derived from recruitment investigations, as current

populations are a direct consequence of successful recruitment from the previous generation.

4.1.2 *POPULATION STUDIES OF* ADALARIA PROXIMA *AND* GONIODORIS NODOSA

The Nudibranchiate mollusc *Adalaria proxima* produces planktonic lecithotrophic veligers. *Goniodoris nodosa* (Montagu) produces planktonic planktotrophic veligers. The latter spends an obligatory 10-13 weeks in the plankton before metamorphosing (Todd *et al.*, 1998), whereas the former can metamorphose within 1-2 days (Thompson, 1958; Lambert & Todd, 1994). *A. proxima* has been observed to metamorphose several hours after hatching (Kempf & Todd, 1989), but can also delay metamorphosis for 15-20 days in the absence of a metamorphic cue, irrespective of whether they are fed or starved (Kempf & Todd, 1989). Both species are ecologically similar. Their life cycles are closely associated with their bryozoan prey: *Alcyonidium* spp. and *Electra pilosa* (L.) respectively. The veligers only metamorphose in response to molecular compounds released by the Bryozoa (Thompson, 1958; Kempf & Todd, 1989; Lambert & Todd, 1994; Todd *et al.*, 1998).

Todd *et al.* (1988) studied four populations of *A. proxima* from western Scotland. Three populations had potentially direct tidal contact through tidal currents, and spanned 6 km, the fourth population was located further north within a semi-enclosed sea loch. They analyzed allozyme frequencies within the individual populations in order to determine the degree of temporal and spatial genetic variation. Contrary to expectations, they discovered different allozyme frequencies in all four populations; the variations were stable over three consecutive generations. Todd *et al.* (1988) attributed the high genetic diversity to the localized recruitment of the veligers; settlement must occur soon after hatching in order to prevent the dispersal of larvae away from the parent population. Hence populations of *A. proxima* are self-recruiting. This result was further supported in later studies (Todd *et al.*, 1998; Lambert *et al.*, 2003).

Todd *et al.* (1998) compared the genetic differentiation between populations of *G. nodosa* and *A. proxima.* These two species have a similar niche, but have contrasting larval strategies (see above). *G. nodosa* exhibited allozyme similarities over a scale of 1600 km; hence the populations show evidence of high degrees of connectedness. Todd *et al.* (1998) concluded that the recruitment of larvae came from within the same gene pool over the whole area sampled. The genetic similarity exhibited between the populations of *G. nodosa* was attributed to extensive larval dispersal over substantial distances. Initial investigations

on colour morphology in *A. proxima* sampled from 22 populations located between Menai Bridge (north Wales), around Scotland to Beck Scar (north-east England), indicated a "broad east-west differentation", suggesting population homogeneity over 10^2-10^3 km (Todd *et al.*, 1998). The yellow morph dominated the west coast (from Menai Bridge to Oldany Island, Scotland), whereas the white morph dominated the east and northern Scottish coasts with some intermingling at Scapa Flow, Scotland (Todd *et al.*, 1998). Allozyme data supported this finding by showing populations of *A. proxima* to be heterogenous, exhibiting high levels of genetic differentiation (differences in allele frequency) over even smaller spatial scales. Todd *et al.* (1998) found significant differentiation over 100 km, and evidence of differentiation over 10^2 m, this was reduced to < 1 km in an area dominated by fast tidal flow. These populations were described as demographically "closed" (Lambert *et al.*, 2003). Genetic differentiation was attributed to bottlenecks, random genetic drift, and the extinction of populations which have minimal larval influx from elsewhere (Todd *et al.*, 1998). Populations of *A. proxima* inhabiting exposed shores exhibited reduced genetic differentiation; conversely, in areas of low wave energy genetic variation was greater, and recruitment between populations 10^2-10^3 m apart was considered negligible (Lambert *et al.*, 2003). Thus the populations of *A. proxima* studied by Todd *et al.* (1998) and Lambert *et al.* (2003), exhibited high degrees of genetic differentiation over small spatial scales, which were "more or less stable" over consecutive generations. Populations sampled over 26 km conformed to the isolation by distance pattern; however those populations sampled over 1600 km did not (Todd *et al.*, 1998). Despite the ability of *A. proxima* to delay metamorphosis for between 15-20 days (Kempf & Todd, 1989), the majority of larvae appeared to settle quickly (some larvae were able to metamorphose within 24 hours of release), and within the vicinity of the parent population (Todd *et al.*, 1998; Lambert *et al.*, 2003). However some larvae were incompetent to metamorphose for several days post-hatching (Todd, Bentley & Havenhand, 1991); these larvae may play an important role in the colonization of suitable habitats elsewhere. The increased differentiation over small spatial scales, despite *A. proxima* having a free swimming planktonic stage, was attributed to a behavioural constraint which prevented the larvae from becoming pelagic (Todd *et al.*, 1998; Lambert *et al.*, 2003).

Todd *et al.* (1998) addressed the possibility of recruitment by "rafting" in *A. proxima.* On sheltered coasts rafting was considered likely as nudibranchs were found on deracinated

Fucus. Despite this, Todd *et al.* (1998) discovered little genetic differentiation between those found on the deracinated *Fucus* to those found within the vicinity. They concluded that the apparently "rafted" individuals had moved onto the *Fucus* after it had stranded. Todd *et al.* {, 1998 #169} failed to address the possibility, that on sheltered shores, the deracinated *Fucus* is carried out on the tide but is then washed back up onto the shore. This may lead to gene flow between otherwise isolated populations. On exposed coasts, *A. proxima* were concentrated beneath rocks thereby reducing the likelihood of becoming dislodged (Todd *et al.*, 1998).

Lambert *et al.* (2003) transplanted both *G. nodosa* and *A. proxima* from one population to a recipient population exhibiting different allelic frequencies. They hypothesized that if the recipient population exhibited a shift in allele frequencies towards the donar population, genetic drift is occurring. However, if the transplanted animals exhibited a shift in allele frequencies towards the recipient population, selection is occurring. Subsequent generations recruited from the transplanted *G. nodosa* site exhibited no shift in allele frequencies; Lambert *et al.* (2003) concluded, similarly to Todd *et al.* (1998), that the genetic composition is homogenous spatially and temporally over 1600 km. The long pelagic phase of *G. nodosa* results in extensive larval dispersal and significant gene flow between populations over consecutive generations. The recruitment of individuals comes from the same gene pool which homogenizes genetic variation (Lambert *et al.*, 2003), this increases the effective population size. Of the three transplant experiments with *A. proxima*, only one exhibited inexplicable variation. In this instance the expected allele frequencies from the transplanted animals failed to become expressed in subsequent generations. Contrary to expectations, some allele frequencies expected to increase, decreased (Lambert *et al.*, 2003). However, in the other two transplant experiments, the recipient populations exhibited increases in allele frequencies in alleles that were fixed in the transplanted animals. There was also the elimination of one polymorphism in a recipient population as the transplanted animals were fixed with a different allele at its corresponding locus (Lambert *et al.*, 2003).

Further investigations of colour morphs by Lambert *et al.* (2003) supported the limited dispersal theory of Todd *et al.* (1988) and Todd *et al.* (1998). Yellow morphs from Oldany Island were transferred to the exclusively white morph population at Kylesku Bridge (north west Scotland). Following transplantation, six yellow morphs were recruited at Kylesku Bridge the following season and nine the year after. Lambert *et al.* (2003)

discovered a direct association between the yellow phenotype and the *Fum* allele 1. The yellow morphs at Kylesku Bridge exhibited the *Fum* allele 1, further indicating that *A. proxima* populations have reduced larval dispersal and localized recruitment (Lambert *et al.*, 2003).

The ecological implications of reduced larval dispersal, and hence restricted gene flow, within populations of *A. proxima* were investigated by Lambert *et al.* (2000) and Jones *et al.* (1996). Growth rate and reproductive allocation between six populations exhibiting distinct allele frequencies were found to be different (Lambert *et al.*, 2000). Each population exhibited a specific size, and each had a specific zygote size that was consistent between generations (Lambert *et al.*, 2000). They concluded that both characteristics had a "strong genetic component". Jones *et al.* (1996) studied the embryonic and larval traits in six populations of *A. proxima.* The mean egg size of the first spawned mass for each population showed significant differences between populations; however there was no significant difference in individual mean egg size between populations. There were also significant differences in the number of embryos within a spawn mass, and the mean larval shell length between populations (Jones *et al.*, 1996). These differences were attributed to the high degree of genetic heterogeneity displayed between the *A. proxima* populations (Jones *et al.*, 1996).

Overall, the distribution of populations of *A. proxima*, though attributable to the distribution of *Electra pilosa*, was described to be "small, spatially discrete, and persistent" (Lambert *et al.*, 2000). The genetic differentiation exhibited over consecutive generations within a population was found to be stable. Between populations, the genetic differentiation was highly variable indicating the significance of localized recruitment; changes were unpredictable and attributed to genetic drift (Todd *et al.*, 1988; Todd *et al.*, 1998; Lambert *et al.*, 2003). Genetic differentiation was observed on scales as small as <1 km in areas of fast tidal flow (Todd *et al.*, 1998). Restricted larval dispersal resulted in genetically discrete populations with their own characteristic ecological and embryological traits (Jones *et al.*, 1996; Lambert *et al.*, 2000). Contrary to *A. proxima*, *G. nodosa* exhibited low levels of genetic differentiation over 1600 km (Todd *et al.*, 1998). The duration spent within the plankton is sufficient to result in extensive dispersal, thereby increasing the effective population size.

4.1.3 THECACERA PENNIGERA

Thecacera pennigera (Montagu) is a member of the Family Polyceridae. It is a strict semelparous species; there is no generation overlap (personal observation). In the three populations sampled in this study there was only one cohort per year, the timing of which is linked to the growth of the adult prey, *Bugula plumosa* (Pallas). The adult duration is short; they occur from the end of July to September. Previous studies (see Chapter 3) have shown that when reared at 20°C the obligate planktotrophic veliger stage is at least 16 days in duration. Other species of nudibranchs which have planktotrophic larvae have veliger stages which can last 8 days in *Phestilla melanobranchia* Bergh (Harris, 1975), to 58-59 days for *O. muricata* (Müller) at 10°C (Todd & Havenhand, 1985). The larval duration of *T. pennigera* is likely to be longer than 16 days when in its natural environment due to lower water temperatures. Todd (1981) reared *Onchidoris bilamellata* (L.) in approximately 32 days, however he estimated that at an average ambient temperature of 6°C veliger development could take up to 69 days. The obligatory time *T. pennigera* spends within the plankton will be considerably longer than that of the lecithotrophic *A. proxima*, and more comparable to that of the planktotrophic *G. nodosa* which requires 10-13 weeks at ambient temperature (Todd *et al.*, 1998).

Discrete populations of *T. pennigera* along the south Wales coastline at Porthcawl, Mumbles (Swansea) and Pembroke Dock were sampled. They span the coastline over a distance of 172 km. The tidal flow is governed by the passage of water into and out of the Bristol Channel. The Gower peninsula deflects water during the ebb around Pembrokeshire and into the Irish Sea away from the fast moving current passing Ilfracombe (Fig. 4.1A). During the flood tide, the water is channeled between the Gower peninsula and Ilfracombe (Fig. 4.1C). The maximum water speed during the ebb spring tide past Porthcawl and Swansea reaches 1.5 knots and past Milford Haven 2.2 knots, during neaps the maximum tidal flow is 0.7 knots and 1.0 knot respectively (Featherstone & Lambie, 2005). The maximum water speed during the flood spring tide at Porthcawl and Swansea reaches 1.3 knots and past Milford Haven 2.5 knots, during neaps the maximum tidal flow is 0.6 knots and 1.2 knots respectively (Featherstone & Lambie, 2005). Due to the flushing of water through the Bristol Channel there is a high degree of mixing, the current speeds are sufficiently strong enough to transport larvae between the three populations, and most probably to populations further afield.

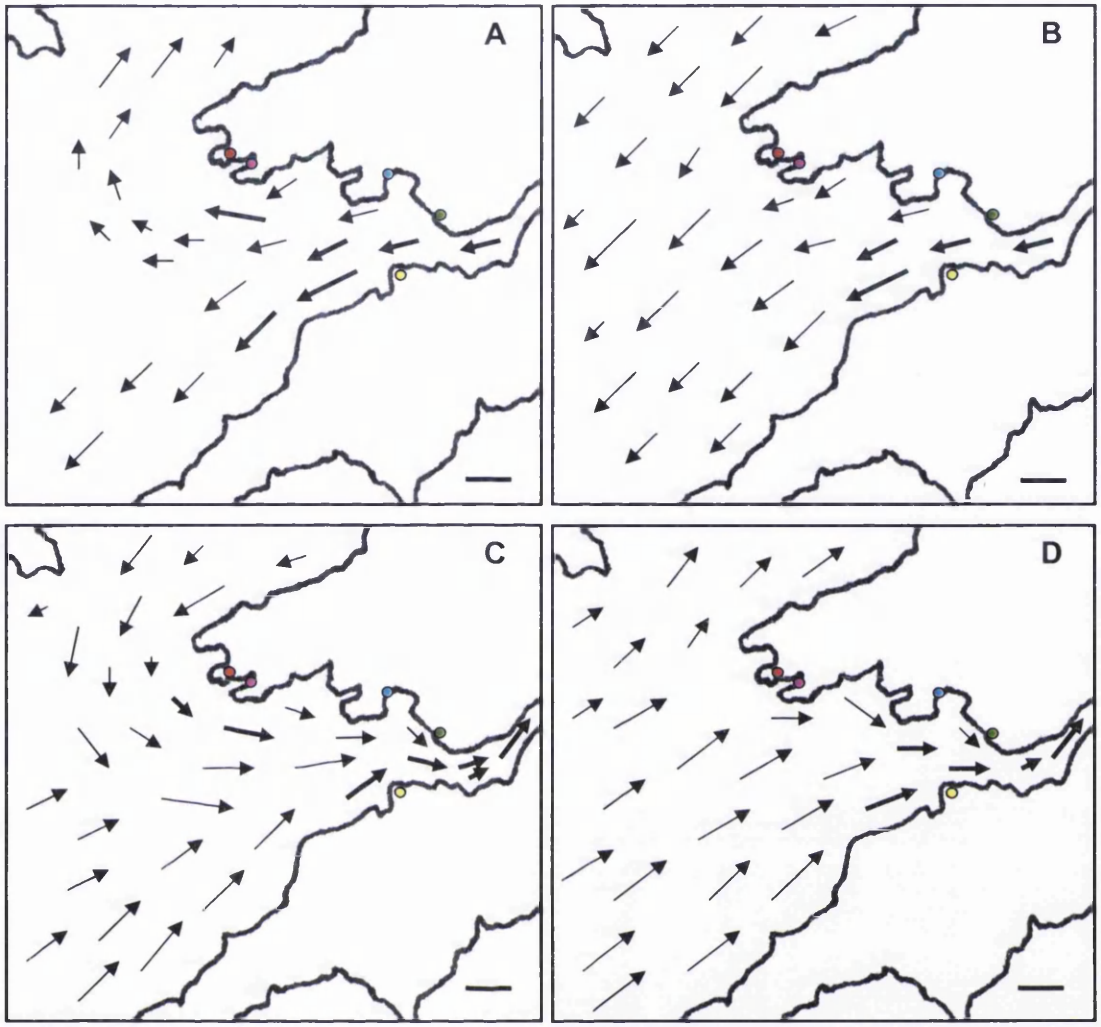

Figure 4.1. Water flow at different stages of the tidal cycle around south west Wales and southern England. The thicker arrows indicate the strongest currents. Red dot shows the location of Milford Haven; pink, Pembroke Dock; blue, Mumbles; green, Porthcawl; and yellow, Ilfracombe. **A.** The direction of the water currents 3 hours after high water at Milford Haven. **B.** 5 hours after high water at Milford Haven. **C.** Approximately 4.5 hours before high water at Milford Haven. **D.** Approximately 1.5 hours before high water at Milford Haven. Modified from Anonymous (2005). Scale bars: **A-D** = 20 km.

4.1.4 *AIMS OF STUDY*

In order to investigate larval dispersal between populations of *T. pennigera* around the south Wales coastline mtDNA analysis was performed with the intention of developing mtDNA techniques for future studies using *T. pennigera.* The original intention was to sample three Welsh and a foreign population at Woods Hole, Massachusetts. However, the latter population did not appear in 2003 or 2004 (A. Shepard, personal communication). Another recorded population of *T. pennigera* from Europe, at Oosterschelde in the Netherlands, was common until 2001, since then the population has been absent (P. H. van Bragt, personal communication). Hence, only the three populations from Porthcawl, Mumbles and Pembroke Dock were sampled and analyzed.

4.2 *MATERIALS AND METHODS*

Three *Thecacera pennigera* populations were sampled: Mumbles (003°58'4"W 051°34'2"N) in August 2002, Pembroke Dock (004°58'00"W 051°41'00"N) in September 2003 and Porthcawl (003°42'02"W 051°28'06"N) in July 2004. The animals were transported back to the University within a vacuum flask. They were kept for five days in small plastic tanks (30×20×8 cm) containing WOL sea water (see Chapter 3 for details regarding the types of sea water used); an inverted perforated plastic Pasteur pipette was modified to function as an air stone. During this period, they were starved to clear the digestive gland, thus preventing DNA contamination from *Bugula.* The animals were preserved directly in 100% ethanol.

4.2.1 *DNA EXTRACTIONS*

The following DNA extraction and PCR amplification protocol used was modified from Thollesson (2000):

The posterior region of the slug was used in all extractions to avoid DNA contamination by allosperm contained within the receptaculum seminis. The tissue was initially washed in TRIS-HCl (pH 8) to remove excess ethanol, then homogenised with a sterile micropestle in pre-heated 60°C CTAB buffer (2% CTAB, 1.4M NaCl, 20mM EDTA, 100mM TRIS-HCl pH 8, 0.2% β-mercaptoethanol) with 100μg ml^{-1} Proteinase-K. After one hour incubation at 60°C, the proteins were removed by adding an equal volume of chloroform-isoamyl alcohol (24:1). The solution was then centrifuged at 7700 × g for 10 minutes; the aqueous phase was retained and the process repeated. Approximately 200 μl of

100% iso-propanol (⅔ the original volume) was added and mixed by gentle inversion. The solution was left over night at room temperature to allow the nucleic acids to precipitate. The solution was centrifuged at 7700 × g for 10 minutes, the supernatant was discarded, and the remaining DNA pellet was washed using 300 µl of 76% ethanol in 10 mM ammonium acetate. The pellet was agitated gently for 30 minutes. The supernatant was discarded, and the pellet was allowed to air dry for approximately 15 minutes (depending on the degree of residue left within the microcentrifuge tube). The DNA was hydrated in 50 µl of nuclease free H_2O and placed within a water bath at 65°C for one hour.

The DNA was quantified using a Beckman-Coulter spectrophotometer, and was diluted to a 60 ng template using nuclease free H_2O.

4.2.2 *POLYMERASE CHAIN REACTION (PCR) AND SEQUENCING*

PCR was performed in a 25µl final volume using a 60 ng template of DNA. Promega™ *Taq* DNA polymerase in Storage Buffer A (catalogue number M1861) containing 500 mM KCl and 100 mM TRIS-HCl was diluted in the final volume to 50 mM and 10 mM respectively; to this, 2.5 mM NaCl, 100 µM of each dNTP, 0.3 µM of each primer and 2 units of *Taq* polymerase were added.

A BioRad™ i-cycler was used with the following program: initial denaturation for 2 minutes at 94°C, followed by 40 cycles of 30 seconds at 94°C, then 30 seconds at 50°C, and 1 minute at 72°C, with an extension of 7 minutes at 72°C, and final holding at 4°C.

Initially the COI gene was amplified using the universal COI primers LCO1490 [5'-ggtcaacaaatcataaagatattgg-3'] and HCO2198 [5'-taaacttcagggtgaccaaaaaatca-3'] (Sigma-Genosys) (Folmer, Black, Hoeh, Lutz & Vrijenhoek, 1994). Three months into the analysis the PCR failed, as a result primers were designed from Thollesson's (2000) *T. pennigera* COI sequence using the internet-based program Primer3, and were used from that time forward. They were checked for hairpins, G/C content, primer dimer formation and melting point using the internet-based program NetPrimer. The resulting primers read: forward [5'-ttgggatatggtgtggtttagt-3'] and reverse [5'-ccccagctaatacaggtaatga-3'] (Sigma-Genosys).

The PCR product was run on a 1% agarose gel, made with a 1% TBE solution and 6.3% ethidium bromide. Each PCR product was run against a 2-log DNA ladder (0.1-10.0 kb) (New England BioLabs™, catalogue number N3200S). The gels were run at 100V for approximately 90 minutes. The bands were viewed under a BioRad™ transilluminator and photographed. Prior to sequencing the PCR product was cleaned using a Qiagen™

QIAquick PCR purification kit (catalogue number 28104) as per manufacturer instructions. It was quantified using a Beckman-Coulter™ spectrophotometer.

Initially a Beckman-Coulter CEQ™ 8000 was used to sequence the PCR product. The required DNA sequencing reaction was prepared as per manufacturer instructions using CEQ 2000 dye terminator cycle sequencing with quick start kit (Beckman Coulter). The universal COI primers were initially used for sequencing; however these were then changed to the designed primers when the universals failed. The PCR product varied in length from 549 base pairs (using the designed primers) to 700 base pairs (using the universal primers). Towards the end of the study the sequencing was performed by The Sequencing Service (School of Life Sciences, University of Dundee, Scotland) using Applied Biosystems Big-Dye Version 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer.

4.2.3 *ANALYSIS*

The sequences were loaded into the NCBI web-site to ensure they corresponded to Thollesson's (2000) COI sequence for *T. pennigera*. The sequences were then translated into an amino acid sequence using the internet program EMBOS and re-loaded into the NCBI web-site to determine the reading frame. Each forward and reverse sequence was individually aligned by eye. Consequent alignment and editing of populations was performed using the program ClustalX (Thompson, Gibson, Plewniak, Jeanmougin & Higgins, 1997). The sequences from the three different populations were separated into haplotypes and their frequencies statistically tested using Chi-squared (Nass, 1959). Phylogenetic tree construction was performed on MEGA version 3 (Kumar, Tamura & Nei, 2004), using the bootstrap neighbour joining (NJ) and the bootstrap maximum parsimony (MP) methods. The COI sequences of members of the Polyceridae: *Polycera quadrilineata* (Müller), *P. aurantiomarginala*, *Polycerella emertoni*, *Limacia clavigera* (Müller), and *Palio dubia* (M. Sars), were used as outgroups (all sequences obtained from NCBI). An AMOVA (analysis of molecular variance) was performed using Arlequin version 2.001 (Schneider, Roessli & Excoffier, 2001) (Appendix pg. 235).

4.3 *RESULTS*

Initially PCR (Fig. 4.2) and sequencing were successful using the universal COI primers. However, PCR failed approximately three months into the experiments (Fig. 4.3).

An alternative DNA extraction method was performed following Sokolov (2000), however this methodology failed (Fig. 4.4). Primers were then designed from Thollesson's COI sequence for *Thecacera pennigera* which worked considerably better than the universal primers (Fig. 4.4).

In total, 30, 17, and 21 sequences were used in the analysis from Porthcawl, Mumbles, and Pembroke Dock populations respectively. Extracting good quality DNA from samples over twelve months old was difficult despite preservation in ethanol. Consequently PCR failed in many of the Mumbles specimens; hence the smaller number of sequences for Mumbles than for Porthcawl and Pembroke Dock. Each sequence was edited to a length of 303 base pairs. 16 different haplotypes were identified from the three populations. No significant differences were observed in the haplotype frequencies between the populations (Chi-squared, χ^2 = 148.957, df = 129.312, $P>0.05$), and between the frequency and variation in sequences of the 16 haplotypes observed within and between the three populations (AMOVA, $P>0.05$) (Table 4.1). The mean number of pairwise differences (nucleotide diversity) for the 16 haplotypes was very low (π = 0.0096). Thus it can be concluded that there is no significant difference in the haplotype frequencies and in haplotype sequences within and between the three populations. All three populations show high degrees of genetic similarity (homogeneity) and connectedness over a distance of 107 miles.

The maximum parsimony (Fig. 4.5) and neighbour joining (Fig. 4.6) phylogenetic trees both showed high degrees of consensus. Despite the majority of branches having low bootstrap values, good support exists amongst two clades which join haplotypes 9 & 10, and 16 & 14, with bootstrap values of 81 and 66 respectively for MP, and 86 and 68 respectively for NJ. Haplotypes 9 and 10 were only observed within the Pembroke Dock population, and haplotypes 14 and 16 were only expressed within the Pembroke Dock and Mumbles population respectively. The strongest support was between the grouping of Thollesson's (2000) Spanish *T. pennigera* sequence (bootstrap value 100) and the different haplotypes obtained from the three Welsh populations.

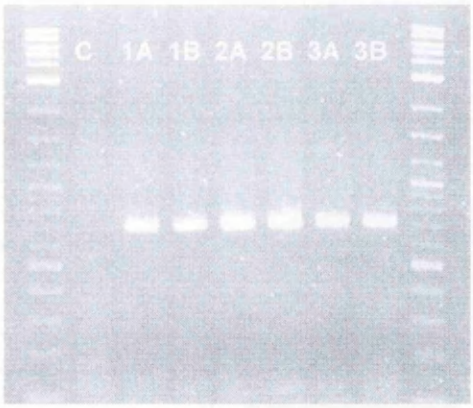

Figure 4.2. A 1% agarose gel showing three PCR products (two replicates of each and a control) using the universal COI primers. The product is approximately 700 bp.

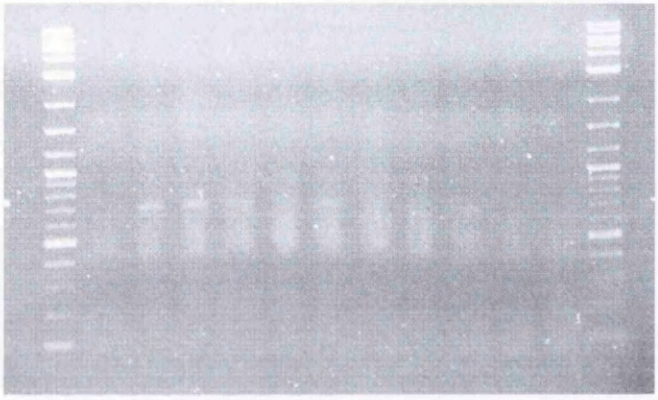

Figure 4.3. A failed PCR using the universal primers.

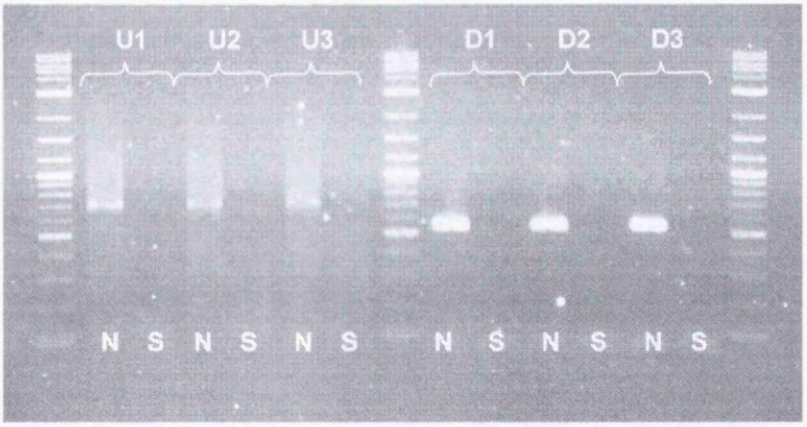

Figure 4.4. A 1% agarose gel showing three PCR products (1-3) extracted using the normal CTAB buffer (N) and a protocol by Sokolov (2000)(S), using universal primers (U) and the designed primers (D).

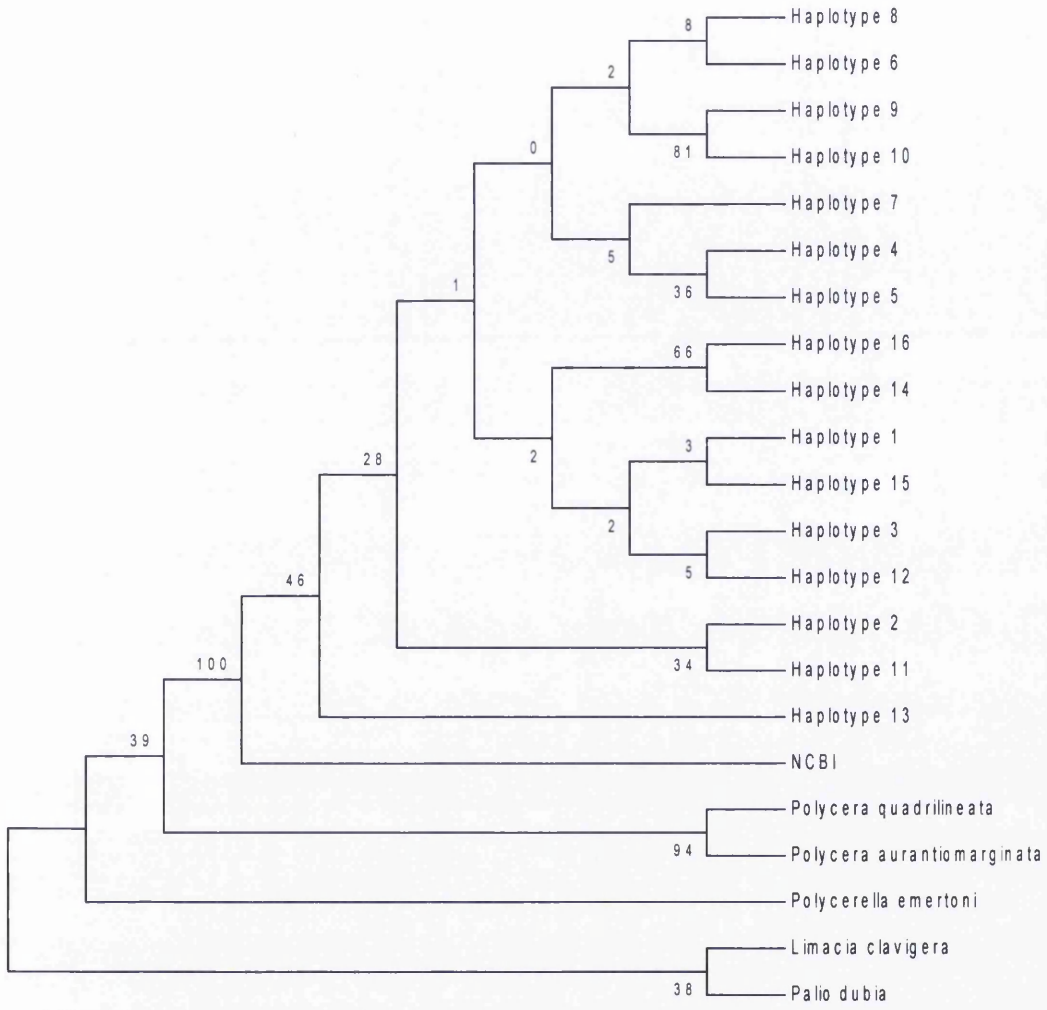

Figure 4.5. A maximum parsimony constructed tree of the 16 haplotypes from the three Welsh populations showing bootstrap values from 250 replicates. Included are Thollesson's (2000) *Thecacera pennigera* sequence (NCBI) and five other species of Polycerid (NCBI) as outgroups.

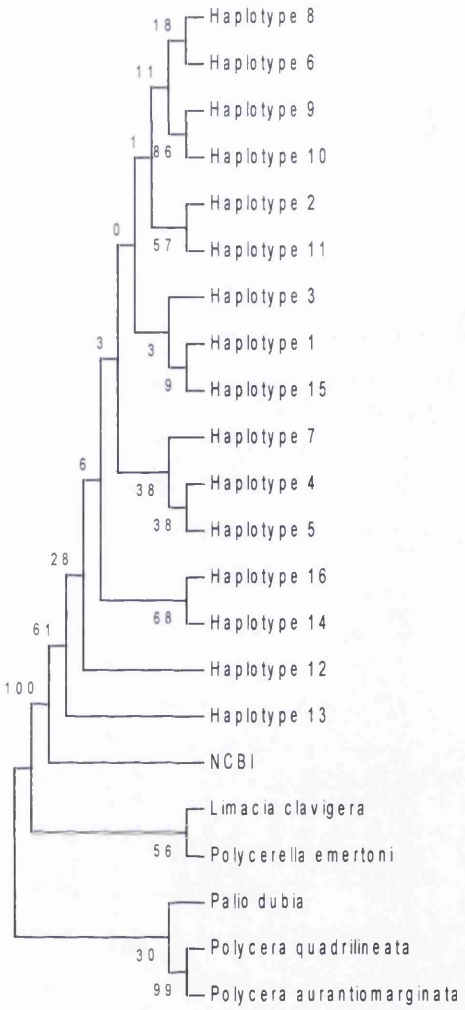

Figure 4.6. A neighbour joining constructed tree of the 16 haplotypes from the three Welsh populations showing bootstrap values from 250 replicates. Included are Thollesson's (2000) *Thecacera pennigera* sequence (NCBI) and five other species of Polycerid (NCBI) as outgroups.

SOURCE OF VARIATION	DF	SUM OF SQUARES	VARIANCE COMPONENTS	% VARIATION
AMONG POPULATIONS	2	1.817	0.01552	2.66
WITHIN POPULATIONS	65	36.845	0.56684	97.34
TOTAL	67	38.622	0.58236	
F_{ST}	0.02665			
P	0.11535 ± 0.00722			

Table 4.1. The AMOVA results showing the source and percentage variation between the three Welsh populations of *Thecacera pennigera*.

4.4 *DISCUSSION*

There are several advantages in using mtDNA techniques rather than allozymes to study population genetics. The main advantage is that mtDNA is maternally inherited and therefore is more likely to show genetic drift and splits within clades e.g. Quesada *et al.* (1995); hence the effective population size (N_e) is smaller in comparison to allozyme studies. Another advantage is the ability to trace back individuals which may be of particular interest e.g. if they have an unusual DNA sequence.

Despite the presence of 16 different haplotypes found over the three populations, the Chi-squared and AMOVA tests showed that there was no significant genetic differentiation within or between the populations. None of the mtDNA sequences obtained from the three populations differed in protein sequence after translation. All base changes were synonymous. However, all 16 Welsh haplotypes exhibited one non-synonymous substitution when compared to Thollesson's (2000) *T. pennigera* sequence. In Thollesson's (2000) sequence one valine was coded instead of leucine. These amino acids are chemically similar, leucine has an extra $-CH_2$ group. This substitution is unlikely to have significantly affected the function of the COI gene. The phylogenetic analysis showed a close association between haplotypes 9 & 10, and 14 & 16. Both pairs were similar in base composition. Pairs 9 & 10, differed from haplotype 1 by the three same synonymous substitutions; however, haplotype 9 had one extra synonymous substitution. A similar situation arose with haplotypes 14 & 16, both had the same two synonymous substitutions from haplotype 1; however haplotype 14 had one other synonymous substitution. Both haplotypes 9 & 10 were exhibited within Pembroke Dock only. It is possible that the one individual which contained haplotype 9 and the three individuals which contained haplotype 10 had a common ancestor; hence they could have been self-recruited from the previous generation. Due to the semi-enclosed area in which Pembroke Dock is situated (within the Cleddau estuary), larval dispersal may have been restricted. However this is speculation, and would require further investigation. Haplotypes 14 & 16 were contained within one individual from Pembroke Dock and one individual from Mumbles respectively. Other tentative links from the phylogenetic analysis implicate a link between haplotypes 2 & 11 and 4 & 5, with bootstrap values of 34 and 36 respectively for maximum parsimony, and 57 and 38 respectively for the neighbour joining methods. The mean number of pairwise differences

between the 16 haplotypes was small; this supports the previous results of genetic homogeneity within the three populations.

The results of this study show that the three populations (spanning 172 km along the south Wales coastline) of *T. pennigera* exhibit genetic homogeneity; these results support the findings of Todd *et al.* (1998) for *G. nodosa.* The period of time the planktotrophic veligers of *T. pennigera* spend in the plankton is unknown, as attempts to rear the veligers through metamorphosis failed (see Chapter 3). In Wales, *T. pennigera* is an annual semelparous species (personal observation). Its life cycle is closely associated with *Bugula plumosa* on which the adults are exclusively found feeding from the end of July to the start of October. This annual life cycle is similar to that of *G. nodosa* which has only one discrete spawning period (Garstang, 1890), although a second spawning of *G. nodosa* has been reported in favourable conditions (see Thompson & Brown (1984)). It seems likely therefore, that *T. pennigera* has a similar larval duration, at ambient conditions, as *G. nodosa* i.e. between 10 and 13 weeks. An extended larval phase however, does not necessarily generate genetic homogeneity within populations. *Alcyonidium mytili* Dalyell produces pelagic larvae, the duration of which is unknown; Porter *et al.* (2002) discovered a high level of differentiation within subpopulations of *A. mytili. A. gelatinosum* (L.) has short lived lecithotrophic larvae; contrary to expectation, when compared to *A. mytili* it was found to exhibit a higher level of migration. The retention of *A. mytili* larvae within areas of reduced water flow, and the potential for the *A. gelatinosum* to be rafted by deracinated *Fucus* (on which it is found), were implicated in the results. As well as an extended larval phase, the widespread transport and mixing of larvae is required to create the genetic homogeneity exhibited amongst the populations of *T. pennigera* sampled in this study. The action of the tides, forcing water in and out of the River Severn generates extensive flushing in the Bristol Channel. During the ebb, currents which run along the north side (those that dominate the Welsh coast) can either be entrained around Pembrokeshire and north into the Irish Sea, or are entrained south west into the Atlantic (Fig. 4.1A, B) (Featherstone & Lambie, 2005). During the flood, currents flow south from the Irish Sea around Pembrokeshire and turn eastwards into the channel (Fig. 1C, D). Water also flows north east up from Devon and Cornwall past Ilfracombe and into the channel (Featherstone & Lambie, 2005). A spring ebb tide would be sufficient to transport larvae in a net westerly direction (from Mumbles) approximately 8.0 km over six hours. A spring flood tide would be sufficient to transport

the larvae in a net easterly direction (from Mumbles) approximately 7.2 km over six hours. The diurnal tidal flushing and the extended larval phase of *T. pennigera* are likely to disperse the larvae over extensive distances resulting in high levels of gene flow and connectedness.

There is however the possibility that the differentiation within the three *T. pennigera* populations is a result of adult and/or spawn dispersal. This is deemed unlikely for several reasons. All three populations were found within areas of low wave activity therefore the deracination of *B. plumosa* by wave action would be minimal. Todd *et al.* (1998) concluded that the likelihood of "rafting" for both *G. nodosa* and *A. proxima* adults is increased on sheltered shores. The short tufted colonies of *B. plumosa* attach directly to the substratum, and are found in rocky overhangs or attached to pier pilings. Very infrequently are they found occupying space on top of rocks. These features serve to protect against dislodgement, therefore reducing the likelihood of adult and/or spawn dispersal. If however dislodgement were to occur, the chances of the displacement of spawn and/or adults, followed by relocation into a favourable environment in numbers which would be sufficient to ensure recruitment the following year, is unlikely. This displacement of adults and/or spawn would then have to occur every year thereafter to ensure that the homogeneity was maintained, which is considered even more unlikely. Once deracinated, *B. plumosa* survives only a few days within the laboratory, even when provided with phytoplankton (personal observation). When *B. plumosa* is in a poor condition the adult *T. pennigera* will not feed (personal observation). Despite remaining alive for several weeks without feeding (personal observation), any period of starvation would ultimately affect reproductive effort even if the adults survived dispersal into a habitat with *B. plumosa.* Experiments performed by Smith & Sebens (1983) found that when *Onchidoris aspera* (Alder & Hancock) were starved for a period of 16 days, the adults failed to spawn. Adult rafting has however, been indicated in the dispersal of *T. pennigera* worldwide. Thompson & Brown (1984) postulated that shipping, which is thought to have resulted in the spread of *B. flabellata* Grey has also contributed to the spread of *T. pennigera.* The populations of *T. pennigera* found world-wide are notoriously sporadic, and typically only a few specimens are discovered at any one time (Barnard, 1933; Macnae, 1957; Baba, 1960; Swennen, 1961; Willan, 1976). One notable exception was the population Marcus (1957) found in Brazil, thus demonstrating the unpredictability of populations established by adult "rafting".

Within the past five years the population of *T. pennigera* at Oosterschelde, Netherlands, has disappeared and subsequently failed to reappear (P. H. van Bragt, personal communication). It was first recorded off the Netherlands by Swennen (1961), a second observation was recorded 25 years later by Dekker (1986). The extinction event which recently occurred off the Dutch coast could be attributed to a recruitment failure of its prey species *Bugula* and consequently a recruitment failure of *T. pennigera* (either earlier or later than *Bugula*, or the larvae settling in too small a number to allow the population to become established). The population at Mumbles has recruited successfully for many generations (P. J. Hayward, personal communication) and, since discovery, has not suffered an extinction event. When the Dutch population returns it would be interesting to carry out genetic analysis on the individuals to establish the source of recruitment.

In order to fully understand the extent of larval dispersal within *T. pennigera*, it would have been necessary to sample and analyze from localities further away from Wales. Despite best efforts, the unpredictability of the majority of *T. pennigera* populations made this impossible. However, the result from this study does provide support to other findings, notably Todd *et al.* (1998), that the inclusion of a long-term larval phase within a life cycle increases gene flow, and hence genetic homogeneity, between populations.

Chapter 5

Chapter 5

The histology of *Ancula gibbosa* (Risso)

5.1 *INTRODUCTION*

Ancula gibbosa (Risso) is an enigmatic member of the Goniodorididae. Despite having "one of the widest geographical ranges of any nudibranch", Thompson & Brown (1984) also stated that it was "an uncommon species". It was first described in 1818 from Nice (Risso, 1818), since then it has been reported all round the British Isles. Alder & Hancock (1845-1855) recorded densities of 15 m^{-2} off Northumberland. Thompson & Brown (1984) reported sightings from Orkney, Clyde, St. Andrews, and the Isle of Man. Garstang (1889) recorded two specimens at Plymouth, and then four additional animals a year later (Garstang, 1890). He also reported Herdmann & Clubb's observations of many *A. gibbosa* at Hilbre Island. In Wales, *A. gibbosa* has been found in Pembrokeshire from Martins Haven and Skokholm (Hunham & Brown, 1975), at Pembroke Dock from Carrs Rocks (personal observation), in Mumbles, Swansea (personal observation), and from the Welsh south east coast at Kenfig and Sully (Boyden, Crothers, Little & Mettam, 1977). Further afield, is has been reported from Ireland, off Galway and Greenisland (Thompson & Brown, 1984). In Europe, it has been reported from the Bay of Biscay to the White Sea and Murmansk (Thompson & Brown, 1984). Swennen (1961) recorded it from the coast of the Netherlands, and Lemche (1929) recorded it from Iceland and the west and south coast of Norway. Franz (1970) recorded *A. gibbosa* from northern and southern New England, USA, and described its amphiatlantic distribution as boreo-subartic. Clark (1975) reported it from Connecticut. Another species, *A. pacifica* MacFarland, has been recorded from the west coast of America, although Thompson & Brown (1984) suggested that it could be *A. gibbosa*. The only obvious morphological difference is extra pigment on the dorsum, however further evidence is needed to substantiate Thompson & Brown's (1984) suggestion.

Little is known about the ecology of *A. gibbosa*, and until recently, even its diet was unknown. Swennen (1961) offered specimens several different species of hydroid, algae and two species of polychaete worms, but concluded that "the animals could fast nearly two months". Clark (1975) reported it feeding on *Bowerbankia* sp. Todd (1981) (through personal communication with B. Picton), stated *A. gibbosa* fed mainly upon Entoprocta. However, Thompson & Brown (1984) later stated that the ascidians, *Botrylloides leachi* (Savigny), *Botryllus schlosseri* (Pallas) and *Diplosoma listerianum* (Milne Edwards) constitute its prey; they made no mention of Entoprocta. More recently, Picton & Morrow (1994) state the entoproct, *Pedicellina cernua* (Pallas) is consumed.

Records of the breeding season vary depending on location. Alder & Hancock (1845-1855) reported spawn in Northumberland from late spring to summer. Off the coast of the Netherlands, Swennen (1961) found a few juveniles in November and December, and in the following year found large numbers of spawning adults from the latter part of February to the beginning of July. Franz (1970) recorded spawn in winter and spring at Connecticut. Clark (1975) found spawn from April to June, and concluded that the adult life span was approximately four months. Additionally Clark (1975) reported that the population at Connecticut was not self sustaining. The time between the disappearance of the adults and the appearance of juveniles was estimated at 5 months. Clark (1975) assumed the veliger stage could not last 5 months in the plankton, and therefore concluded that recruitment must come from populations further north. At Mumbles, adult *A. gibbosa* were found sporadically from February until November (see Table 1.1), spawn however was only found in April and November. Since August 2003, no *A. gibbosa* have been found at Mumbles, this could be due to the high prevalence of *Splanchnotrophus willemi* Canu (see Chapter 6).

Two colour morphs of *A. gibbosa* are frequently encountered: the pallial papillae and rhinophoral filaments are tipped with either white or yellow (Fig. 5.1). The majority of individuals Swennen (1961) recorded were white morphs. Contrary to this, Thompson & Brown (1984) commented that the yellow morph is more common. Colour is not dependent upon the age of the individual as originally speculated (Thompson & Brown, 1984).

There are nine genera that constitute the Family Goniodorididae, they are: *Akiodoris* Bergh, *Ancula* Lovén, *Ancylodoris* Dybowski, *Armodoris* Minichev, *Goniodoris* Forbes & Goodsir, *Hopkinsia* MacFarland, *Okenia* Brown, *Teshia* Edmunds, and *Trapania* Pruvot-Fol

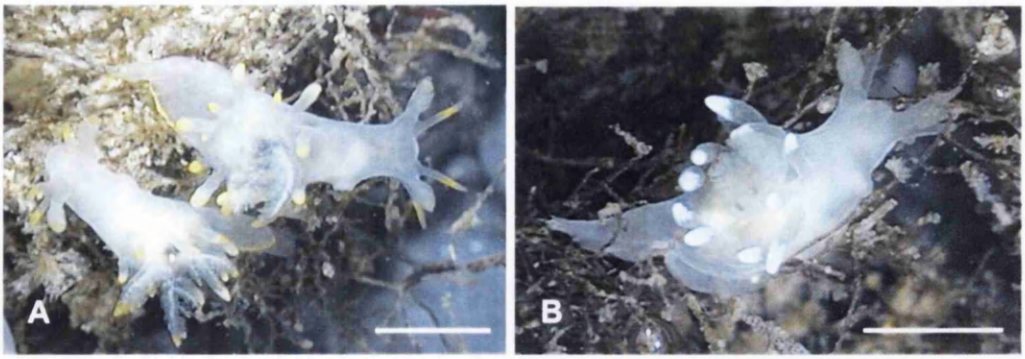

Figure 5.1. The two north eastern Atlantic colour morphs of *Ancula gibbosa*. **A.** Yellow morph. **B.** White morph. Scale bars: **A-B** = 10 mm.

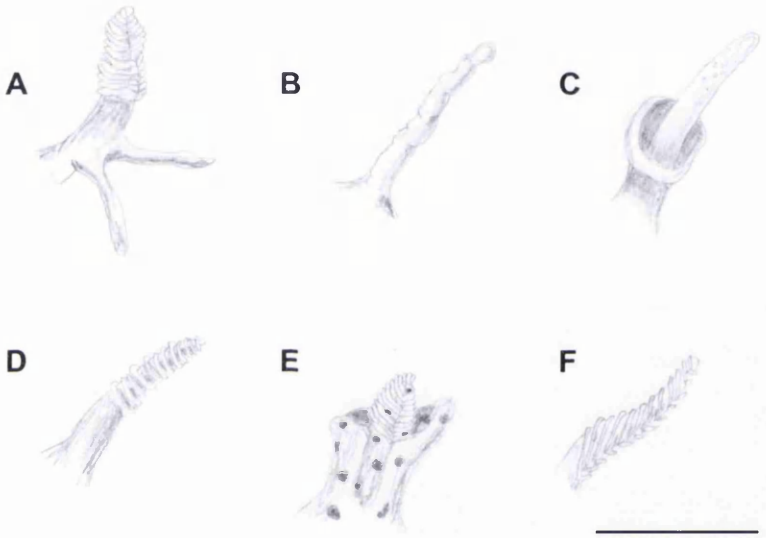

Figure 5.2. Rhinophore morphology found in nudibranch molluscs. **A.** *Ancula gibbosa*. **B.** *Coryphella pedata*. **C.** *Doto coronata*. **D.** *Facelina auriculata*. **E.** *Thecacera pennigera*. **F.** *Janolus cristatus*. Scale bar: **A-B, D-F** = 3 mm; **C** = 1 mm.

(Wägele & Willan, 2000). Extensive studies on the internal anatomy of the goniodorids are scarce; the few that have been published mainly involve members of the genus *Goniodoris*. Wägele & Cervera (2001) documented the internal morphology of *Goniodoris castanea* Alder & Hancock, and Hancock & Embleton (1852) published a detailed account on the anatomy of three species of dorids. Although the latter contains a few minor morphological misidentifications, both provide good descriptions of the internal morphology.

The following paragraphs outline the significant aspects of nudibranch anatomy. There are major variations in morphology between species, too many of which to list;

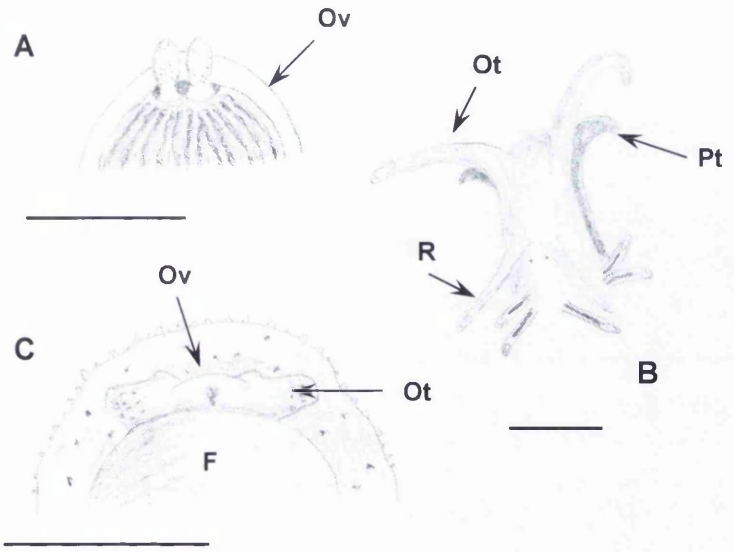

Figure 5.3. Anterior region of several nudibranch molluscs. **A**. Dorsal view of *Armina loveni* (redrawn from Thompson (1988)). **B**. Dorsal view *Coryphella pedata*. **C**. Ventral view of *Acanthodoris pilosa* (redrawn from Thompson (1988)). Abbreviations: Ov, oral veil; Ot, oral tentacles; Pt, propodial tentacles; R, rhinophores, F; foot. Scale bars: **A** = 5 mm; **B** = 4 mm; **C** = 6 mm.

however, a few species are included to highlight the differences exhibited within the Nudibranchia.

5.1.1 *EXTERNAL MORPHOLOGY*

The anterior region of nudibranchs is composed of several sensory structures. Often the most prominent feature is the rhinophores, which are chemosensory in function and can be traversed with lamellae (Fig. 5.2). In some species, the proximal part of the rhinophore is smooth and the distal lamellate, as for *G. castanea* (Wägele & Cervera, 2001), although they can be completely smooth. In some species rhinophores possess bulb-like swellings, or have longitudinal lamellae which extend along their length. Sheaths are occasionally present, into which the rhinophores can be withdrawn for protection. The sheaths can have a rounded or flared edge. In some species the sheath may not completely surround the rhinophore; in *Thecacera pennigera* (Montagu) the sheaths open mesially and the collar is raised anteriorly. Wägele & Willan (2000) noted the presence of annulate rhinophores in some Aeolidoidea. A few species lack rhinophores altogether. The oral veil can extend anteriorly in front of the rhinophores, to surround the mouth; however it is separate from the notum and foot (Fig. 5.3). Often a pair of oral tentacles, formed from

extensions of the oral veil, are located above the mouth; and in some species they possess grooves (Wägele & Willan, 2000). In others, the veil is reduced to a semicircular shape e.g. *Tenellia adspersa* (Nordmann). When the oral veil is overgrown by the notum, it is only visible from the ventral surface.

Propodial tentacles, which are formed from the foot, are present in the Facelinidae (Wägele & Willan, 2000). In species which lack propodial tentacles the anterior foot region is rounded. The border of the foot is divided into two lips which are characterised by subepidermal glands (Wägele & Willan, 2000). Members of the Anthobranchia possess a "philtrum", a small notch in the upper lip of the foot border. Wägele & Willan (2000) suggested this was a "specialisation related to a particular food type". Wägele & Cervera (2001) documented a small notch in the foot beneath the mouth in *G. castanea*, although it is not known if this is a philtrum.

The notum, or mantle, constitutes the dorsal surface of the nudibranch, and can form a characteristic skirt which covers the oral veil and mouth; typically it is separate to the foot. In contrast to this, the notum can be fused with the foot. In other species, the notum may be smaller than the foot, for example *G. castanea.* In this species the notum does not cover the oral veil, forming instead a ridge anteriorly to the rhinophores, further posteriorly the notum fuses with the foot (Wägele & Cervera, 2001). Usually the rhinophores are continuous with the notum, although occasionally they can arise separately from beneath it, as in *Armina* (Wägele & Willan, 2000). Wägele & Willan (2000) classified all projections arising from the notum of nudibranchs as "papillae". The papillae can take the form of simple structures: small and wart-like, or more complex cerata, which are finger-like projections containing branches of the digestive diverticulum. The papillae can completely cover the notum, form discrete bands, or arise from just the margin. In Tritoniidae, the papillae are branched and extend from the notal margin, where they act as gills. In the Bathydorididae,the papillae can autotomize due to a band of muscle located at their base (Wägele & Willan, 2000). More complex papillae are present in *Jorunna tomentosa* (Cuvier) and *Rostanga rubra* (Risso) which are called carophyllidia. Each carophyllidium has a central sensory bulb surrounded by spicules which project through the notum. In *R. rubra*, the spicules are connected together to form a collar, in *J. tomentosa* the spicules are separate (Kress, 1981). *G. castanea* has spicules within the foot, oral veil, rhinophores, notum, and the

base of the gills, however they are absent from the margin of the notum (Wägele & Cervera, 2001).

The dorsal notal epithelium of *G. castanea* is composed of cuboidal cells with large vacuoles; adjoining these are cells containing heavily staining granules. This epithelium also constitutes the rhinophores and the oral veil (Wägele & Cervera, 2001). The pedal epithelium is composed of tall columnar cells fringed with cilia. Beneath it glandular follicles are present, these increase in abundance towards the anterior region of the pedal epithelium. The follicles are also present in the oral veil (Wägele & Cervera, 2001).

The last prominant feature often present on the notum of the Nudibranchia are the gills. They can be situated medio-dorsally around the anus; there can be as few as two and as many as 30. In species with certain types of papillae (notably cerata), gills are often absent as the papillae act as the respiratory structures. The gills of *G. castanea* have tall columnar gland cells, with basally-lying nuclei and few muscle fibres. Only a small number of species have the ability to fully retract the gills into a branchial cavity, and those that can possess large retractor muscles at the base of the gills to facilitate this (Wägele & Willan, 2000).

The location of the anus varies; in the Dendronotoidea it is situated anteriorly on the right. However, typically it is located posteriorly beneath the notum. In apomorphic species the anus is situated dorso-posteriorly (Wägele & Willan, 2000).

5.1.2 *ALIMENTARY CANAL*

The first part of the alimentary canal in Nudibranchia is called the "oral tube", this extends from the mouth to the pharynx (Wägele & Willan, 2000). In most species the cells constituting the oral tube have mucopolysaccharide follicles underlying them, and lack a cuticle (Wägele & Willan, 2000). Collectively these follicles form the oral glands. In some species, the cells open directly into the oral tube; or, in *Armina*, the glands join to a common duct which opens medio-ventrally into the oral tube (Kolb, 1998). In *Flabellina affinis* Gmelin the oral glands have separate ducts, one from the gland on the right, the other from the left, which discharge independently (Schulze & Wägele, 1998). In some species the oral glands are absent, e.g. *G. castanea* (Wägele & Cervera, 2001) and *Bathydoris* spp. (Wägele, 1989). Marcus (1957) described the oral glands of *Okenia evelinae* Marcus as "long clusters" which discharge on both sides of the oral tube. The oral glands of *Armina* contain two types of glandular cells, one type has a basally-lying nucleus with non-staining vacuoles; the other

lacks an obvious nucleus and contains one large grey vesicle along with other small vesicles (Kolb, 1998).

The pharynx extends to the start of the oesophagus, and is typically separated from the oral tube by a cuticularised labial disc. The labial disc protrudes into the oral tube as a papilla (Wägele & Willan, 2000). The pharynx is highly muscularised and is often lined with a cuticle. In *G. castanea* it is composed of radial, longitudinal, and circular muscle fibres (Wägele & Cervera, 2001). Marcus (1957) described the "inner mouth" of *O. impexa* Marcus as having only a thin cuticle and an unarmed labial disc, and *O. evelinae* as having a thick cuticle with a "bilabiate beak". Opening into the pharynx, in the majority of Nudibranchia, are salivary glands. The morphology of the glands differs; Wägele & Willan (2000) considered a "ribbon-like" morphology to be plesiomorphic. In the Dendrodorididae and Tethydidae the glands have become reduced and rounded (Wägele & Willan, 2000). In *Armina* the glands are large and consist of muticellular follicles; the cells constituting the glands have basally-lying nuclei and spherical granules (Kolb, 1998). The salivary glands in *G. castanea* lack ducts; Wägele & Cervera (2001) concluded that their secretion must be acid mucopolysaccharides due to their staining properties. In *O. impexa* and *O. evelinae*, Marcus (1957) described the salivary glands as short sacs that insert at the posterior end of the pharynx.

The jaws, if present, are situated posterior to the labial discs, they are composed of rodlets or polygonal platelets. In all the Dendrodorididae and Phyllidiidae the jaws and radula are absent. The platelets can be orientated in "generative grooves". Some species possess aliform jaws, in which the platelets are fused to form a solid jaw with some separate, or a complete absence of platelets (Wägele & Willan, 2000). Denticles can be present on aliform jaws; they are thought to have arisen from the platelets that constitute the solid jaws (Wägele & Willan, 2000). Marcus (1957) described the labial disc of *Trapania maringa* Marcus as being covered with "reticulate cuticula" which took the form of "pointed pegs". Members of the Onchidorididae, Corambidae, and Goniodorididae posses a specialized sucking organ called a buccal pump, a dorsal muscular extension to the pharynx situated distally to the jaws (Wägele & Willan, 2000). In *G. castanea*, the pharynx opens into a triangular cuticularised buccal pump. In *Goniodoris* the pump is divided into two halves by a peripheral muscle, however in *Ancula* this division is absent (Wägele & Willan, 2000). Marcus (1957) described the buccal pump in *T. maringa* as "insignificant".

The oesophagus leads from the pharynx into the stomach. It is cuticularised in only a few species of Nudibranchia (Wägele & Willan, 2000) e.g. *Bathydoris clavigera* Thiele (Wägele, 1989). The oesophagus of *G. castanea* is convoluted, and surrounded by a thin muscle layer (Wägele & Cervera, 2001). In *Armina,* the cells lining the oesophagus are columnar and ciliated (Kolb, 1998). In *O. evelinae* it is described as "strongly muscular and dilatable" (Marcus, 1957). The oesophagus leads into an enlarged area recognized as the stomach, this transition is not always distinct e.g. *G. castanea* (Wägele & Cervera, 2001), *F. affinis* (Schulze & Wägele, 1998) etc. Typically the stomach is lined with ciliated cells (although rarely it can be cuticularized), with branches connecting it to the surrounding digestive gland. In *Armina* the stomach is lined with cuboidal cells which have basally-lying nuclei; cilia are not present (Kolb, 1998). In *G. castanea* the first two branches are located anteriorly and connect to the right and left "holohepatic" (compact) digestive gland; additional openings located posteriorly lead from the stomach into the digestive gland (Wägele & Cervera, 2001). When the digestive gland is composed of a series of tubes it is described as "cladohepatic". In some species, the transition between the stomach and the left digestive gland is difficult to distinguish due to their apparent amalgamation, however their epithelia are distinct. Generally however, the stomach has a ciliated epithelial lining whereas that of the digestive gland is glandular (Wägele & Willan, 2000). Typically, the stomach has two distinct openings into the digestive gland, although this can vary. *B. clavigera* has only one (Wägele, 1989), whereas *Notaeolidia schmekelae* Wägele has four (Wägele, 1990). In *Armina* the digestive gland forms a canal; with branches leading off into small follicles. The number of branches varies depending upon the species, in *A. neapolitana* (Delle Chiaje) five branch off from the left and six from the right (Kolb, 1998). A small bulb-shaped caecum, formed from the right digestive diverticulum, is present in some species of Nudibranchia. It is lined with small undifferentiated ciliated cells and lacks the mucous cells found within the digestive gland (Wägele & Willan, 2000). The caecum is absent in *G. castanea* (Wägele & Cervera, 2001), *O. evelinae*, and *T. maringa* (Marcus, 1957). Branching anteriorly from the stomach is the intestine. In *G. castanea* the intestine is distinct, and is reinforced by cuticularised bands and thick connective tissue. The typhlosole is located in the proximal area of the intestine, this area is convoluted and ciliated, but lacks a cuticular lining. The typhlosole does not extend the whole length of the intestine in *G. castanea*; further distally

the intestine is lined with columnar ciliated cells (Wägele & Cervera, 2001). A similar morphology was described by Marcus (1957) for *O. evelinae.*

5.1.3 *REPRODUCTIVE SYSTEM*

The reproductive organs in the Nudibranchia lie anteriorly on the right. All members are hermaphrodite; the majority exhibit protandry prior to becoming simultaneous hermaphrodites. Two genital formations are exhibited within the Nudibranchia: androdiaulic (diaulie I or diaulie II) or triaulic. There are two morphological variations of the ovotestis that exist within the Nudibranchia. In *Armina, Tritonia, Flabellina* etc. they are arranged so that the male and female gametes are within the same follicle; the oocytes are located peripherally and the spermatozoa distally (Thompson, 1961b; Kolb, 1998; Schulze & Wägele, 1998 respectively). Alternatively, the oogonia are located within separate acini, several of which adjoin one male acinus, as in *O. impexa* and *O. evelinae* (Marcus, 1957). The ovotestis of the Nudibranchia is closely associated with the digestive gland, the degree of association being dependent upon the species (see Chapter 2).

Leading from the ovotestis, a hermaphroditic duct passes into the ampulla, this acts as a storage organ for autosperm. The ampulla varies considerably in size and shape depending on the species and sexual maturity of the individual (Wägele & Willan, 2000). In *G. castanea*, the ampulla is a prominent structure, and is lined with flat cells (Wägele & Cervera, 2001); in *Armina* it is an elongate coiled structure (Kolb, 1998); in *O. impexa* and *O. evelinae* it is "globular" and "bean-shaped" respectively (Marcus, 1957). The post-ampullary duct leads from the ampulla, typically it bifurcates into the vas deferens and the oviduct. In the triaulic condition the oviduct bifurcates again into the nidamental gland complex and vagina. The vagina supports the receptaculum seminis and the bursa copulatrix. In the androdiaulic condition, diaulie I, the vagina leads only to the receptaculum seminis. In diaulie II, the vagina is absent and consequently the receptaculum seminis (or bursa copulatrix or both) branches off the oviduct proximally or distally to the nidamental glands (Schmekel & Portmann, 1982).

The male region is composed of a vas deferens which passes through with an elongate cylindrical prostate. The transition from prostate to vas deferens is not always distinct; as a result Wägele & Willan (2000) noted that some authors have failed to identify the prostate. Despite this, they state a prostate is always present. In *G. castanea* the prostate is located

proximally; it is lined with tall columnar glandular cells and is secretory in function. It forms a distinct junction with the vas deferens; the latter is lined with ciliated cells (Wägele & Cervera, 2001). A similar morphology is present in *O. impexa*, *O. evelinae*, and *T. maringa* (Marcus, 1957). However in *Armina*, the prostate is located further distally (Kolb, 1998). The vas deferens terminates at the penis, often in the Nudibranchia it is armed with spines. *Eubranchus farrani* (Alder & Hancock), *E. pallidus* (A. & H.), *E. cingulatus* (A. & H.), and *E. doriae* (Trinchese) all possess a single stylet at the tip of the penis (Edmunds & Kress, 1969). Some have an unarmed penis, for example *A. neapolitana* (Kolb, 1998). *G. castanea* has a penis armed with small spines which is surrounded by a muscular sheath (Wägele & Cervera, 2001). The penis of *O. evelinae* resides in a small orifice with convoluted walls. It lacks spines but is covered by a cuticle, and has a bulb located at the subterminal orifice (Marcus, 1957). In *G. brunnea* Macnae, a true penis is absent; instead the copulatory organ is merely an evaginated continuation of the vas deferens (Macnae, 1957).

During copulation the vagina receives the penis. In the traulic condition the vagina branches into two blind vesicles: the bursa copulatrix (spermatheca) and the receptaculum seminis (spermatocyst). The presence of both structures is found only in the Doridoidea (Wägele & Willan, 2000). The bursa copulatrix is typically located distally. It serves to break down stray allosperm and redundant oocytes, the lining of which, in *Hypselodoris messinensis* (Ihering), is composed of cells with highly polarized cytoplasm (Medina, Griffond, Garcia-Gomez & Garcia, 1988). Further proximally, the receptaculum seminis is located; its wall is convoluted and muscular, and the epithelium is composed of small cells. The allosperms received during copulation are orientated perpendicular to the receptaculum seminis wall, with their heads embedded within the wall. However their cell membranes never fuse, thus the cytoplasm remains separate. In Marcus' (1957) descriptions of *O. impexa* and *O. evelinae*, the former had sperm present within the spermatheca, the latter within the spermatocyst, despite the presence of both structures within each of the species. Schulze & Wägele (1998) detailed two allosperm vesicles which they termed "receptacles" in *Flabellina* spp. These receptacles could be analogous to Marcus' (1957) spermatheca and spermatocyst. Schmekel & Portmann (1982) documented the reproductive organs of *O. impexa*, and drew both a bursa copulatrix and a receptaculum seminis. However, they did not illustrate the location of the nidamental glands, for reasons unknown. In *T. maringa*, Marcus (1957) described the receptaculum seminis as having a separate entrance and exit into the vagina. In some

species, prior to the vagina joining the nidamental glands there is a fertilization chamber, e.g. *T. pennigera* (Willan, 1976), *F. affinis* (Schulze & Wägele, 1998) etc. In the diaulie I condition, e.g. *Bathydoris clavigera* and *B. hodgsoni* Eliot, the bursa copulatrix is located in a pocket at the distal end of the oviduct, and does not connect to the post-ampullary duct (Wägele, 1989). In contrast in *Notaeolidia* the vaginal duct terminates at the receptaculum seminis (Wägele, 1990). In *E. farrani* and *E. cingulatus* the vaginal duct ends with the receptaculum seminis, and mid-way along the vagina (between the gonopore and the receptaculum seminis), the bursa copulatrix is simple, comprising an enlarged region of the duct (Edmunds & Kress, 1969). In *Armina*, the vagina terminates at the allosperm receptacle (Kolb, 1998). In *G. castanea* a sphincter muscle is present near the opening of the vagina (Wägele & Cervera, 2001).

The female gland mass is composed of three main areas: the capsule, membrane, and mucous glands. Klussmann-Kolb (2001) thoroughly investigated the structure of the nidamental glands in 11 species of Nudibranchia, the details of which are documented below. The oviduct leads into the proximal region of the nidamental gland mass: the capsule gland. This region is characterized by its narrow, highly coiled structure. It is lined with columnar granulated cells with basally-lying nuclei. These cells are interspersed with ciliated supporting cells, which have their nuclei located apically (Klussmann-Kolb, 2001). The transition from the capsule gland to membrane gland can be continuous, for example in cladobranch species, or a small discrete duct can connect the glands (Klussmann-Kolb, 2001). The cells that constitute the membrane gland vary in shape from prismatic to columnar, and have "heterogeneous mucous filaments" within the cytoplasm (Klussmann-Kolb, 2001). They are interspaced with inverted prismatic supporting cells, with apical nuclei and very long cilia. Klussmann-Kolb (2001) observed that the supporting cells often overlapped the glandular cells at the apex. The last section of the nidamental gland is the mucous gland. The transition from membrane to mucous gland can be continuous; but, occasionally the membrane gland opens directly into the oviduct and the mucous gland branches off the oviduct further distally (Klussmann-Kolb, 2001). Proximally, the gland is lined with squat columnar cells, which become more columnar the further distally they are located. In *Onchidoris bilamellata* (L.), some cells contain "mucus…(in) the form of small vesicles embedded in heterogeneous, filamentous mucus" along the whole length of the gland. After staining with toluidine blue, the glandular cells lining the mucous gland along

the proximal length in *Adalaria proxima* (Alder & Hancock) contain "large filamentous mucous patches", and further distally the cells are filled with a "homogeneously violet staining mucus", thus indicating that the cells contain acidic mucopolysaccharides (Klussmann-Kolb, 2001). The glandular cells are interspersed with ciliated supporting cells, which have apical nuclei (Klussmann-Kolb, 2001). This arrangement of mucus varies significantly between the species (Klussmann-Kolb, 2001). At the distal end of the nidamental gland there is a small glandular area called the "adhesive region", this region is analogous to Wägele & Willan's (2000) "vestibular glands". The glandular cells that constitute this region are columnar and contain mucus "droplets". In *A. proxima*, *Acanthodoris pilosa* (Müller) and *Lomanotus veriformis* Eliot the droplets are orientated towards the lumen (Klussmann-Kolb, 2001). The interspaced supporting cells are ciliated. The absence or presence of the adhesive region varies among genera.

Details on the ultrastructure of the nidamental glands are given by Klussmann-Kolb (2001).

5.1.4 *CIRCULATORY AND EXCRETORY SYSTEM*

The circulatory system in the Nudibranchia consists of two major structures located dorso-anteriorly to the gills: the ventricle and auricle. They are surrounded by a thin membrane called the pericardium, and together they constitute the "heart". In the Doridoidea the auricle receives oxygenated haemolymph from the notum via two lateral sinuses and from the gills via a branchial vessel, which join to the auricle posteriorly. The auricle is joined to the smaller ventricle by a valve. Leading anteriorly from the ventricle is the aorta anterior and the aorta posterior. They serve to distribute oxygenated haemolymph around the visceral organs. The aorta anterior supplies the foot, the genital complex, notum and the entire nervous system; it also leads into the blood gland (Schmekel & Portmann, 1982). In *Goniodoris* the aorta posterior supplies the kidney, digestive gland, the left branch of the gonads and the right branch of the intestine (Schmekel & Portmann, 1982). In species without gills, the branchial vessels are absent; however the lateral sinuses remain; they transport oxygenated haemolymph from the viscera to the auricle (Wägele & Willan, 2000). Schmekel & Portmann (1982) documented a "vena posterior" leading from the posterior viscera into the auricle, and two "vena anterior" leading from the anterior viscera into the auricle for the Aeolidoidea.

The blood gland is an organ associated with the circulatory system; it is only present in the members of the Bathydoridoidea and Doridoidea (Wägele & Willan, 2000). It is composed of loosely connected cells and haemolymph vessels (Schmekel & Portmann, 1982). Posteriorly it is connected to the circulatory system via the anterior aorta. Its function is not fully understood, although Hyman (1967) suggested that it could be a source of amoebocytes. Other authors have postulated its involvement in haemocyanin storage or production (Schmekel & Wechsler, 1973; cited in Wägele & Willan (2000)). In some species the blood gland is situated immediately anteriorly to the pericardium, although it can be located between the pericardium and the cerebropleural complex, or dorsally to the cerebropleural complex (Wägele & Willan, 2000). The latter position is found in dorids (Wägele, 1989). In *G. castanea* the blood gland is located near to the pericardium, above the reproductive system (Wägele & Cervera, 2001).

The renal chamber, or kidney, lies dorsally above the reproductive system and the visceral mass, and ventrally to the pericardium; anteriorly the walls of the chamber are highly convoluted, posteriorly it is thin and flat. In *G. castanea* the flattened renal chamber is located dorsally to the gonad and digestive gland. The renopericardial duct connects the kidney to the pericardium which it joins via a syrinx (Wägele & Cervera, 2001). The syrinx has ciliated convoluted walls and can take the form of a bulb (Wägele & Willan, 2000). The ureter leads from the renal chamber to the external milieu, it terminates at the nephroproct (Wägele & Willan, 2000).

5.1.5 *NERVOUS SYSTEM AND SENSORY ORGANS*

The main feature of the Nudibranchia nervous system is the fusion of the cerebral ganglia to the pleural ganglia to form the "cerebropleural complex", and the close association of the cerebropleural complex with the pedal ganglia. In *O. impexa* and *O. evelinae* the cerebral and pleural ganglia are completely fused (Marcus, 1957). In primitive species the fusion of the ganglia constituting the cerebropleural complex is not as advanced. In *Bathydoris* the cerebral and pleural ganglia are separate, and the cerebral ganglia are separated into two parts by the insertion of the rhinophoral ganglia (Wägele, 1989). The cerebropleural complex is located posteriorly and slightly dorsally to the pharynx. Typically the pleural ganglia are joined together by the visceral loop. In the Doridoidea, a visceral ganglion is present on the visceral loop. When the visceral ganglion is absent it is assumed

to have been incorporated into the cerebropleural complex (Hyman, 1967). The pedal ganglia are found closely associated with the cerebropleural complex. The pedal and parapedal commissures (collectively termed the subcerebral commissures) join the pedal ganglia together, and are often associated with the visceral loop. In *O. impexa* and *O. evelinae* the visceral loop is separate from the subcerebral commissures, and an abdominal ganglion is present on the visceral loop, which is situated behind the right cerebropleural complex (Marcus, 1957). The buccal ganglia are situated away from the main cerebropleural complex on the ventral side of the oesophagus (Wägele & Willan, 2000). Two cerebrobuccal commissures, which innervate the radula, join the buccal ganglia to the cerebropleural complex. In some species, the two commissures fuse shortly after leaving the buccal ganglia (Wägele & Willan, 2000). The buccal ganglia can either be totally separated (joined only by a connective) or closely associated with one another. In *Ancula* the ganglia are separate, but the connective is short (Wägele & Willan, 2000). There is often a rhinophoral ganglion located at the base of each rhinophore which is connected via the rhinophoral nerve to the cerebral ganglia. In some species two rhinophoral ganglia are present for each rhinophore, however there may be none. Wägele & Willan (2000) suggested that the latter condition could be due to the fusion of the rhinophoral ganglia to the cerebral ganglia, rather than a compete loss.

Most Nudibranchia possess eyes, which are often located near to the base of the rhinophores, and can be located at various depths beneath the epidermis (Hyman, 1967). They connect to the cerebral ganglia by optic commissures. Wägele & Willan (2000) described some species as possessing "sessile eyes". In this condition they are located dorsally to the cerebropleural complex, this has resulted in the loss of the optic commissure. Wägele & Willan (2000) attributed this relocation as a result of the development of other sensory organs i.e. the rhinophores, oral, and propodial tentacles. The eyes of *G. castanea* are "sessile". They are composed of a lens surrounded by a layer of pigment and measure 125 μm (Wägele & Cervera, 2001).

The statocysts in the Nudibranchia are located next to the pedal ganglia, but are connected to the cerebral ganglia. They are composed of a small spherical cavity lined with a flattened epithelium surrounded by connective tissue (Hyman, 1967). The lumen may contain up to 200 otoconia of varying sizes, or one large otolith. In *G. castanea*, the statocyst contains several otoconia (Wägele & Cervera, 2001).

5.1.6 *AIMS OF STUDY*

The aims of this study were to investigate the morphology and histology of *A. gibbosa* using light microscopy, and to compare the findings to other members of the Goniodorididae.

5.2 *MATERIAL AND METHODS*

Thirteen unparasitized *Ancula gibbosa* were collected from Mumbles, Swansea (003°58'4"W 051°34'2"N) between March and April 2003. They were anaesthetized using a 7% $MgCl_2$ solution diluted 50:50 with aquarium sea water (see Chapter 3 for an explanation regarding the source of sea water). Often anaesthesia would take several days, during this time they were left in a fridge until they failed to respond to physical stimuli. The $MgCl_2$ solution was changed daily. A 3.7% formalin solution (Sigma, catalog number F-1635; 37% solution diluted using distilled water) was used to fix the animals. After one week they were transferred into 70% ethanol (Fischer Scientific analytical reagent grade, diluted using distilled water from absolute) for long term storage.

In some instances the animals needed to be anaesthetized quickly, consequently they were placed into aquarium carbonated sea water, generated using a SodaStream™. They were kept in this solution for up to two hours, and then fixed as described above.

For the embedding and staining procedures see Chapter 2.

The slides were examined under an Olympus BH2 compound microscope and a Wild Heerbrugg Makroskop M420. Photographs were taken using a JVC TK1270 digital camera using Image-Pro Plus version 4.1 for Windows 95/NT/98.

5.3 *RESULTS*

5.3.1 *EXTERNAL HISTOLOGY AND MORPHOLOGY*

Ancula gibbosa has two colour morphs: white and yellow. Yellow was the most common morph found at Mumbles; only two white morphs were collected over the period of this study. Common to some species of the Doridoidea, the notum is continuous over the whole dorsal surface; it is smooth, lacks small papillae and is fused with the foot. It is characterized with between eight and 11 large pallial papillae which surround the gills. They are tipped with either white or yellow pigment and contain only haemolymph. They do not contain pockets of the digestive diverticulum. The pedal epithelium is composed of

columnar cells with basally-lying nuclei, the pedal sole is interspersed with muscle fibres. There are two types of gland cells present, one stains dark violet with Cole's haematoxylin and eosin, and the other light violet; both are found beneath the epidermis (Fig. 5.4A). Both types of gland cells are grouped in small follicles, which discharge their contents directly into the epidermis (Fig. 5.4B). Towards the oral aperture (the oral veil) the numbers of dark violet stained gland cells increases. Typically the dark violet cells are situated adjacent to the pedal epithelium and the light violet cells are located further dorsally. The notal epithelium is composed of simple columnar cells with basally-lying nuclei, with a layer of loose connective tissue beneath. There are large vacuoles present which appear empty (Fig. 5.4C). This epithelium is continuous with the pallial papillae and rhinophoral filaments, however the vacuoles are absent from these areas. The rhinophores (Fig. 5.4D) are lamellate with a smooth proximal section; their epithelium is composed of columnar cells with basally-lying nuclei. Thin muscle fibres run along their length. The rhinophores are not fully retractile. Two rhinophoral filaments project from the base of each rhinophores (Fig. 5.2A), and two small blunt oral tentacles are present. Dense purple staining glands are present in the distal section of the rhinophores, which are very similar in structure to the glands found in the gills.

The epithelium constituting the gills is similar in composition to that of the notum (Fig. 5.4E). However, the gills possess glands which are visible in living animals as dense white structures. They are composed of spherical clusters of cells (Fig. 5.4F). The glands are found along the whole length of the gill.

5.3.2 *ALIMENTARY CANAL*

The initial region of the alimentary canal, the oral tube, extends from the oral aperture to the labial disc. This area lacks a cuticle and is lined with columnar cells with basally-lying nuclei and densely packed cilia (Fig. 5.5A). The oral glands are located proximally (close to the oral aperture), around the oral tube. In *A. gibbosa* they are ductless, thus the contents are discharged directly into the oral tube (Fig. 5.5B). The labial disc separates the pharynx from the oral tube; it is highly muscularised and is lined with thick cuticle (Fig. 5.5C). Situated just behind the labial disc is a pair of cuticularized jaws, these are characterized by the presence of rodlets. Further distally the radula is located (Fig. 5.5D, E). Thompson & Brown (1984) provided details of radula formation. A pair of small, compact, purple staining salivary

glands are located on either side of the pharynx behind the radula; they are joined to the pharynx by two short ducts (Fig. 5.6A). Each gland has a discernible lumen. Situated dorsally above the pharynx is a cuticularized muscular buccal pump (Fig. 5.6B). The uncuticularized oesophagus extends from the back of the pharynx into the uncuticularized stomach. It does not have a defined convoluted surface but is not smooth either. It is lined with columnar ciliated cells surrounded by a thin muscle layer (Fig. 5.6C). The transition of the oesophagus into the stomach is indistinct (Fig. 5.6D). The holohepatic digestive gland completely surrounds the stomach which branches extensively into the former, the epithelium of which is marked by the abundance of very large glandular cells (Fig. 5.6E). A caecum is absent. The uncuticularized intestine emerges dorsally from the stomach, with a distinct transition. The epithelium is characterised by small cuboidal cells, interspersed with ciliated supporting cells. The area of intestine immediately leaving the stomach forms a distinct typhlosole (Fig. 5.6F). It has a laterally convoluted ciliated surface which extends along its length until it turns posteriorly, at which point the typhlosole ends and the intestine narrows to form a convoluted crescent (Fig. 5.7A). It is lined with columnar cells which have medially-lying nuclei. Towards the anus, the intestine becomes circular with a fluted lumen; it is surrounded by a thin muscle layer and is lined with long cilia (Fig. 5.7B).

5.3.3 *REPRODUCTIVE ORGANS*

As in all other nudibranchiate molluscs, *A. gibbosa* is a protandric hermaphrodite. Unusually however, it exhibits the androdiaulic condition, diaulie II (Fig. 5.9). The ovotestes are situated amongst the digestive gland and are composed of separate male and female acini. The developing oocytes are located in acini which branch off male acini; the latter contains the developing spermatozoa (Fig. 5.7C). The autosperm pass into a distinct U-shaped flattened ampulla via a ciliated hermaphroditic duct. The ampulla is located dorsally to the nidamental gland system and ventrally to the prostate. It has a very thin epithelium. Here the autosperms are stored randomly orientated (Fig. 5.7D). After leaving the ampulla, the post-ampullary duct runs anteriorly and then turns posteriorly to lie in close association with the oviduct. The former is narrow and lined with a thin ciliated epithelium; the latter is much wider, and has a convoluted surface which is surrounded with a thin muscular layer (Fig. 5.7E). They are joined together by a thin membrane. The post-ampullary duct then

turns anteriorly and as it does so bifurcates to form the oviduct (which continues to run anteriorly) and the prostate (which runs posteriorly).

The male region of the reproductive system is composed of a large elongate cylindrical prostate which, to accommodate its length, is folded several times. It is lined with large, densely staining, columnar glandular cells (Fig. 5.7F). The transition from prostate into the distal vas deferens is distinct; the glandular cells are replaced by smaller cuboidal cells which are underlain with a thin muscular layer. The vas deferens loops twice before terminating at the armoured invaginated penis (Fig. 5.8A).

The oviduct stretches anteriorly for a short distance before leading into an elongate, flattened highly ciliated chamber. Branching off from this proximal region of the female reproductive system is the bursa copulatrix and the receptaculum seminis. The spherical receptaculum seminis is located distally to the bursa copulatrix. It is characterized by a thin convoluted inner epithelium and the presence of allosperm (Fig. 5.8B). The allosperm are orientated with their heads pointing towards and embedded within the epithelium; the aperture into the receptaculum seminis is extremely small and is surrounded by a muscular sphincter. The larger spherical bursa copulatrix is lined with cuboidal cells with very large medially-situated nuclei (Fig. 5.8C), its lumen contained degrading gametes. Branching off distally from the ciliated chamber is the initial division of the nidamental gland, the capsule gland (Fig. 5.8D). This gland is fairly short and takes the form of a coiled tube. It is characterized by a tall columnar epithelium with densely staining cytoplasm and basally-lying nuclei; the lumen is lined with short cilia. The nidamental glands all lead into a single small chamber; they are not connected in sequence. In several of the *A. gibbosa* sectioned this chamber contained large amounts of sperm; presumably allosperm. The membrane gland is short and lined with columnar cells (Fig. 5.8E). However, the nuclei are not always basal and the lumen is lined with long cilia. The last and largest section of the nidamental gland mass is the mucous gland. In *A. gibbosa* the proximal and distal regions are distinct (Fig. 5.8F). The former is composed of cuboidal cells, whereas the latter region is composed of columnar cells; both have basally-lying nuclei. A small adhesive region is present at the aperture of the oviduct. The cells constituting the adhesive region are columnar with basally-lying nuclei, the lumen is sparsely ciliated.

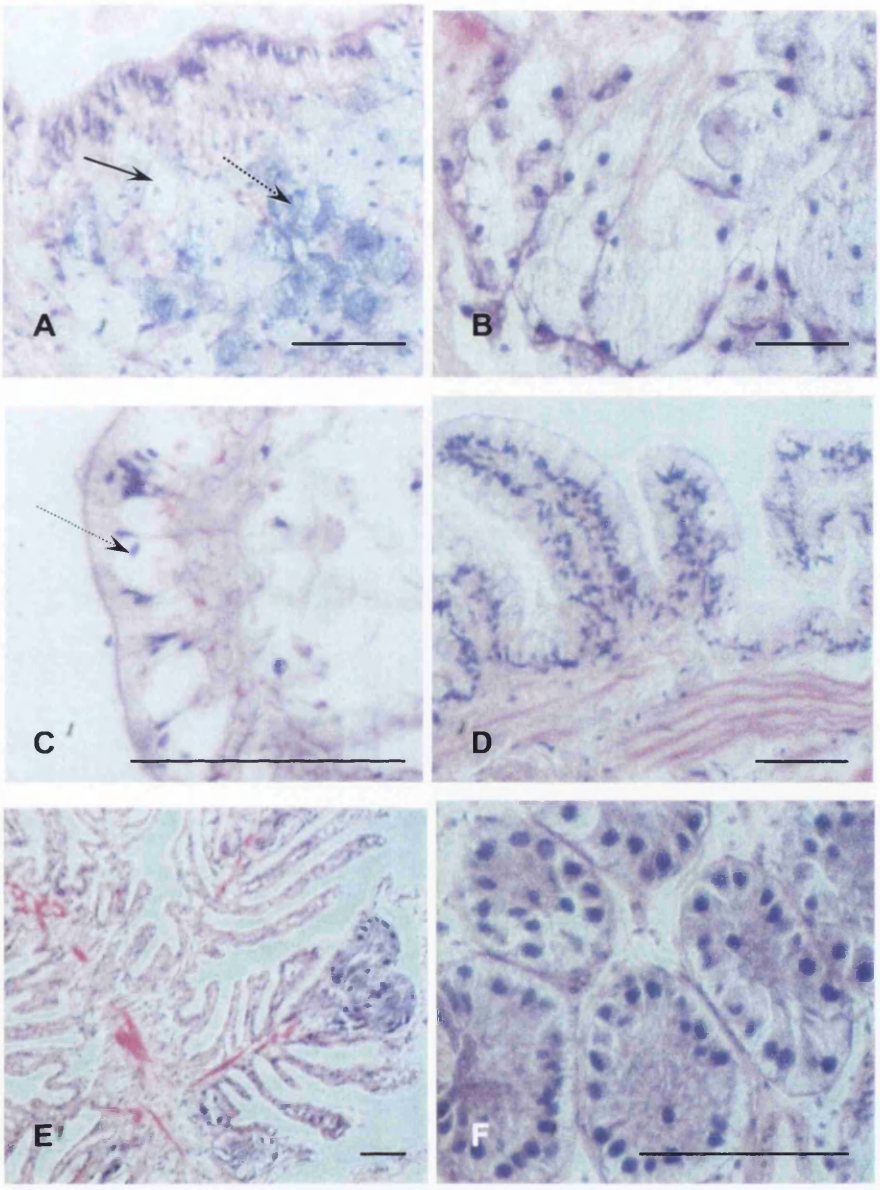

Figure 5.4. *Anaula gibbosa*. **A.** The pedal epithelium showing two types of gland cells: dashed arrow and solid arrow. **B.** An enlarged light violet pedal gland. **C.** The notum with large vacuoles (dashed arrow). **D.** A section of a rhinophore showing muscle fibres. **E.** A gill with gill glands. **F.** An enlarged view of the gill glands. Scale bars: **A-F** = 50 μm.

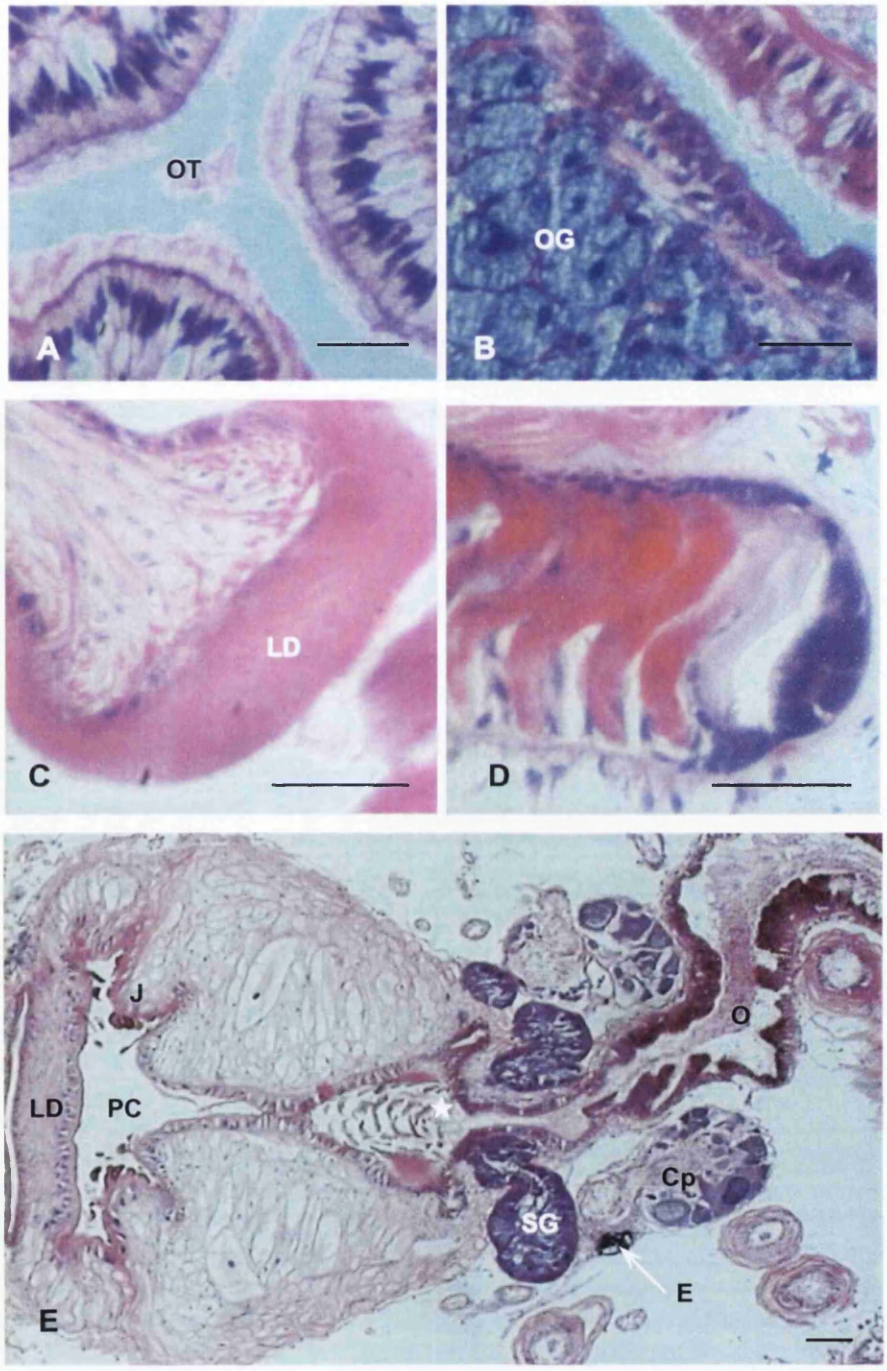

Figure 5.5. *Anaula gibbosa*. **A**. The ciliated cells lining the oral tube. **B**. The ductless oral glands. **C**. A cuticularized labial disc. **D**. The generative region of the radula. **E**. A transverse section of the initial region of the alimentary canal, ★ the radula. Scale bars: **A-D** = 50 μm; **E** = 100 μm. Abbreviations of labels given on page 139.

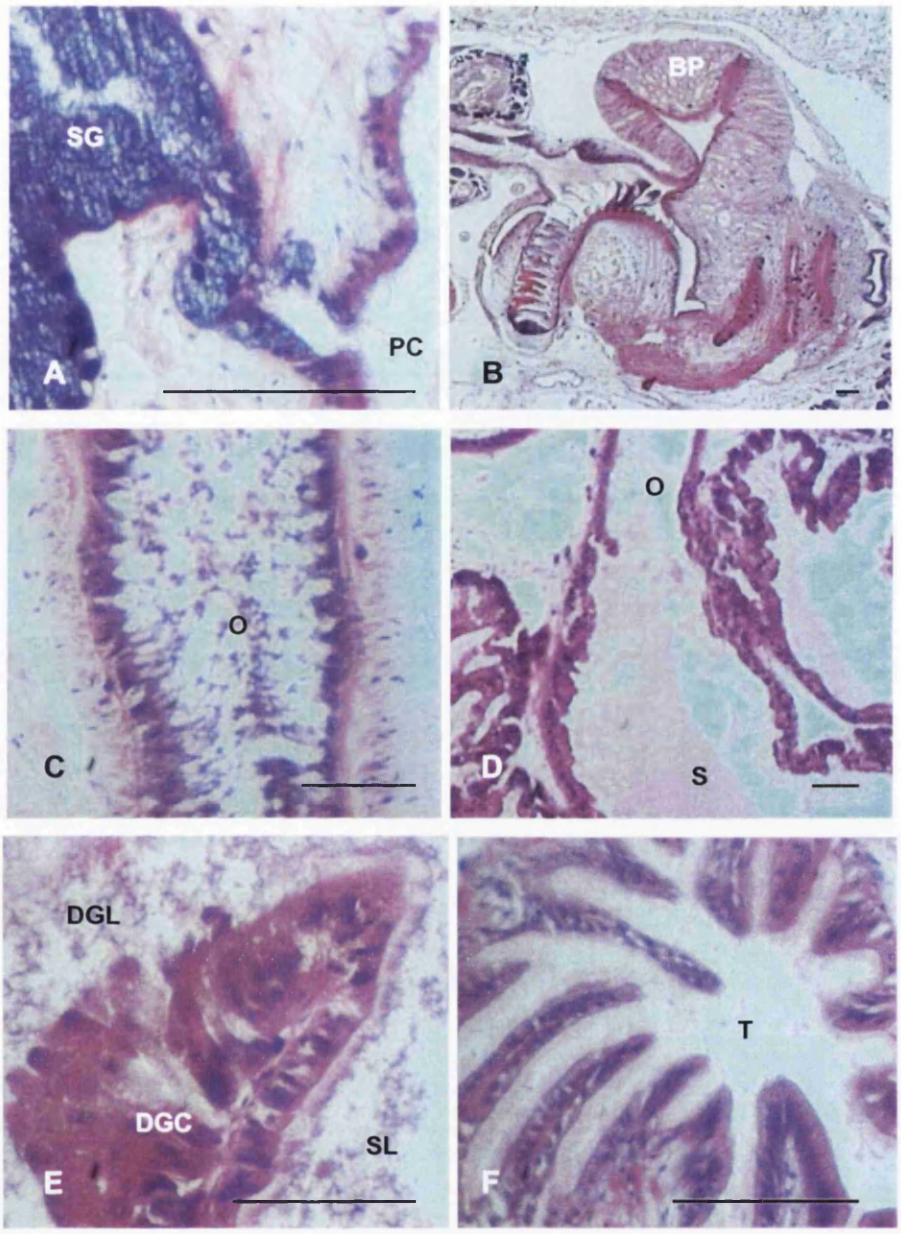

Figure 5.6. *Ancula gibbosa*. **A.** A salivary gland joining the pharyngeal cavity. **B.** The buccal pump situated dorsally to the pharynx. **C.** The oesophagus. **D.** The indistinct transition of the oesphagus into the stomach. **E.** The transition of the digestive gland into the stomach, note the large glandular cells on the left and the smaller cuboidal ciliated cells on the right. **F.** The convoluted typhlosole. Scale bars: **A**; **C-F** = 50 μm; B = 100 μm.

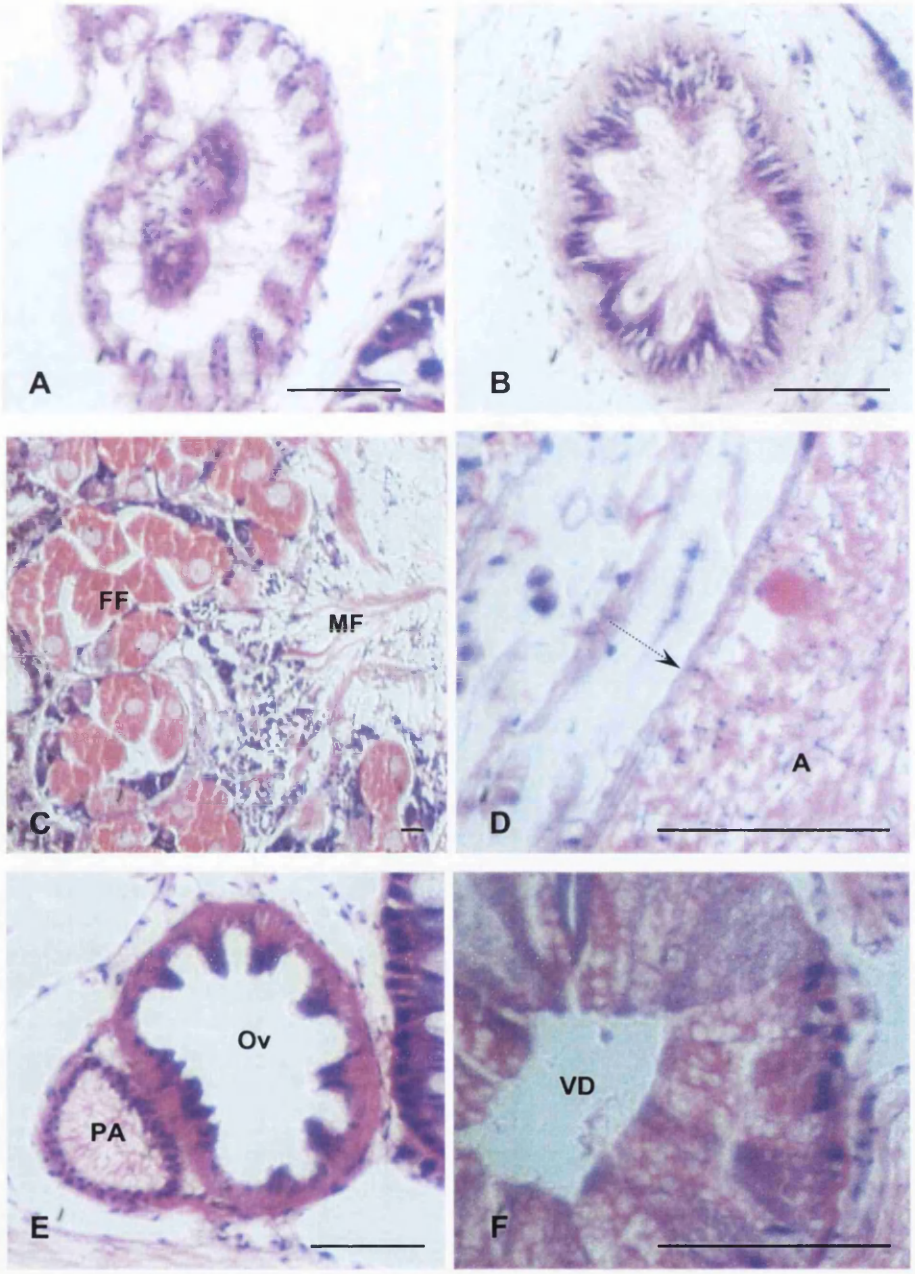

Figure 5.7. *Anaula gibbosa*. **A**. The proximal part of the intestine. **B**. The distal part of the intestine. **C**. The ovotestis orientated with the oogonia separate to the spermatogonia. **D**. The ampulla containing autosperms, the dashed arrow points to the epithelium. **E**. The ciliated post ampullary duct lying adjacent to the oviduct. **F**. The prostate lined with tall glandular cells. Scale bars: **A-F** = 50 μm.

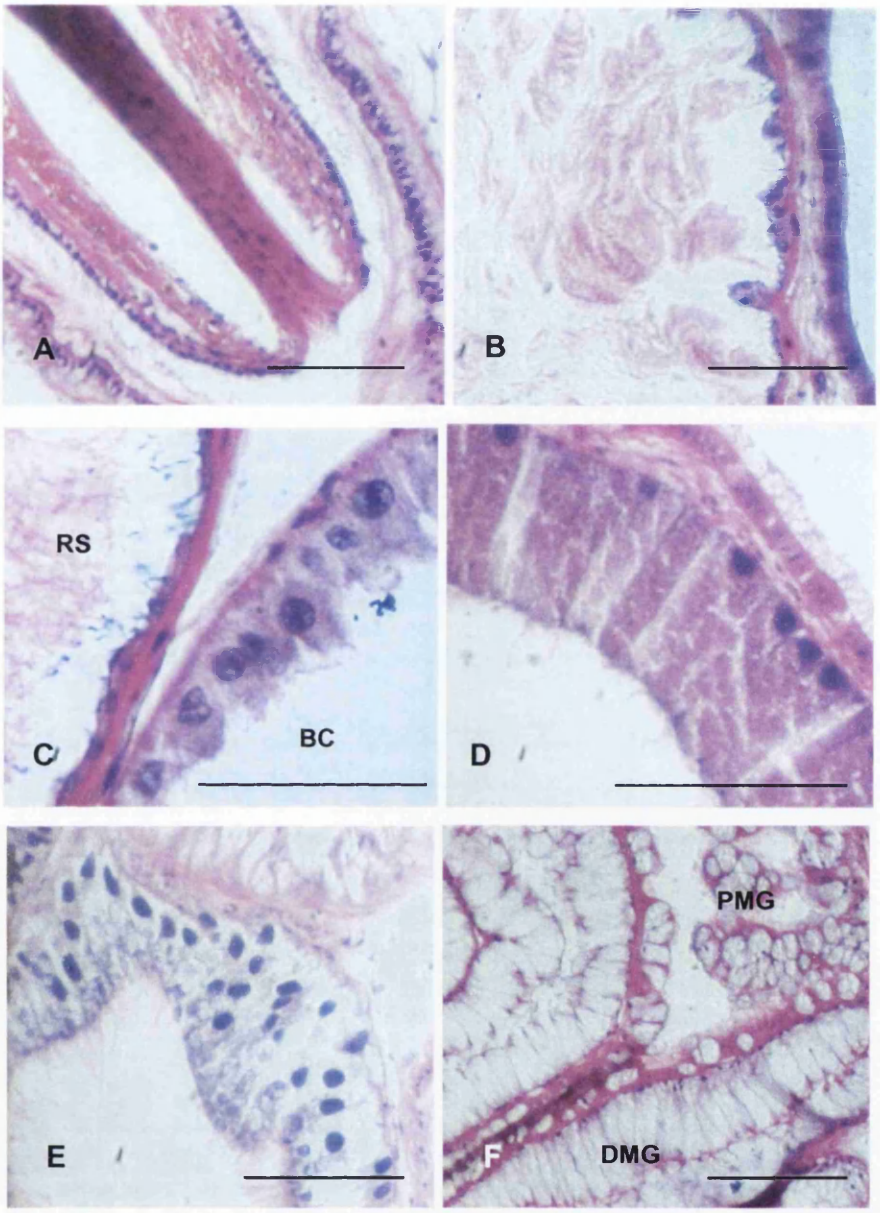

Figure 5.8. *Anaula gibbosa.* **A.** The invaginated penis. **B.** The receptaculum seminis containing allosperms. **C.** An enlarged image of the receptaculum seminis and the bursa copulartix, showing the fine detail of the epithelium. **D.** The capsule gland. **E.** Membrane gland. **F.** Mucous gland with the distinct proximal and distal part. Scale bars: **A-F** = 50 μm.

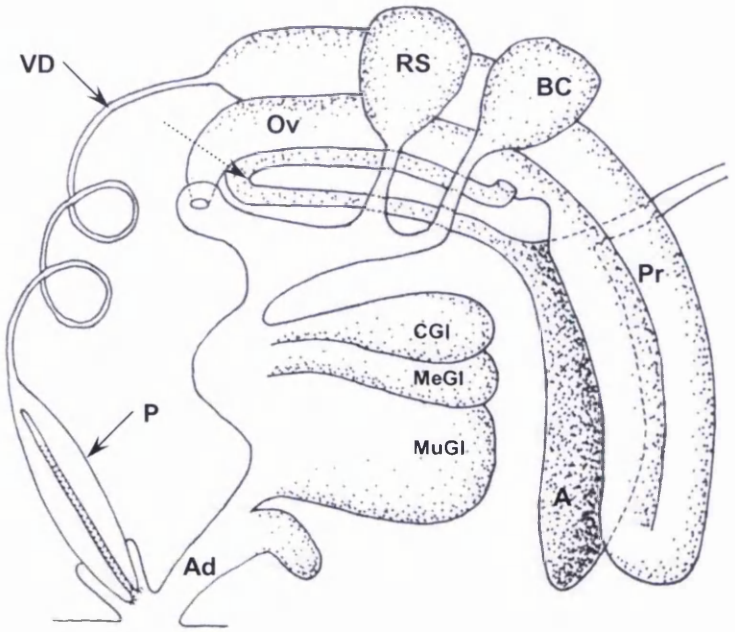

Figure 5.9. The reproductive system of *Anaula gibbosa* exhibiting diaule II. Dashed arrow points to the post ampullary duct.

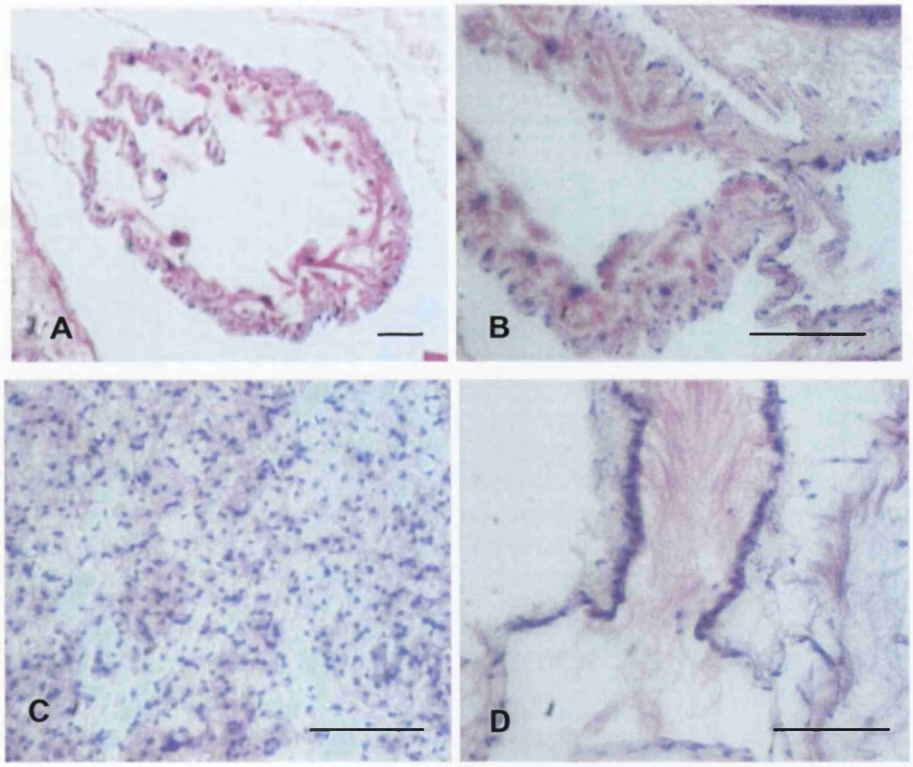

Figure 5.10. *Anaula gibbosa*. **A**. The ventricle with valve, connecting it to the auricle. **B**. The transition from the ventricle to the aorta. **C**. The blood gland. **D**. The syrinx fusing with the pericardium. Scale bars: **A-D** = 50 μm.

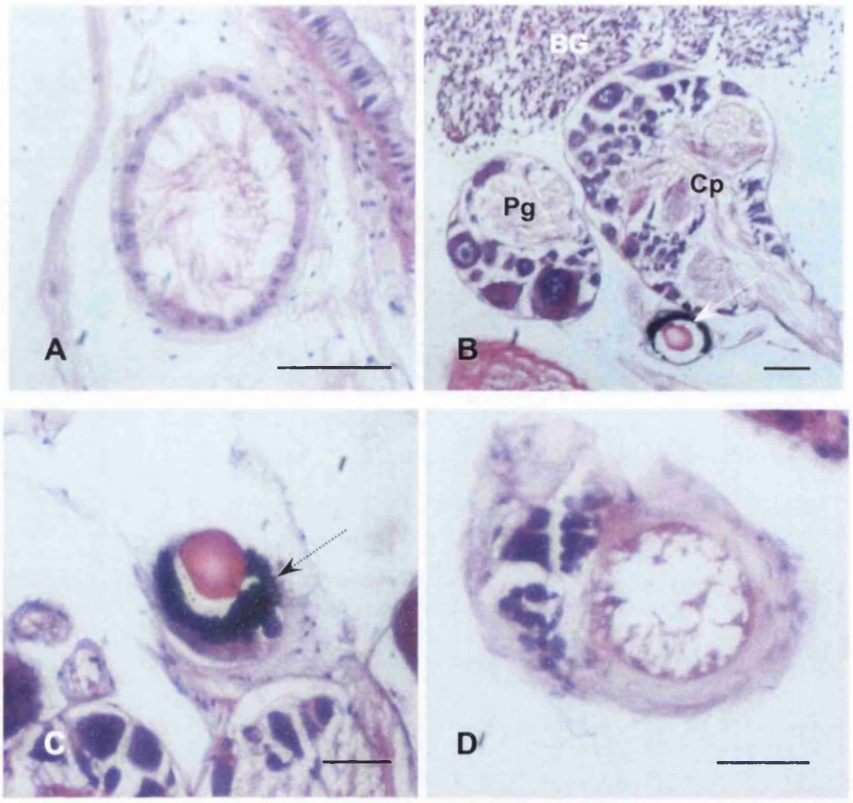

Figure 5.11. *Ancula gibbosa*. **A.** The ureter. **B.** The cerebropleural complex adjacent to the pedal ganglion, dashed arrow points to the eye. **C.** An enlarged view of the eye, dashed arrow points to the pigment layer. **D.** The statocyst, the otolith is not visible. Scale bars: **A-D** = 50 μm.

Abbreviations to figures:

A	Ampulla
Ad	Adhesive region
BC	Bursa copulatrix
BG	Blood gland
BP	Buccal pump
CGl	Capsule gland
Cp	Cerebropleural complex
DGL	Digestive gland lumen
DGC	Digestive gland cells
DMG	Distal mucous gland
E	Eye
FF	Female follicles
J	Jaws
LD	Labial disc
MeGl	Membrane gland
MF	Male follicles
MuGl	Mucous gland
O	Oesophagus
OG	Oral gland
OT	Oral tube
Ov	Oviduct
P	Penis
PA	Post ampullary duct
Pg	Pedal ganglia
PMG	Proximal mucous gland
Pr	Prostate
PC	Pharyngeal cavity
RS	Receptaculum seminis
S	Stomach
SG	Salivary glands
SL	Stomach lumen
T	Typhlosole
VD	Vas deferens

In all *A. gibbosa* studied there was a small blind sac originating from the female aperture. Using light microscopy, the cells lining the pocket appeared undifferentiated, and no organs or gland cells were present.

5.3.4 *CIRCULATORY AND EXCRETORY SYSTEM*

The circulatory system of *A. gibbosa* is similar to that of other nudibranchiate molluscs. The heart is located dorso-anteriorly to the pallial papillae and gills. It is composed of a muscularized ventricle joined by a valve to the auricle (Fig. 5.10A), surrounded by the pericardium. Anteriorly the ventricle leads into the aorta (Fig. 5.10B), which bifurcates into the aorta anterior and the aorta posterior. The aorta anterior joins to the blood gland (Fig. 5.10C). The blood gland is located above the cerebropleural complex and is composed of loosely associated cells. There is a ciliated syrinx which connects the pericardium to the kidney (Fig. 5.10D). The kidney of *A. gibbosa* is located ventrally to the circulatory system, and dorsally to the digestive gland and reproductive system. The long ureter is lined with small ciliated cuboidal cells (Fig. 5.11A). It was found alongside the intestine, and it discharges into the pallial papilla.

5.3.5 *NERVOUS SYSTEM*

A detailed investigation of the nervous system of *A. gibbosa* was not undertaken; however the basic features were identified. The cerebropleural complex is located on either side of the oesophagus (Fig. 5.5E); the pedal ganglia are situated alongside. The eyes are located adjacent to the cerebropleural complex so there is a very reduced optic nerve (Fig. 5.11B). It is composed of a spherical lens surrounded by a layer of pigment. It is not clear as to whether the pigment is located within the retina cells (Fig. 5.11C). The statocysts are positioned adjacent to the cerebropleural complex, and each contains one large otolith (Fig. 5.11D).

5.4 *DISCUSSION*

To date, there has been no published account of the morphology and histology of the goniodorid *Ancula gibbosa.* This is most likely due to its sporadic occurrence. The following discussion provides detail for the first time of the morphology of *A. gibbosa* compared to other members of the Goniodoridídae. Despite other species having been described, many

accounts lack detail and do not provide accurate descriptions (Macnae, 1957; Marcus, 1957; Kress, 1970); the exception is Wägele & Cervera's (2001) paper on *Goniodoris castanea.*

There are some interesting morphological differences between *A. gibbosa* and other members of the Goniodorididae. Most noticeable is the diaulic reproductive system with the absence of a vagina. The morphology of the reproductive system has been used by many authors to try to reconstruct the phylogeny of the Nudibranchia, notably by Ghiselin (1965). Since then, the increased use of molecular techniques combined with morphometrics has improved our understanding of the phylogeny of the Nudibranchia. Certainly, what is conclusive from this study is that the family Goniodorididae needs to be phylogenetically re-assessed using both morphological and molecular techniques to substantiate Thollesson's (1999) theory that *A. gibbosa* is the most primitive member of the group.

A. gibbosa has a completely smooth notum which lacks small papillae, carophyllidia, and spicules. I observed eight to 11 pallial papillae located around the anus of the *A. gibbosa* collected from Mumbles. Thompson & Brown (1984) reported up to seven on each side and Pruvot-Fol (1954) ten to 12. The notal epithelium is simple, and is composed of columnar cells underlain with connective tissue. Wägele & Cervera (2001) found vacuolated cells in the epithelium of *G. castanea*, similar vacuoles were found in the notum of *A. gibbosa.* The function of these vacuoles is unknown. However, in epithelium covering the rhinophores, gills, rhinophoral filaments, and pallial papillae these vacuoles were not present. Morphologically the rhinophores are typical of the Goniodorididae with a smooth proximal region and lamellate distal region (Fig. 5.2). Subepithelially they were characterized by gland cells which stained similar to the gill glands. The gill glands of *A. gibbosa* extend the whole length of the branchial plume. Wägele & Cervera (2001) described the glandular cells (of the rhinophores, notum, and gills) of *G. castanea* as having "brown to greenish grana" when stained with toluidine blue. They suggested their function involved the secretion of noxious compounds to deter predators. It is possible that the glands present in the rhinophores and gills of *A. gibbosa* have a function related to detection of sensory information and gaseous exchange respectively. The absence of such glands on the notum, and the absence of spicules and defensive papillae, however, suggests that *A. gibbosa* is not well defended against predators. It is possible that *A. gibbosa* possesses some other form of defense mechanism which is currently unknown. The pedal sole of *A. gibbosa*, as in *G.*

castanea, contains two types of glandular cells which are distinguishable using light microscopy. In *G. castanea* Wägele & Cervera (2001) noted the gland cells surrounding the oral veil were the same as those found in the foot. Marcus (1957) also described "Long clusters of labial glands open on both sides of the outer mouth" in *O. evelinae*. It is possible that these are homologous to the glands found around the oral aperture in *A. gibbosa* and *G. castanea*. Forrest (1953) labeled the oral glands of *G. nodosa* as labial glands, but failed to comment on their function or their morphology. It appears that the labial glands of Forrest (1953) and of Marcus (1957) are different. The former author used the term to describe the oral glands, and the latter to describe the glands found surrounding the oral aperture which are continuations of the pedal glands. The exact functions of the gland cells around the oral veil are unknown. However Forrest (1953) described a glandular epithelium surrounding the mouth of *Archidoris pseudoargus* (Rapp). He ascertained that these cells lubricated food particles prior to ingestion, thereby protecting the cells lining the alimentary canal from the spicules contained within the consumed sponge. *A. gibbosa* is a suctoral feeder; it is assumed that its buccal pump is used in a similar manner to that of *G. nodosa* and *G. castanea*. In the latter two species, only the soft tissue of ascidians is consumed, the harder outer epithelium is left (Forrest, 1953). It therefore seems unlikely that secretions from the gland cells would need to protect the alimentary canal from soft food. Perhaps the gland secretions assist in the transportation of food along the alimentary canal with the aid of the buccal pump.

The ciliated oral tube in *A. gibbosa* is uncuticularized, a feature normal for the majority of the members of the Nudibranchia (Wägele & Willan, 2000). The presence of diffuse ductless oral glands, as found in *A. gibbosa*, is not typical of the Goniodorididae. They are completely absent in *G. castanea*, and Marcus (1957) fails to comment on their existence in *Okenia impexa*, *O. evelinae*, and *Trapania maringa*. The pharynx starts at the cuticularized labial disc; further distally the jaws are located. Jaws are present in both *A. gibbosa* and *G. castanea*. Marcus (1957) failed to mention the presence of jaws in *O. impexa* and *T. maringa*. However, he described *O. evelinae* as having a "bilabiate beak", and the labial disc of *T. maringa* as having a "reticulate cuticula". Previous authors have described the members of the Doridoidea as having "jaw-like structures" which have been identified as part of the labial cuticle, however, Wägele & Willan (2000) consider of them as separate jaws. It is therefore likely that the labial armature Marcus (1957) described are homologous to jaws. In both *G. castanea* (Wägele & Cervera, 2001) and *A. gibbosa* the jaws are composed of rodlets. The

cuticulartized buccal pump is situated dorsally to the pharynx. It is only present in members of the Onchidorididae, Corambidae, and Goniodorididae (Wägele & Willan, 2000). There are two morphological forms of the buccal pump. The more primitive form is present in *Ancula*, in which the musculature is not divided (Fig. 5.6B). The more evolved form is exhibited in *Goniodoris*, in which the pump is divided into two regions by a peripheral muscle (Wägele & Willan, 2000). Marcus (1957) did not mention the presence of a buccal pump in *O. impexa*, *O. evelinae* or *T. maringa*. However, he did record the presence of a crop in all three species, which he drew as a dorsal bulge off the pharynx. It seems likely that this structure is analogous with the buccal pump. Macnae (1957) recorded a buccal mass for *G. mercurialis* Macnae, and *G. brunnea*, but failed to describe one in *Okenia amoenila* Bergh. A full description of the functioning of the buccal pump is given by Forrest (1953).

A pair of salivary glands discharges into the distal region of the pharynx in almost all Opisthobranchia. In both *G. castanea* and *A. gibbosa* they take the form of compact spherical glands. In *G. nodosa* they are lobed (Forrest, 1953). Forrest (1953) also stated that the salivary glands of *G. castanea* and *G. nodosa* discharge via ducts, but this was later contradicted for *G. castanea* (Wägele & Cervera, 2001). Certainly, within *A. gibbosa* both ducts are very short due to salivary gland cells extending along the majority of their length (Fig. 5.6A). However, there is a discernible region lacking gland cells in each gland which constitutes a distinct duct. Both glands possess a distinct lumen. Marcus (1957) described *O. evelinae* as having salivary glands of similar morphology to that described above for *A. gibbosa* and *G. castanea*. No reference is made of the salivary glands in *O. impexa* or *T. maringa*.

The uncuticularized oesophagus extends from the back of the pharynx into the stomach. Marcus (1957) documents the presence of a crop in the posterior region of the oesophagus in *O. impexa*, *O. evelinae* and *T. maringa*. From his descriptions, I believe what he identified as the crop was actually the buccal pump. A crop is only present in members of the Cephalaspidea and Anaspidea (Wägele & Willan, 2000). The epithelium of the oesophagus in *A. gibbosa* is similar to that of *G. nodosa* described by Forrest (1953) i.e. it is ciliated and lacking the pronounced convolutions recorded for the closely related species: *G. castanea* (Wägele & Cervera, 2001) and *O. evelinae* (Marcus, 1957). Gland cells are not present. The occurrence of a muscular oesophagus is typical, however, the degree of musculature differs. As in *G. castanea*, *A. gibbosa* has a thin muscle layer, whereas the oesophagus of *O. evelinae* was described as highly muscular (Marcus, 1957). In *A. gibbosa* the

transition of the oesophagus into the stomach is not readily distinguished. In the Nudibranchia, the intestine branches off the stomach together with either two or three chambers of the digestive gland. However, in *Bathydoris clavigera* only one branch leads from the stomach into the digestive gland (Wägele, 1989) while *Dendrodoris nigra* has several openings (Wägele, Brodie & Klussmann-Kolb, 1999). In *A. gibbosa* the first branch of the digestive gland is located dorsally and joins the stomach almost immediately after the oesophagus begins to widen to form the stomach. This position is similar to that described by Forrest (1953) for *G. nodosa* and *G. castanea.* Other branches, if present, could not be distinguished; neither could the thin walled ventral region or the thicker walled dorsal region of the stomach, described by Forrest (1953) in *G. nodosa* and *G. castanea.* The epithelium of the digestive diverticulum was composed of distinctive pear-shaped cells. Forrest (1953) described four different cells constituting the digestive epithelium, two secretory cells, one digestive cell and an intersititial cell. None of these cells could be distinguished in *A. gibbosa*, this was probably due to poor resolution of the light microscope and inappropriate staining. *A. gibbosa* lacks the cuticularized lining in the proximal region of the alimentary canal, observed in *G. castanea* and *G. nodosa* (Forrest, 1953; Wägele & Cervera, 2001). The location of the cuticle has been disputed; Wägele & Cervera (2001) reported it within the proximal part of the intestine in *G. castanea*, whereas Forrest (1953) reported it within the dorsal region of the stomach. It seems likely that Forrest (1953) misidentified this region of the intestine as a region of the stomach. Leading anteriorly from the stomach in *A. gibbosa* is the typhlosole, after a short distance it turns to run posteriorly, at this point the typhlosole ends. The lumen of the intestine reduces in width and forms a convoluted ciliated crescent shape; further distally the lumen narrows and becomes circular. As in *G. castanea* (Forrest, 1953; Wägele & Cervera, 2001), but contrary to *G. nodosa* (Forrest, 1953), no gland cells were present along the length of the intestine. The intestine finishes at the anus, which is located at the centre of the branchial plume.

Many morphological and phylogenetic studies in the Opisthobranchia concentrate on the arrangement of the reproductive organs. Hence, they are often the most thoroughly investigated feature of a species; despite this there has been no published account of the structure of the reproductive organs in *A. gibbosa.* Here, for the first time, a detailed description is given. The ovotestes lie at the periphery of the digestive gland. However, some follicles do penetrate into the digestive gland. The developing oocytes are located in

acini, several of which adjoin a male acinus. This arrangement is common to *G. castanea* (Wägele & Cervera, 2001), *T. maringa* and *O. impexa* (Marcus, 1957), and most other nudibranch species (Wägele & Willan, 2000). The follicles lead into the pre-ampullary duct which leaves the visceral mass dorsally. The duct is simple, ciliated, and short; it leads into the dorsal region of the ampulla. The ampulla is a simple U shaped bag structure. In all specimens examined it was full with autosperm, even when mature oocytes were not present within the female acini, thereby confirming that similar to all other nudibranch species, *A. gibbosa* undergoes a period of protandry prior to becoming hermaphrodite. This distal region did not contain any features which were unusual within the Nudibranchia.

The female region of *A. gibbosa* does not correspond to that documented for other members of the Goniodorididae. It is similar to that described by Klussmann-Kolb (2001) for the cladobranch *Lomanotus veriformis* Eliot with the exception of having both a bursa copulatrix and a receptaculum seminis (Fig. 5.9). Contrary to other Goniodorididae, *A. gibbosa* has only two genital openings (diaulie II). The unusual feature of the oviduct is its close association with the post-ampullary duct. These run alongside one another in opposite directions until the post-ampullary duct bifurcates into the vas deferens and the oviduct. The oviduct then leads into a heavily ciliated chamber from which branch both the bursa copulatrix and the receptaculum seminis. Further distally, the chamber leads into the nidamental gland complex. The nidamental gland mass is composed of the capsule, membrane, and the mucous glands. They are similar in structure to that described for other species (Klussmann-Kolb, 2001). The distal region of the oviduct has a small glandular area called the adhesive region. No sphincter muscle was present at the gonopore, which has been described for *G. castanea* (Wägele & Cervera, 2001).

The male region is typical of that described for other members of the Goniodorididae: *G. castanea* (Wägele & Cervera, 2001), *O. impexa* (Marcus, 1957), *T. maringa* (Marcus, 1957), *G. mercurialis* (Macnae, 1957), *O. amoenula* (Macnae, 1957), and *T. fusca* (Kress, 1970). The long prostate, which in *A. gibbosa* is folded several times in order to accommodate its length, has a distinct junction with the vas deferens. The vas deferens loops twice before terminating at an armoured invaginated penis.

The diaulie II condition of *A. gibbosa* indicates that this species is plesiomorphic compared to other members of the Goniodorididae. However, only the genus *Goniodoris* has been thoroughly investigated from this family, so it is not known if the diaulic condition of

A. gibbosa is common to any other members of the Goniodorididae. It is however, exhibited within *Bathydoris* (Wägele, 1989), and most members of the Cladobranchia (Wägele & Willan, 2000). The discovery that *A. gibbosa* has a diaulie II reproductive system could place *Bathydoris* and *Ancula* phylogenetically closer together than otherwise assumed. This however is purely speculative, and would require further investigation. The presence of a small blind pouch leading from the genital vestibulum was consistent for all *A. gibbosa* animals investigated. It lacks association with the nidamental gland complex, the adhesive region, and the penis. In literature documenting the reproductive morphology within the Nudibranchia, no mention is made of a blind sac in any of the species. Perhaps the pouch is everted hydrostatically during copulation, contributing to the gonadal collar, and thereby assisting in "stabilizing" copulation.

The circulatory system of *A. gibbosa* is typical of most nudibranchiate mollusca. The auricle is located posteriorly to the ventricle; they are both surrounded by the pericardium. The pericardial complex is situated dorso-posteriorly, a condition common within the Doridoidea (Wägele & Willan, 2000). The blood gland is located above the cerebropleural complex. This is different to the position in *G. castanea*, where the blood gland is located slightly in front of the heart but dorsally to the genital system (Wägele & Cervera, 2001). The ciliated syrinx connects the pericardium to the kidney. In *A. gibbosa* the syrinx does not form the typical siphon shape but is similar to the "elongate" morphology found in *G. castanea* (Wägele & Cervera, 2001).

The cerebropleural complex of *A. gibbosa* is similar to that described for other nudibranchs. The complex consists of two fused cerebral and pleural ganglia, which are positioned on either side of the pharynx. The finer details of the nervous system were not recorded in this study. The eyes are sessile, and are positioned next to the cerebropleural complex. The statocyst contains one large otolith.

Wägele & Willan (2000) did not include *Ancula* in their phylogenetic analysis, instead they used a species of *Trapania*; however, Thollesson (1999) did. He discovered ambiguity amongst the Goniodorididae (using 16S mtDNA), and could only conclude a close association between *Goniodoris* and *Okenia*. *Ancula* was found to be the most primitive member of the Goniodorididae (Thollesson, 1999). What is certain is that the Goniodorididae requires further investigation using mtDNA techniques and morphological comparisons to clarify the Family's systematics.

Chapter 6

Chapter 6

The effects of *Splanchnotrophus willemi* Canu infecting *Ancula gibbosa* (Risso)

6.1 *INTRODUCTION*

Parasitism within the Opisthobranchia is little known. Members of the copepod family Splanchnotrophidae are exclusive parasites of opisthobranch molluscs. To date, most of the literature concerns the genus *Ismaila*, which has 11 known species. Haumayr & Schrödl (2003) contributed significantly to our understanding by publishing descriptions of eight new species of *Ismaila* along with details of their respective hosts, however very little research has been published on the boreo-mediterranean genus *Splanchnotrophus*. There are only four described species: *S. angulatus* Hecht, *S. dellachiajei* Delamare Deboutteville, *S. gracilis* Hancock & Norman, and *S. willemi* Canu recorded from 11 species of opisthobranch mollusc. Huys (2001) in his extensive review places the two Western Australian species: *S. sacculatus* O'Donoghue and *S. elysiae* Jensen within the genera *Ceratosomicola* and *Arthurius* respectively.

Members of the Splanchnotrophidae are endoparasitic, penetrating the body of the host after a period of time as pelagic nauplii larvae. The mature females are orientated within the haemocoel around the digestive gland, kidney or the pericardium. In aeolids they can be found within the host's cerata. When ovigerous, the female penetrates the host's body wall for the second time exposing the urosome, which supports the egg sacs (Huys, 2001). Often the most obvious indication of parasitism is the presence of the egg sacs protruding from the host's integument. The males are located throughout the host, and are able to move freely within the visceral cavity (Hancock & Norman, 1863; Haumayr & Schrödl, 2003). Despite this, they are often found closely associated with the female, often just beneath the host's integument (Hancock & Norman, 1863; Ho, 1981). Males of *S. gracilis* have only been found in a host which has been infected with a female and can vary in

number from one to 12; however a typical rate of infection is between three or four (Hancock & Norman, 1863).

6.1.1 ISMAILA *SPP. PREVALENCE AND INCIDENCE OF INFECTION*

Schrödl (2002) identified eight species of Chilean opisthobranchs as hosts for *Ismaila* spp. With four other host species described elsewhere (Bergh, 1898; Marcus, 1959 cited in Schrödl (2002)) the incidence of infection totals 12 out of a possible 65 opisthobranch species found from Chile (Schrödl, 2002). Despite these eight Chilean species having been documented as being infected by *Ismaila* spp., Schrödl (2002) noted a low prevalence of parasitism. In three of the identified host species, only one animal was infected. In one case this amounted to 10% prevalence due to the small number of animals collected. Schrödl (1997; 2002) noted an average 39% infection rate in *Flabellina* sp. 1. The prevalence fluctuated depending on the time of year the nudibranchs were collected: January 1994, April 1992 and September 1996 had 40%, 21% and 48% infected individuals respectively. Haumayr & Schrödl (2003) however, noted a 66.6% infection rate in *Flabellina* sp. 1 when describing the species of *Ismaila* from the same material collected by Schrödl (2002). Schrödl (1997; 2002) remarked that none of the *Flabellina* sp. 1 collected in March 1994 were infected with *Ismaila*. Despite this, Haumayr & Schrödl (2003) described *I. damnosa* Haumayr & Schrödl gathered from its host *Flabellina* sp. 1 collected in March 1994 from the same location. In *Okenia luna* Millen, Schrödl, Vargas, & Indacochea the prevalence of *Ismaila* varied from 50% in January 1994 (n = 8) to 70% in March-April 1992 (n = 122) (Schrödl, 2002). The prevalence in January 1994 reached 100% (Haumayr & Schrödl, 2003) despite the collection of *O. luna* from the same location, although in greater numbers: 50 as compared to eight. In *Thecacera darwini* Pruvot-Fol the prevalence was similar ranging from 70 to 100% between April 1992 and February 1995 (Schrödl, 2002). The prevalence of infection in *T. darwini* varied depending on its geographic location. Those found in northern and southern Chile had low infection frequencies, 0 or 2% and 0 to 19% respectively, whereas in central locations the rates recorded were 88% and 96% (Schrödl, 2002). He speculated that hydrographical conditions, density of hosts, and even the heavily populated area around the central Chile coast combined with the inevitable anthropogenic influence could be the reasons behind these fluctuations. Jensen (1990) also noted differences in prevalence depending upon location. *Elysia australis* (Quoy & Gaimard) collected from

Eagle Bay Western Australia and Rottnest Island were infected with *Arthurius elysiae* (Jensen), however, none collected from Princess Royal Harbour were infected.

Ho (1981) discovered several heavily parasitized *Dendronotus iris* Cooper one animal contained: 425 *I. occulta* Ho of which 113 were adults. There was a low incidence of parasitism around Oregon: out of 26 different opisthobranch species Belcik (1981) collected only *Janolus fuscus* O'Donoghue was infected with a copepod parasite (later described as *I. belciki* by Ho (1987)) with a prevalence of 62%.

6.1.2 *EFFECTS OF PARASITISM*

The documented effects of parasitism on the host varies significantly from no apparent consequence to death. When *Flabellina sp.* 1 was infected with *Ismaila*, Schrödl (1997) described the host's gonads as being "absent or strongly reduced" due to the parasite occupying space in the body cavity where the gonads would otherwise have been. Gonads were present where the parasite was absent, albeit they were rudimentary. The parasitised *Flabellina sp.* 1 failed to produce egg masses and had a shorter life span than those which were uninfected. In one instance the *Ismaila* sp. "abruptly destroyed the body integument" which lead to the host's death (Schrödl, 1997). When *I. monstrosa* Bergh was first described, Bergh (1868) noted severe atrophy of the gonads in *Phidiana lynceus* Bergh in which it was discovered. Jensen (1987) found *I. monstrosa* within the sacoglossan *Ercolania funerea* (Costa) in which the parasite had destroyed most of the gonadal tissue. Despite this the host's behaviour and growth were otherwise seemingly unaffected. Haumayr & Schrödl (2003) noted that despite the high infection rate of *I. androphila* within its host *O. luna*, there was no obvious damage caused. Parasitism by *I. aliena* Haumayr & Schrödl in *T. darwini* also resulted in no obvious internal damage, despite the host appearing swollen and exhibiting a "disturbed" behaviour when more than one female parasite was present. However, it was suggested that the male *I. aliena* may cause damage by moving around inside the host's integument (Haumayr & Schrödl, 2003).

Although there has been extensive research on *Ismaila* spp. covering the interactions they have with their hosts, the incidence and prevalence of infection etc., very little research has been carried out on the enigmatic genus *Splanchnotrophus*, except for the review of the family conducted by Huys (2001), who addressed two species of *Splanchnotrophus*, *S. angulatus* and *S. gracilis*.

6.1.3 SPLANCHNOTROPHUS WILLEMI

Discovered in Boulonnais France, *S. willemi* was first described by Canu (1891) in the nudibranch *Facelina auriculata* (Müller). In the original description 21 males and five females were discovered. Canu (1891) noted several features of *S. willemi* which enabled it to be distinguished from other splanchnotrophid species. These were the host species, the long dorsal prolongations of the thorax, and the colour and shape of the egg sacs. The only reference Canu (1899) made regarding the effect of the parasite on its host was that it restricted the growth of the nudibranch's organs when the female parasite reached sexual maturity. The male *S. willemi* were often found within the pallial papillae of the host and were said to move with "rampent avec vivacité" along the hepatic diverticula. This free movement might well have resulted in some internal damage, similar to that described by Haumayr & Schrödl (2003) in *T. darwini* caused by *I. aliena*. However no damage was noted. In his paper however, Pelseneer (1894) reported a splanchnotrophid infecting *F. auriculata* (identified as *S. willemi* by Monod & Dollfus (1932)) having caused significant damage to the digestive gland, resulting in the complete destruction of the stomach. All organs which once connected to it were observed to open directly into the coelomic cavity. No comment was made regarding the condition of the gonads.

The first recorded observation of *S. willemi* infecting *Ancula gibbosa* (Risso) was by Canu (1899), although once again no observations were reported on the effect of the parasite on its newly recorded host. There was an earlier report of a small *A. gibbosa* ($5/16^{th}$ inch) collected from Plymouth by Garstang (1890) which was infected with a splanchnotrophid, however the species was not identified and nothing was noted on the morphology of the parasite. Perhaps Garstang assumed the parasite to be one of the two species described by Hancock & Norman (1863). In his report he commented that after two days the parasite crawled out of the host "which was only half its original size, and was being steadily devoured". I am uncertain as to whether Garstang (1890) meant the parasite or the host was being devoured. However what is unusual is that his report suggests that the unidentified *Splanchnotrophus* sp. can exist outside its host and that it appears to restrict or reduce the body size of its host. The only other comment made on parasitism in *A. gibbosa* within the UK was that by Thompson & Brown (1984) where Garstang (1890) was quoted.

The last published recorded discovery of *S. willemi* was in 1927 by Cuénot (1927) in Arcachon. After Cuénot's (1927) documented discovery, Monod & Dollfus (1932) noted that the lack of host specificity exhibited by *S. willemi* was unusual. Since then no formal documented observation has been made.

6.1.4 *AIMS OF STUDY*

This aims of this study were to report on the discovery, for the first time in UK waters, of the formally identified splanchnotrophid *S. willemi* infecting the nudibranch mollusc *A. gibbosa*; to investigate the effects of parasitism of *S. willemi* on the host tissues; to record the prevalence of infection in the Mumbles population; and to report of the histology and morphology of a mature male *S. willemi.*

6.2 *MATERIALS AND METHODS*

Twenty *Ancula gibbosa* were collected from the intertidal at Mumbles pier, Swansea (003°58'4"W 051°34'2"N), between March and April 2003. They were anaesthetised rapidly using carbonated sea water, fixed in 3.7% formalin solution (Sigma catalogue number F-1635) and then transferred to 70% ethanol after one week. For embedding and staining schedules see Chapter 2.

The slides were examined under an Olympus BH2 compound microscope and a Wild Heerbrugg Makroskop M420. Photographs were taken using a JVC TK1270 digital camera in conjunction with the software Image-Pro Plus version 4.1 for Windows 95/NT/98.

The reconstruction of the male *Splanchnotrophus willemi* was generated using the freeware Reconstruct version 1.0.2.0 © 1996-2004 John Fiala.

6.3 *RESULTS*

Out of the twenty *Ancula gibbosa* collected and sectioned for light microscopy, seven were found to be infected with the parasite *Splanchnotrophus willemi*, identified courtesy of Dr. Rony Huys at the Natural History Museum, London. Those animals infected with the parasite were mature and were at least 14 mm in length (Table 6.1). In every case of infection, the female *S. willemi* was positioned with her large prosome inserted posterior to the digestive gland and the gonadal tissue, with the lateral processes wrapped around the main viscera. The cephalosome was orientated ventrally towards the foot. The urosome was positioned pointing towards the dorsal surface of the host; in two cases the female's urosome had perforated the dorsal integument of its host and was supporting the

developing nauplii (Fig. 6.1). In one host the female *S. willemi* had developing nauplii outside its host, and mature oocytes inside the host supported on the lateral processes (Fig. 6.2A, B, C). In no instance were there more than one female and one male per host. Males were only found in the presence of females. The location of the male *S. willemi* varied from host to host: only one was associated with the female, just beneath the pericardium. The others were found all just beneath the host's integument embedded within the body wall.

An infected *A. gibbosa* with an ovigerous female was left overnight anaesthetising in carbonated seawater. Within twelve hours the nauplii had hatched and were photographed (Fig. 6.2D), however they died shortly after, probably due to exposure to the anaesthetic.

The internal damage caused by *S. willemi* varied considerably. In six infected *A. gibbosa* there was no apparent damage to the gonads or the digestive tissue. In one infected *A. gibbosa* the digestive gland was indistinguishable from gonadal tissue (Fig. 6.2E). The oocytes were randomly orientated throughout the 'stomach', and the typical cellular structure of the digestive viscera was destroyed. One other animal had a similar lack of internal organisation, however it was not infected with *S. willemi* or any other parasite (Fig. 6.2F).

All of the infected *A. gibbosa* had sperm within the ampulla and also within the receptaculum seminis, indicating that copulation had occurred at least once during their lifetime. In one infected host the sperm within the receptaculum seminis had deformed heads (Fig. 6.3A, B).

In all the 20 *A. gibbosa* sectioned (both infected and uninfected) there were strange unidentified cuticular objects (Fig. 6.4A-C). These were found throughout the animal, often encapsulated by the host's tissue, and they varied in size from 42-300 μm. Typically they were associated with the digestive gland. It is possible that these are juvenile *S. willemi* nauplii at various stages of maturity, or another copepod parasite. The nature of the objects is such that it is certain that they are foreign and are not part of the host.

A mature male *S. willemi* was reconstructed *in situ* using the Reconstruct© freeware (Fig. 6.5). The body comprises of a cephalothorax which incorporates the first two pedigerous somites (P1 and P2); the prosomites which bear legs 3 and 4, and which incorporates P3 and P4 pedigerous somites; and the urosome which incorporates the P5 pedigerous somite, the genital somite and the anal somite. The two paired spermatophores are clearly visible in Figures 6.5F and 6.5G.

SIZE OF *ANCULA* (LENGTH IN MM)	MATURE?	PARASITE		LOCATION OF ♂ PARASITE WITHIN *ANCULA*
		MATURE ♂	MATURE ♀	
Unknown	Male	0	0	
15	Yes	1	1	Beneath rhinophores.
17	Yes	0	1	
11	Yes	0	0	
15	Yes	1	1	Ventral surface in foot.
9	Yes	0	0	
15	Yes	0	0	
13	Yes	0	0	
17	Yes	1	1	Dorsal surface anterior to gills.
10	Yes	0	0	
17	Yes	1	1	Dorsal surface beneath the pericardium.
9	Yes	0	0	
15	Yes	0	0	
14	Yes	1	1	Beneath gills.
13	Yes	0	0	
14	Yes	1	1	Within the gill filaments.
10	Yes	0	0	
14	Yes	0	0	
9	Yes	0	0	
4	No	0	0	

Table 6.1. The frequency of infection of *Anaula gibbosa* by *Splandmotrophus villemi*.

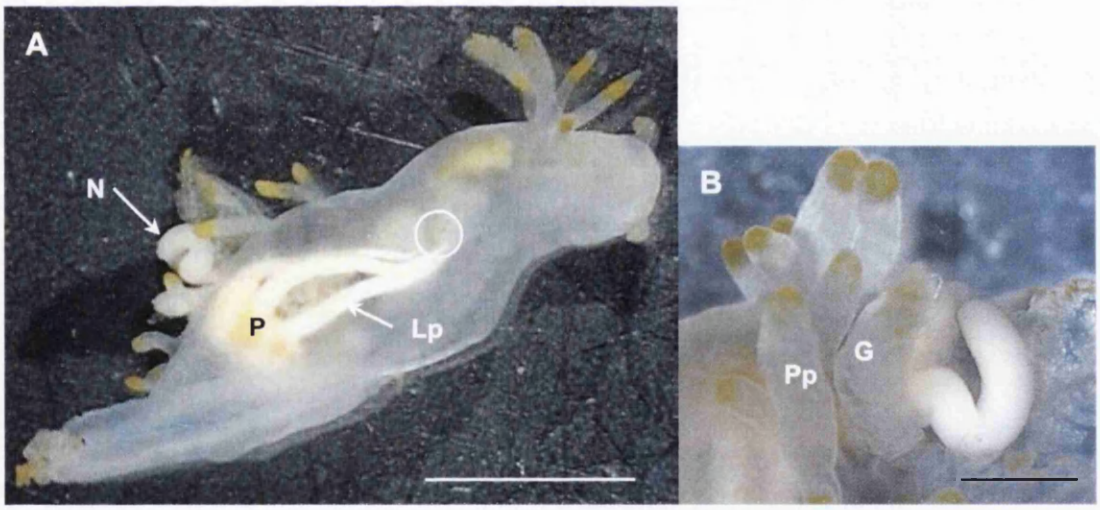

Figure 6.1. Orientation of the female *Splandmotrophus villemi* within *Anaula gibbosa*. **A.** An ovigerous female *S. villemi* with her prosome inserted behind the viscera and the lateral processes surrounding the digestive gland extending up towards the genital pore (circled). Note the developing nauplii located outside the host. **B.** The sickle shape of the egg sacs of *S. villemi* surrounded by the host's pallial papillae and gills. Abbreviations: Pp, pallial papillae; G, gills; Lp, lateral processes; N, nauplii; P, prosome. Scale bars: **A** = 5 mm; **B** = 1 mm.

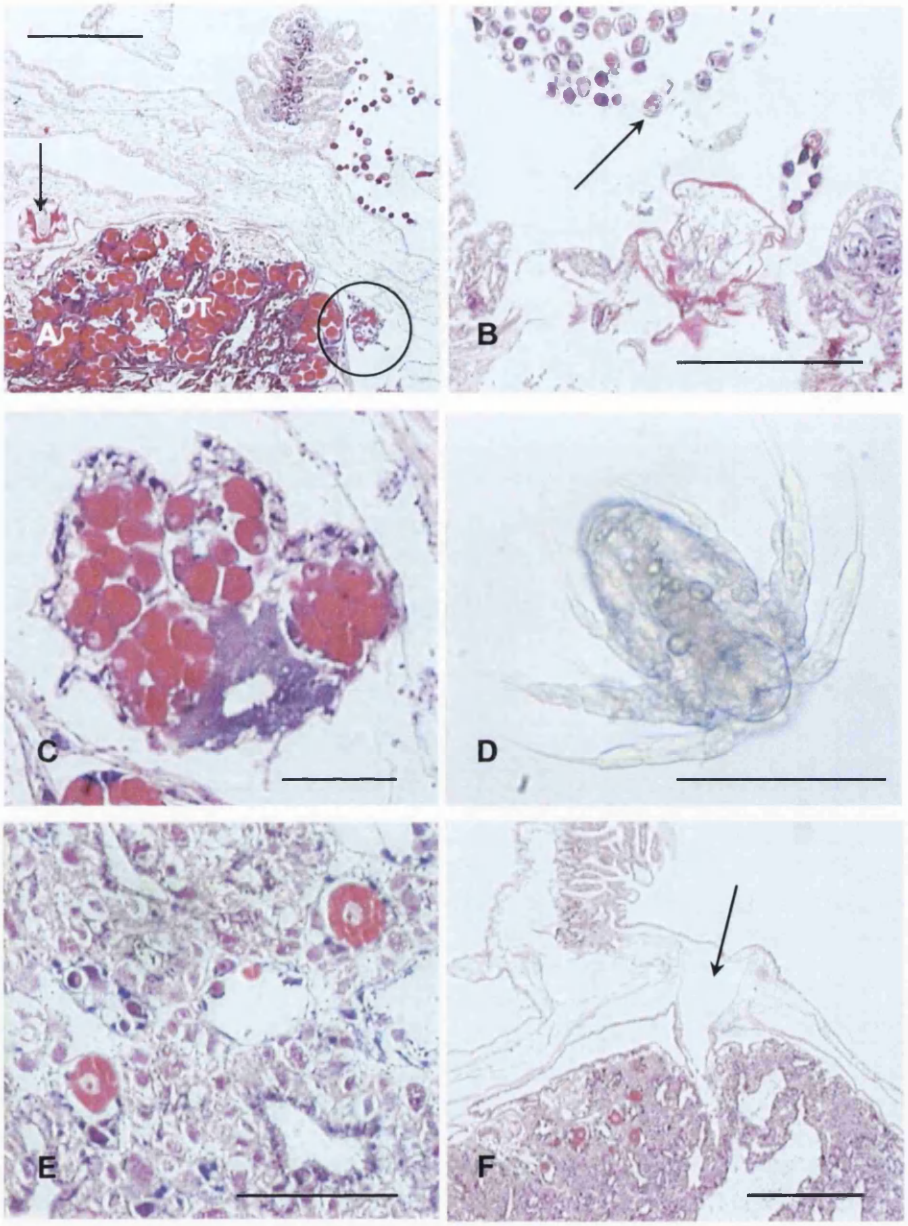

Figure 6.2. *Anadara gibbosa* infected with *Splandnotrophus willemi*. **A.** One lateral process of a *S. willemi* female supporting mature oocytes, circled; and the cephalothorax of the male, arrow. Note the healthy ovotestis of the host. **B.** The urosome of the female *S. willemi* pentrating the host's integument supporting developing nauplius, arrow. **C.** The female *S. willemi* lateral processes supporting mature oocytes. **D.** A *S. willemi* nauplii. **E.** The complete disintegration of the digestive and gonadal tissue of an infected *A. gibbosa*. **F.** *A. gibbosa* with a large cavity (arrow) suspected to have been made by a female S. *willemi*. However, in this host no parasite was found. Note the disintegration of the visceral mass. Abbreviation: OT, ovotestis. Scale bars: **A-B**; **F** = 1 mm; **C-E** = 100 μm.

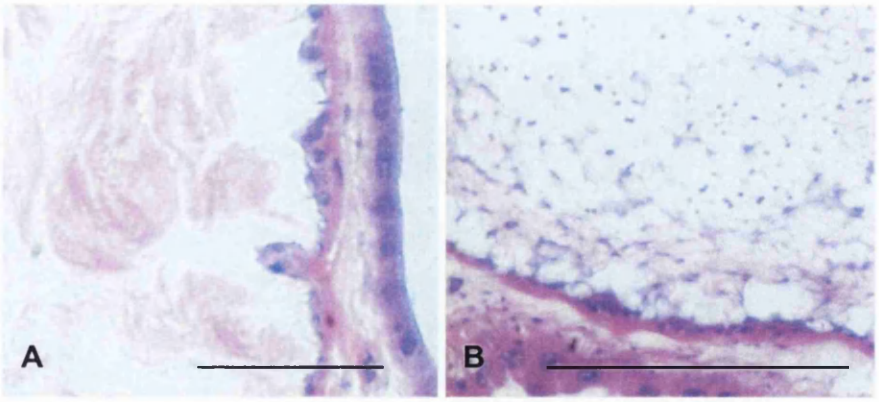

Figure 6.3. *Ancula gibbosa*. **A.** An uninfected *Ancula gibbosa* with healthy sperm imbedded within the wall of the receptaculum seminis. **B.** A parasitised *A. gibbosa* showing the deformed sperm heads. Scale bars: **A-B** = 50 μm.

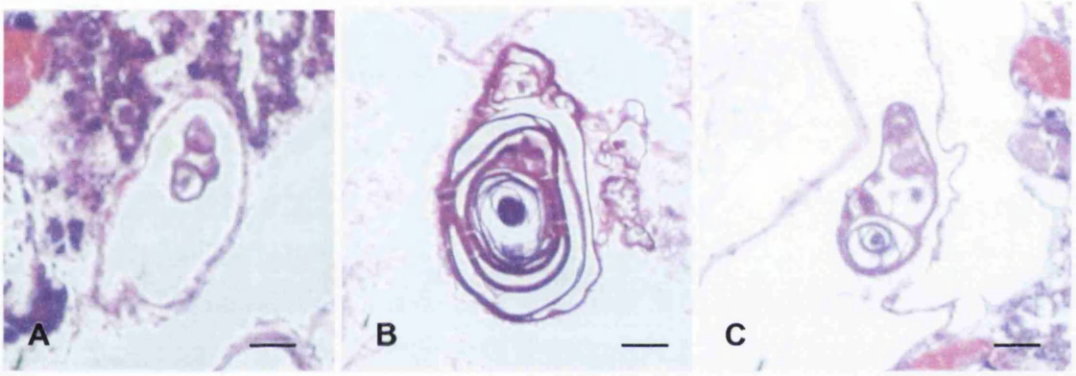

Figure 6.4. Unidentified cuticular objects. The size and shape varies considerably. **A.** The object is encapsulated within a pocket in the digestive gland of *Ancula gibbosa*. **B-C.** The objects are within the haemolymph. Scale bars: **A-C** = 50 μm.

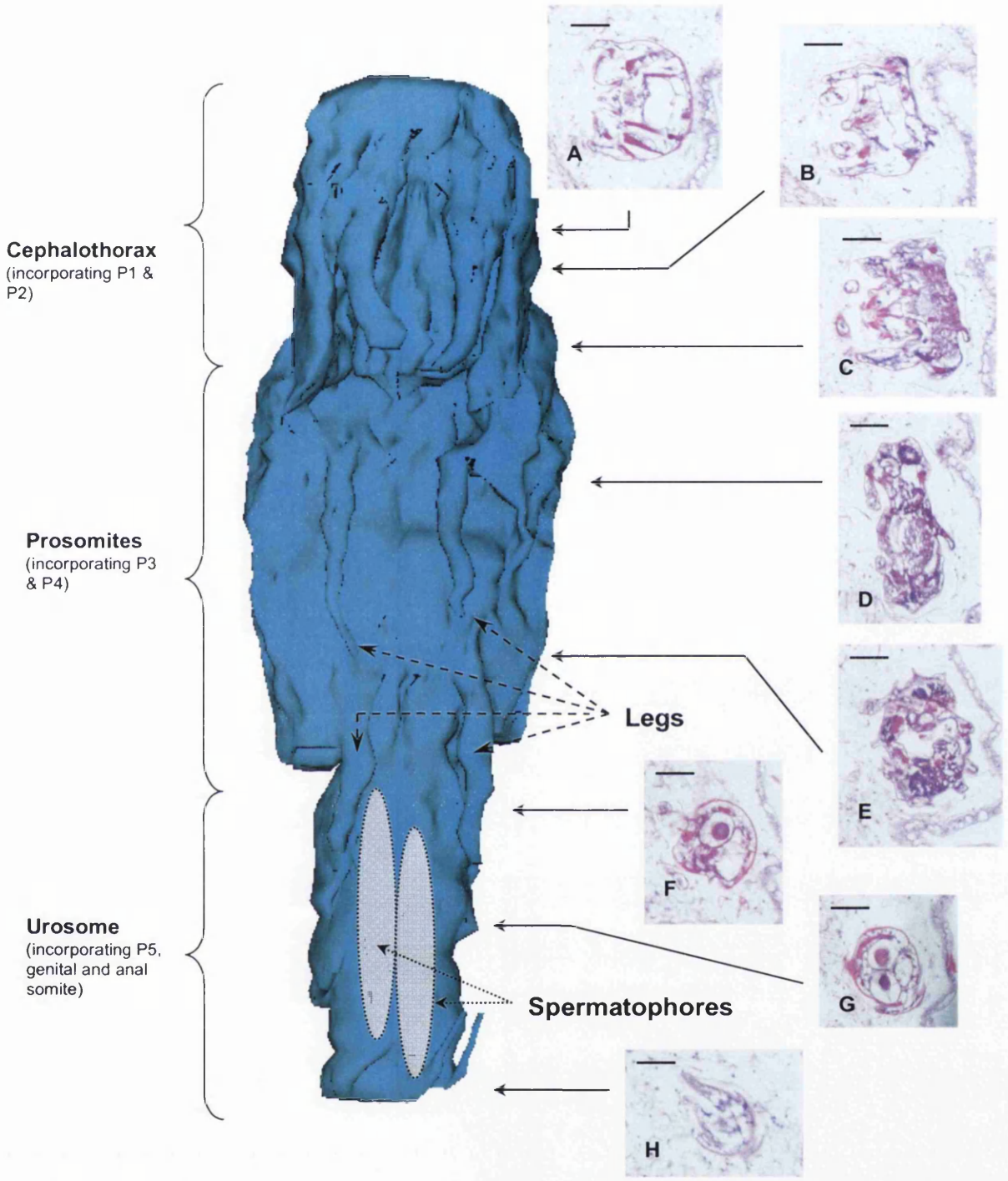

Figure 6.5. A 3D reconstruction of a mature male *Splandmotrophus villerni in situ.* Photographs show transverse sections. **A.** The start of the antenna. **B.** The antenna and the top of the mouth parts. **C.** The labrum and mouthparts. **D-E.** The prosomites. **F-G.** The spermatophores within the urosome. **H.** The caudal rami. Scale bars: **A-H** = 100 μm.

6.4 *DISCUSSION*

This is the first recorded discovery of the splanchnotrophid *Splanchnotrophus willemi* infecting the nudibranch *Ancula gibbosa* in Mumbles, South Wales, UK. This is the latest and most comprehensive report of *S. willemi* since Cuénot (1927).

The orientation of the female *S. willemi* was consistent in all *A. gibbosa* infected. This position is similar to that reported for other splanchnotrophid parasites including *Ismaila* spp., with the cephalosome orientated ventrally and the urosome orientated dorsally. In cases of ovigerous females, the urosome penetrated the host's integument close to the gills within the ring of the pallial papillae. As suggested by Hancock & Norman (1863), the location of the egg masses is such that they are continually washed by the respiratory stream of water induced by the gills of the host. Hecht (1893) commented that the position of the ovigerous sacs in *S. angulatus* alongside the host's papillae provides them with camouflage. It is more likely that the pallial papillae provides protection for the egg sacs, as they do for the gills of *A. gibbosa.*

The location of the mature male parasite was not consistent. They were not found associated with the female, instead they were located within the body wall at various positions (Table 6.1). With the exception of one host, the male *S. willemi* were always found immediately beneath the host's integument. A similar situation was described by Hancock & Norman (1863) for *S. gracilis* and *Lomanoticola brevipes* (Hancock & Norman) males. As reported by Canu (1891), *S. willemi* males exhibit a degree of free movement throughout the body of their host. Movement away from the female might have been induced by the narcosis; this however is unlikely as narcosis was fairly rapid in most cases. Close association of the male to the female is perhaps not as important for *S. willemi* in the *A. gibbosa* studied as it is in *I. androphila* as there is no competition for paternity, the host having only one male parasite and one female parasite. They may well only be associated with the female during sperm transfer.

6.4.1 *PREVALENCE OF* SPLANCHNOTROPHUS WILLEMI

The maximum number of parasites per *A. gibbosa* was two; one mature male and one mature female (Table 6.1). Mature males were never found without a mature female. These results are not consistent with most reports regarding the sex ratio of splanchnotrophid infections. Hancock & Norman (1863) noted that in one *Acanthodoris pilosa* (Müller) only

one female *S. gracilis* was present with up to twelve males, although typically there were three or four. In a single *Dendronotus iris*, 425 *I. occulata* were counted by Ho (1981). However, the large size of the nudibranch was very probably a major factor behind the high prevalence in one individual. Belcik (1981) found 64 mature female and 32 mature male *I. monstrosa* in an unknown number of *Janolus fuscus*. Similar skewed sex ratios were reported by Haumayr & Schrödl (2003). One *I. androphila* female was found associated with one to seven males in an individual *Okenia luna*; several female *I. aliena* were found with numerous males in an individual *Thecacera darwini*; up to five female *I. damnosa* were found infecting an individual *Flabellina* sp. 1 (there was no mention regarding the male prevalence); six female *I. socialis* Haumayr & Schrödl were found along with six males in an individual *Aeolidia papillosa serotina* Bergh and up to five female *I. magellanica* Haumayr & Schrödl were found in an individual *Elysia patagonica* Muniaín & Ortea.

Hemioniscus balani (Bate) is a parasitic isopod which infects the barnacle *Chthamalus dalli* Pilsbry. The planktonic *H. balani* are male, they mate with a sessile female *H. balani* located within a host, then move onto an uninfected host, settle, and become female. Infection with more than one *H. balani* is rare (Blower & Roughgarden, 1989). Blower & Roughgarden (1989) discovered that prevalence increased with host density and host spatial pattern, and hypothesized that the male *H. balani* are attracted to the females by pheromones. It is possible that a similar situation occurs in *S. willemi* in *A. gibbosa*. A resident female may produce pheromones to attract a male and stops releasing them once a male has infected her host. Due to the large size of the female and of the male *S. willemi* when mature, infection by more than one female and one male might result in detrimental effects to both host and the parasite. The cessation of the release of the pheromone may prevent infection by multiple parasites and would therefore increase the host's longevity.

The 35% prevalence of *A. gibbosa* with *S. willemi* is low when compared to the incidence of *Ismaila* spp. off the Chilean coast: *I. androphila* in the host *O. luna* - 100% infection (n = 50), *I. aliena* in *T. darwini* - 84.6% infection (n = 52), *I. damnosa* in the host *Flabellina* sp. 1 - 66.6% infection (n = 33) (Haumayr & Schrödl, 2003). However, the prevalence drops in the aeolidoidean nudibranch *Phidiana lottini* (Lesson) to three in 200 specimens infected with *I. robusta* Haumayr & Schrödl. Canu (1891) in his original description reported 21 males and five females infecting *Facelina auriculata*. However, it is not clear whether they were all found in one *F. auriculata* or if it was a cumulative total from

several hosts. When Canu (1898) reported the second account of infection by *S. willemi*, no males were found despite the common occurrence of females. Nothing was recorded regarding the infection rates in *A. gibbosa* when it was first described as a host species for *S. willemi* by Canu (1899).

6.4.2 *EFFECTS OF* SPLANCHNOTROPHUS WILLEMI *ON* ANCULA GIBBOSA

The effect of parasitism on *A. gibbosa* varied considerably. In one of the infected *A. gibbosa* sectioned, there was a complete breakdown of cellular structure within the digestive gland and the gonadal tissue. Moreover there were no obvious ovotesties; instead unassociated oocytes appeared throughout the remaining viscera. There were large cavities and no sign of spermatogenesis (Fig. 6.2E). Despite this, the ampulla and the receptaculum seminis both contained sperm. Another *A. gibbosa* displayed similar cellular damage, however there was no parasite present. In this uninfected individual there was a large cavity located on the dorsal surface which could have resulted from the expulsion of *S. willemi* (Fig. 6.2F). Observations made by Garstang (1890) indicate that it is possible for a splanchnotrophid to "reject" its host. In his description he noted the reduction in body size of *A. gibbosa* over a period of two days, perhaps this individual was near death causing the splanchnotrophid to crawl out, to avoid the same fate as its host. It is possible that a similar situation occurred with the *A. gibbosa* in this study. This animal may have been infected with *S. willemi* which had become disassociated from its host during a time of stress, or perhaps the parasite had died and consequently been expelled by the host. The cellular degradation seen within these two individuals is similar to that described by Pelseneer (1894) who noted that *F. auriculata* infected with *S. willemi* had no stomach, although there was no mention of the condition of the ovotestes. In other genera of the Splanchnotrophidae the effect of parasitism can be just as devastating. Schrödl (1997) noted the gonadal tissue next to the *Ismaila* sp. in *Flabellina* sp. 1 was either "absent or reduced", but was present in areas away from the parasite, albeit in a rudimentary form. Jensen (1987) noted a similar consequence of parasitism in the sacoglossan *Ercolania funerea* in which, the gonadal tissues were described as "almost absent" and the host as likely to have been "castrated" by its parasite. Bergh (1868) noted *I. monstrosa* within *Phidiana lynceus*, occupying the area where the sexual gland should have been, which was described as "severly atrophied". The two *A. gibbosa* described above, in their current condition would have been unable to produce viable oocytes or sperm, but had at some point been sexually active as evidenced by the presence

of sperm within their ampulla and receptaculum seminis. Schrödl (1997) discovered that once infected with *Ismaila*, *Flabellina* sp. 1 did not produce any egg masses and died prematurely. It was impossible to determine whether sexual activity continued after the infection of the *A. gibbosa* collected in this study, or whether they produced any viable egg masses prior to infection.

The remaining six infected *A. gibbosa* had no damage to the digestive gland and ovotestes were unaffected, with all stages of oogenesis and spermatogenesis occurring. Both the ampulla and receptaculum seminis contained sperm. The other sexual glands, both male and female, were present and appeared normal. However, in one infected individual the sperm within the receptaculum seminis was not embedded within the wall, instead were randomly orientated (Fig. 6.3B). The sperm heads appeared deformed and were probably sterile. Jensen (1990) noted apparently healthy reproductive organs in *Elysia australis* infected with *Arthurius elysiae*. Despite this they showed a complete disinterest in copulating even when uninfected individuals attempted to copulate with them. In the original description of the genus *Splanchnotrophus*, Hancock & Norman (1863) commented that the hosts were seemingly "unconscious" of their parasites, and Hecht (1893) described similarly unaffected behaviour when a host was infected with *Splanchnotrophus*, albeit accompanied by a lack of coordination by the host. The behavioural effects of *S. willemi* on *A. gibbosa*, were not in this study, and have not previously been observed.

The presence of sperm within the ampulla and the receptaculum seminis proved that all hosts had had sex at least once during their lifespan. They would almost certainly have been able to produce viable spawn had copulation occurred prior to infection, thereby ensuring at least some reproductive success at Mumbles. It is possible that, as with *I. monstrosa* and *A. elysiae*, once infected with *S. willemi*, spawning by *A. gibbosa* ceases, and or that its behaviour is adversely affected thus preventing future sexual encounters. This would have an inevitable impact on the numbers of viable offspring recruiting into the next generation.

Contrary to other findings, the size of infected *A. gibbosa* was similar to those uninfected. Canu (1891) noted that the female *S. willemi* impaired the growth of the mollusc's internal organs as the host approached sexual maturity. Garstang (1890) noted a dramatic reduction to half its original body size of a parasitized *A. gibbosa* over two days. In this study the infected *A. gibbosa* displayed no signs of reduced internal organ growth. The

only indications of infection by *S. willemi* were the female's prosome and the lateral processes inserted around the viscera which were visible through the translucent epidermis, and, the protruding egg masses between the pallial papillae when the female *S. willemi* was ovigerous. A similar observation was made by Jensen (1987) in *E. funerea* in which the somatic growth was unaffected despite the host lacking gonadal tissue. Hancock & Norman (1863) commented that those *Doto coronata* (Gmelin) infected with *L. brevipes* were of a normal size, and that they should live the typical life expectancy regardless of infection.

6.4.3 *MALE* SPLANCHNOTROPHUS WILLEMI

A 3D reconstruction of a mature male *S. willemi in situ* was generated using the freeware Reconstruct©. Despite some lack of detail due to the limitations of the programme and the likelihood of minor deformations caused by the preservation and sectioning within its host, it is similar in morphology to the male *S. angulatus* drawn by Huys (2001). In his paper he commented that *S. willemi* and *S. angulatus* could be conspecific. However the new material obtained in this study, shows that this is extremely unlikely. The cephalothorax cannot be compared due to the low resolution. However, the location of the spermatophores within the prosome extending a considerable way through the urosome is not seen in the *S. angulatus* male figured by Huys (2001). When describing the mature male *S. dellachiajei*, Debouttevillle (1951) documented elongated spermatophores that extend through the urosome, similar to *S. willemi.*

6.4.4 *AMBIGUITY OF EARLY PAPERS DESCRIBING* SPLANCHNOTROPHUS WILLEMI

When *S. willemi* was first described, Canu (1891) used several criteria to define the species, including the shape and the colour of the egg masses. In the *S. willemi* infecting *A. gibbosa* from Mumbles, the egg masses were white, not the pale rose reported by Canu (1891), and they were sickle shaped rather than the crescent shape he described. However, they were joined to the urosome midway along the length of the mass, a feature which is characteristic of splanchnotrophid species (Huys, 2001). Canu's (1891) original description of *S. willemi* lacks detail; important morphological features of the parasite are missing, and no drawings were made, thus the description should be interpreted with caution.

It is interesting to note that for each *Ismaila* spp., *Ceratosomicola sacculatus* and *Arthurius elysiae* the host-parasite relationship is very specific, with only one host species identified. However with regards to other members of the Splanchnotrophidae, this is not the case. *S.*

angulatus is known to infect three species of aeolid nudibranch, *S. dellachiajei* is known to infect four species, and *S. gracilis* is known to infect two. *L. brevipes* has the widest known host range, infecting seven different host species. In their review Monod & Dollfus (1932) commented that *S. willemi* is not host specific, and that this is unusual for a parasite. During this study several *F. auriculata* were collected at the same time as *A. gibbosa*, and sectioned for light microscopy. *S. willemi* was first described in the former species. If prevalent at Mumbles; one might assume that the parasite would have also been discovered in *F. auriculata.* However, the *F. auriculata* sectioned were free from splanchnotrophid infection. The lack of detail in the first description by Canu (1891) is such that the two species could be parasitized by two different *Splanchnotrophus* spp., although until the parasite can be sampled from *F. auriculata* and an accurate description made, the identity of Canu's splanchnotrophid will remain uncertain

The understanding of the relationship between the enigmatic poecilostomatoids *Splanchnotrophus* spp. and marine opisthobranch molluscs is still in its infancy; *S. willemi* infecting *A. gibbosa* is no exception to this. With a moderate prevalence of 35%, compared to that of other Splanchnotrophidae, *S. willemi* may have a significant effect on the population at Mumbles, despite the apparent sexual activity of the *A. gibbosa* sectioned. If these infected animals produced a minimum of one viable spawn mass, it is unlikely that this would be enough to ensure the continuation of the population into the next generation. *A. gibbosa* was once noted as being the most common nudibranch of Northumberland, England (Alder & Hancock, 1845-1855). More recently, however, it has been noted for its sporadic occurrence around the UK (Thompson & Brown, 1984). This could, in part, be attributable to infections by *S. willemi.* A similar situation has occurred at Mumbles where, since August 2003, there has been no population of *A. gibbosa* despite regular extensive searches. Perhaps the evolutionary relationship between *A. gibbosa* and *S. willemi* is still young and the effect of the parasite on its host results in significant fluctuations in the numbers of adults spawning. In an extreme case this may result in local populations becoming extinct; these habitats would then rely upon recruitment from other populations to become reestablished, thus resulting in sporadic occurrence. The population genetics of *A. gibbosa* would prove a valuable insight into understanding the recruitment and gene flow of these animals. However until the population is once again present at Mumbles, and/or

other populations of *A. gibbosa* are discovered, the full implications of *S. willemi* on the reproductive effort of its host will remain a mystery.

Chapter 7

Chapter 7

Akera bullata (Müller) veliger settlement preferences and juvenile development.

7.1 *INTRODUCTION*

Until recently the biology and classification of *Akera bullata* (Müller) was contested even after evidence on the alimentary canal presented by Morton & Holme (1955) demonstrated that it should be placed within the Anaspidea rather than the Cephalaspidea. Today it is recognised as a member of the Anaspidea, albeit a primitive one. To date there have been two described variants or subspecies, var. *nana*, the dwarf variety from Balta Sound Shetland, originally described by Jeffreys (1867), and var. *farrani* from Galway Ireland, described by Norman. The former is distinguishable from var. *farrani* only by its small size (Jeffreys, 1867). Populations of *A. bullata* have been found throughout the British Isles from the English Channel, Portland Harbour, Plymouth Sound, Wales to Scotland (Thompson, 1976; Thompson & Seaward, 1989). Further afield they have been reported from Norway, Denmark, Baltic Sea, North Sea, Atlantic, the Mediterranean coasts of France, Italy, and Spain; the Greek Ionian and the Aegean Sea (Thompson, 1976; Thompson & Seaward, 1989). The existence of the two variants has resulted in a great deal of controversy and confusion, not helped by *Akera* exhibiting intermediate morphologies which can often be found in the same habitat (Thompson & Seaward, 1989).

Morton & Holme (1955) doubted that size alone was sufficient to prove the existence of the two forms var. *nana* and var. *farrani*. They ascribed the *A. bullata* found at Devonport Plymouth, which they used in their study, as the species originally described by Müller. Thompson & Seaward (1989) and Thompson (1988) list several distinct features of the two variants that enable them to be distinguished. Var. *nana*, found at Langton Hive Point (LHP), east Fleet, reach sexual maturity at 6-10 mm and rarely exceed 16 mm shell length; they only swim as juveniles, possess an obtusely rounded posterior pallial lobe (usually without the posterior pallial tentacle), and lack the ability to produce purple ink (they die

before the gland develops). Var. *farrani* is larger, reaching 40 mm shell length, swims in the adult stage, often posses a long finger-like posterior pallial lobe and is able to secrete purple ink. Despite these listed differences, Thompson & Seaward (1989) comment that all British records of *A. bullata* should be grouped under *Akera bullata* and that the variants *nana* and *farrani* should remain subspecific. Hereafter only *A. bullata* will be used except when in reference to Thompson & Seaward's (1989) and Jeffreys' (1867) animals.

7.1.1 *AKERA BULLATA* BIOLOGY

Jeffreys (1867) found *A. bullata* var. *nana* on "oozy ground" in mudflats and areas of *Laminaria,* often in estuaries; in the Fleet, Thompson & Seaward (1989) documented *Akera bullata* var. *nana* feeding on *Enteromorpha* from May through to summer. After this they were found amongst eel grass, *Zostera* and *Ruppia,* or on the bottom of the lagoon (which is covered by fine muddy sediment). Thompson (1976) stated that *A. bullata* is herbivorous and that fragments of *Zostera* could be found within the faeces. Thompson & Seaward (1989) later contradicted this earlier report when studying *A. bullata* var. *nana* from the Fleet, as they found "no evidence that it attacked the leaves of *Zostera* or *Ruppia*", however they did state that the roots could be eaten. Morton & Holme (1955) noted the *A. bullata* used for their study were grazers of *Ulva* or possibly deposit feeders.

The swimming of *A. bullata* has been documented by Morton & Holme (1955) who provide detailed descriptions of the mechanisms by which is does so. However, once again in this strangely confusing species the period of swimming appears to differ between studies. Morton & Holme (1955) concluded that swimming of both adults and juveniles occurs mainly during the spring when spawning occurs, this link however is only tentatively suggested. They further mentioned that swimming is sometimes observed in the summer. Thompson (1976) noted that Sykes' claim of swimming occurring mainly in juveniles, conflicts with other accounts linking swimming to the breeding season.

One of the main criteria Thompson (1976) and Thompson & Seaward (1989) used to identify var. *nana* was the inability to secrete purple ink. It was suggested by Thompson & Seaward (1989) that the precocious maturation of *A. bullata* var. *nana* in the Fleet resulted in death before the purple gland became functional. Other *A. bullata* collected elsewhere had the ability to produce ink (Jeffreys, 1867; Morton & Holme, 1955; Thompson & Seaward, 1989). Ink production is common to *Aplysia, Akera, Dolabella, Bursatella,* and *Stylocheilus.* Despite its common occurrence, the function of the ink and how it affects predators has

only been recently understood. In *A. bullata* the ink is produced within the purple gland located within the pallial cavity on the dorsal surface. The purpose of ink production and the mechanisms by which it is created in *Akera* has not yet been described, however extensive studies on chemical defense using sea hares, *Aplysia* spp. has been published. In sea hares the purple pigment, aplysioviolin, is an ester derived from phycoerythrobilin which is obtained from their diet of red seaweeds (Chapman & Fox, 1967). When *Aplysia* is fed only brown algae the animals lose their ability to produce aplysioviolin after 14 days. When returned to a red algal diet they regain the ability to produce aplysioviolin after 72 hours. Without a source of phycoerythrobilin in their diet the aplysiids become "facultatively de-inked" (Chapman & Fox, 1967). Thompson & Seaward (1989) make no statement about whether the diet of the var. *nana* collected from LHP contains red seaweeds, the absence of which could be the cause of the ink deficiency rather than the immaturity of the purple gland.

7.1.2 *REPRODUCTIVE BIOLOGY*

Akera bullata is hermaphroditic; but as with to other opisthobranch molluscs they copulate with other individuals and cannot self fertilise. Morton & Holme (1955) suggested that *A. bullata* at Devonport, Plymouth, are semelparous with an annual life cycle, and consequently die after spawning. Evidence gathered by Thompson & Seaward (1989) supports this view. Spawning principally occurs in the spring and early summer, although this is dependent on location. In the English Channel it is reported to occur from April-July (Morton & Holme, 1955); in Ireland spawning has been observed in April, September and November (Renouf (1935) and Sykes (1905); cited in Thompson (1976)).

Thorson (1946) gave the only detailed account of the larval biology of *A. bullata*, in Denmark. Here the adults were reported to spawn throughout the period April-May to October. Thorson (1946) was cited by Thompson (1976) as stating that the embryonic period is 30 days at 15°C or 20 days at 20°C. According to Thompson (1976) the veliger larvae possess a Shell-type 1 and a Development-type 2 (lecithotrophic veligers). Thorson (1946) however, notes that the shell is similar to "Tergipedids" which he classified as having a Type C shell (equivalent to Thompson's Shell-type 2), and that they hatched with a shell length of 300-375 μm. Thorson (1946) also commented that growth as a veliger was "inconsiderable", that the time spent in the plankton is "short", and that the veligers were found from May-December with peaks in July, August and September.

7.1.3 *VELIGER DEVELOPMENT AND SETTLEMENT IN THE ANASPIDEA*

A. bullata along with many other species of opisthobranch mollusc undergoes metamorphosis, a process that changes the free-swimming planktonic veliger into an adult which is suited for life in the benthos. Thorson (1946) described and illustrated veligers and newly metamorphosed *A. bullata.* His specimens were sampled by bottle collectors near Ven in Øresund, Denmark, at all stages of development. However nothing was recorded regarding settlement preferences.

Members of the Anaspidea typically produce planktotrophic veligers which spend approximately one month or more feeding and developing prior to metamorphosis. The exception is *Phyllaplysia taylori* Dall, a direct developer, which undergoes metamorphosis whilst still within the egg mass and therefore settles immediately on hatching (Bridges, 1975). Current literature does not document any member of the Anaspidea producing lecithotrophic veligers except *Akera bullata*, therefore, the development of lecithotrophic nudibranchs as well as the veliger development of aplysiids will be discussed.

On hatching, aplysiid veligers are metamorphically incompetent. They are obligate planktotrophs and need to feed in order to develop the features required for metamorphosis. The time spent in the plankton is divided into two stages: rapid shell growth, followed by no shell growth. During shell growth the velum enlarges, the larval heart develops and the eyes are formed. Once the veliger has reached a species-specific shell size, growth stops and other features required for metamorphosis develop. The propodium is formed, followed by the enlargement of the left branch of the digestive gland and eyes, then the mantle fold pulls back from the shell aperture (Switzer-Dunlap & Hadfield, 1977). Having achieved competency, the veligers change their swimming behaviour and spend more time close to the bottom (Switzer-Dunlap & Hadfield, 1977). They can maintain this state for a prolonged period of time. Switzer-Dunlap (1978) documented *Stylocheilus longicauda* Quoy & Gaimard metamorphosing four weeks after attaining competency, and Kempf (1981) reported on the ability of *A. juliana* Quoy & Gaimard to survive 316 days, although the numbers surviving beyond metamorphosis was low. Once a veliger has detected a suitable substratum, typically that constituting the adults diet, they settle and commence crawling. Metamorphosis commences after a period of approximately one hour. If competency has not been reached, or the substratum is unsuitable for metamorphosis, the veliger recommences swimming (Switzer-Dunlap & Hadfield, 1981). Nadeau *et al.*

(1989) isolated a "competence factor" for *A. californica* Cooper, they failed to identify its molecular formula, however they concluded that it was between 30-100 KD.

It is well documented that for many species of opisthobranch mollusc chemical cues are required to promote settlement (Thompson, 1958; 1962; Hadfield & Karlson, 1969; Switzer-Dunlap & Hadfield, 1977; Strenth & Blankenship, 1978; Hadfield & Scheuer, 1985; Albert Hubbard, 1988; Nadeau *et al.*, 1989; Pawlik, 1989; Gibson & Chia, 1994; Lambert & Todd, 1994 etc). These are often soluble compounds released by the adult's prey. Switzer-Dunlap & Hadfield (1981) noted that for several *Aplysia* spp., the substratum required for metamorphosis was not specific, i.e. that several different species of seaweed would induce metamorphosis. However for long-term survival only one or two species of seaweed were suitable. Settlement experiments by Switzer-Dunlap & Hadfield (1977) showed that the veligers of *A. juliana*, metamorphosed on the algae *Ulva fasciata* Delile and *U. reticulata* Forskål but failed on other closely related algal species; however post larval development was only successful on *U. fasciata. S. longicauda* will settle upon the blue green alga *Lyngbya majuscula* (Lyngbya) and three species of red algae, but only *L. majuscula* induced post larval growth (Switzer-Dunlap & Hadfield, 1977). *A. dactylomela* Rang settled on many different red algae but settlement on *Laurencia* resulted in the greatest post larval success (Switzer-Dunlap & Hadfield, 1977). Kriegstein *et al.* (1974) discovered only *Laurencia pacifica* Kylin induced settlement and metamorphosis in *A. californica.* Pawlik (1989) later contradicted Kriegstein *et al.* (1974) by showing that *A. californica* metamorphosed in response to several different species of algae, however, *Rhodymenia* promoted the highest number of veligers to settle. Unlike earlier studies, metamorphosis occurred without a stimulus (Pawlik, 1989). *A. parvula* was found to metamorphose on *Chondrococcus hornemanni* (Lyngb.) Schmitz (Switzer-Dunlap, 1978). Plaut *et al.* (1995) followed the growth and metamorphosis of *A. oculifera* Adams & Reeve: settlement was most successful on *Dasia* sp. although metamorphosis was induced by six out of the 12 species of algae tested. However, post-larval development was dependent upon the consumption of either *Enteromorpha intestinalis* Link, or *Ulva* spp. Switzer-Dunlap & Hadfield (1977) showed in their study that *Dolabella auricularia* Lightfoot metamorphosed on a variety of different algal species, but post-larval development was most successful when the juveniles fed on an unidentified mat-forming blue-green alga. Paige (1986) managed to rear both *A. brasiliana* Rang and *Bursatella leachii plei* Rang successfully through metamorphosis using an unspecified red alga and a blue-green alga

respectively. Further experiments by Paige (1988) identified the blue-green alga as a member of the family Oscillatoriaceae.

With the exception of *P. taylori* (Bridges, 1975), the process of aplysiid metamorphosis is similar for all species (Kriegstein *et al.*, 1974; Switzer-Dunlap & Hadfield, 1977; Switzer-Dunlap, 1978; Paige, 1988). Once committed to metamorphosis, the veligers stop crawling and retract either partially or completely into their shell. They remain in this state for the majority of the period of metamorphosis, which can last between two and four days (Switzer-Dunlap, 1978). The velar cilia are shed, and the velum is reabsorbed. The oral tentacles start to develop at the site of the velum. Crawling is often resumed one to two days after the commencement of metamorphosis. The adult heart then develops, taking over in function from the larval heart which regresses (Switzer-Dunlap & Hadfield, 1977). During metamorphosis, feeding ceases despite the ability of the juveniles to rotate the radula and buccal mass (Kriegstein *et al.*, 1974). Approximately three days after metamorphosis is complete, the juveniles are able to bite and swallow (Kriegstein *et al.*, 1974; Switzer-Dunlap & Hadfield, 1977; Switzer-Dunlap, 1978). This process is quicker for *B. leachii plei*, in which feeding was observed one day after metamorphosis (Paige, 1988).

7.1.4 *VELIGER DEVELOPMENT AND SETTLEMENT OF LECITHOTROPHIC NUDIBRANCHS*

For lecithotrophic veligers the time spent in the larval stage is reduced, and the features required for metamorphosis are typically attained shortly after hatching. Feeding is not required for metamorphosis to occur, and the generation time is usually short. Hadfield (1978) and Bonar & Hadfield (1974) investigated settlement in the tropical nudibranch *Phestilla sibogae*, Bergh. The veligers hatched out eight days post-fertilisation and became metamorphically competent between one and four days later. This is strongly temperature dependent (Hadfield, 1978). After hatching the propodium becomes inflated and the mantle fold retracts away from the shell wall. During this time the shell of the veliger does not grow (Bonar & Hadfield, 1974).On attaining competency, the veligers alter their swimming behaviour and become negatively phototactic (Bonar & Hadfield, 1974). They will only metamorphose when exposed to a soluble cue released by two species of the coral *Porites*, the prey of the adult *P. sibogae*. The cue is very specific and without it the larvae will not settle and eventually die (Hadfield, 1978; Hadfield & Scheuer, 1985). If the eggs are exposed to the settlement cue prior to hatching and for one day after, the larvae become

desensitized to the cue and fail to metamorphose. This desensitization can be reversed when placed into "clean" sea water and then re-exposed to the cue (Hadfield & Scheuer, 1985). Exposure to *Porites compressa* Dana for a period of 12 hours will stimulate metamorphosis. Metamorphosis starts with the ingestion of the velar cilia and the absorption of the velar lobes. Following this, the veliger begins to pull out of the shell by contracting the larval retractor muscle violently, this severs the connection between larva and shell. During this process crawling may occur, although, typically they remain stationary whilst the shell is cast off. Attachment to the substratum is essential for shell casting to occur. The process of detorsion follows which includes the thickening of the definitive epidermis and the orientation of the visceral mass ventrally (Bonar & Hadfield, 1974; Hadfield, 1978). The adult features including the rhinophores, cerata, and oral tentacles develop later. Several abiotic compounds have been shown to induce metamorphosis in *P. sibogae*: many compounds with a choline constituent are effective inducers namely, succinyl choline chloride, actinomycin D, puromycin and cycloheximide (Hadfield, 1978).

Carroll & Kempf (1990) working on the nudibranch *Berghia verrucicornis* (Costa) found that the hatching of the lecithotrophic veligers occurred between nine and 14 days after oviposition. Following a period of one to three days as a swimming veliger, they stopped swimming and commenced crawling. Settlement onto a substratum is not required for metamorphosis to occur. During metamorphosis the velum was lost and the shell cast off. The body then assumes the adult vermiform shape. Greater numbers of veligers metamorphosed in the presence of the anemone *Aiptasia pallida* (Verrill) or a bacterial film. *B. verrucicornis* exhibits poecilogony under certain culture conditions, resulting in the formation of lecithotrophic and direct developing offspring within the same egg mass. Those masses agitated by aeration produced only lecithotrophic veligers, however those left undisturbed produced a mixture. Carroll & Kempf (1990) suggested that agitation could influence metamorphosis directly, or could stimulate factors intrinsic in the larvae which are responsible for competence and or metamorphosis.

7.1.5 *AIMS OF STUDY*

In this report the settlement cues of *Akera bullata* gathered from LHP, the Fleet Dorset, were investigated using three different species of algae, along with four different species of phytoplankton, and combinations of both. Here, for the first time, a member of the Anaspedia is reported to produce Development-type 2 larvae (Thompson, 1967). The

veligers were followed through metamorphosis and their survivorship recorded. Common documented features supporting the case for var. *nana* being considered a subspecies were also investigated.

7.2 *MATERIALS AND METHODS*

Akera bullata spawn was collected from Langton Hive Point, Fleet, Dorset (050°37'51"N 002°33'28"W), in June 2003 along with their associated algal substrata: *Ulva lactuca* L., *Nemalion helminthoides* (Velley) and *Chondrus crispus* Stackhouse. They were maintained in an aquarium containing re-circulated sea water at a salinity of 35.5 which originated from Swansea bay. The temperature in the wet room varied from 18 to 27°C. The L:D cycle was 12:12 hours.

7.2.1 SPAWN CULTURE

Each spawn mass was placed into a sterile 100 ml glass beaker containing 50 ml White Oyster Ledge filtered sea water (see Chapter 3). It was filtered using a Sartorius™ pressure filter composed of a 293 mm sanitary flange holder connected to a 40 litre pressure tank. Cellulose acetate membrane filters were used with a pore size 0.45 μm (cat. number 11106-293). In accordance with the methodology of Switzer-Dunlap & Hadfield (1981) 0.6 g Penicillin G (Sigma™ P-7794) and 0.5 g Streptomycin Sulphate (Sigma™ S-9137) were added to 100 ml of 0.45 μm filtered sea water. 1 ml aliquots of the antibiotic solution were frozen and defrosted when needed. 0.5 ml was added to each beaker after every water change to create a final concentration of 60 μg ml^{-1} Penicillin G and 50 μg ml^{-1} Streptomycin Sulphate. A 0.41 μm Millipore™ nylon mesh (Ø 47 mm, catalogue number: NY4104700) was carefully floated on the meniscus to prevent rafting of the veliger larvae, thus reducing mortality. Parafilm™ was used to cover the beakers to prevent evaporation. The water was changed every alternate day. The beakers were kept in a constant temperature room maintained between 18 and 20°C, with a L:D cycle of 12:12 hours.

Once hatched, the veligers swam in the water column. They were observed under an Olympus S40 binocular microscope; the healthy veligers were removed using a Pasteur pipette and transferred to sterile Sterlin™ 60 ml plastic containers. Each vessel contained a different substratum (listed below), to investigate the cues required for settlement.

7.2.2 *SETTLEMENT EXPERIMENTS*

To investigate whether *A. bullata* veligers prefer certain substrata for settlement, and whether post larval success is dependent upon particular substrata, each of the plastic vessels contained a different variable. The species of algae used for the study were dominant in the Fleet at the time of the spawn collection, and the phytoplankton species were the same as those used in Todd's (1981) culture of *Onchidoris bilamellata* (L.). See Table 7.1 and 7.2 for the experimental conditions used. For details regarding phytoplankton culture see Chapter 3. Experiment 1 was replicated, the replicates were called group A and group B. Experiment 2 was only performed once, due to a lack of veligers.

All culture vessels were left in a constant temperature room maintained at between 18 and 20°C with a L:D cycle of 12:12 hours. Every third day the water was changed and dead animals removed. The vessels were observed daily under an Olympus S40 binocular microscope for evidence of metamorphosis. The metamorphosed *A. bullata* were easily distinguished from the free swimming veligers as they had settled on the bottom of the vessel or directly onto the substratum, and had begun to absorb their velum. The juveniles were carefully transferred to sterile Nunclon™ multidishes with six wells (catalogue number 152795). A maximum of four individuals were placed into each well with their respective substratum. Phytoplankton was added to those veligers in experiment 2, at a concentration of 25 cells μl^{-1}. The multidishes were placed back into the constant temperature room and the sea water was replaced twice a week. At each water change the multidishes were vigorously cleaned to remove the biofilm. The sea water used thereafter was taken from the aquarium and was not filtered. The algal substratum was changed when necessary, and dead animals were removed immediately. Once the animals reached approximately 1 mm in body length they were transferred into Sterilin™ 60 ml sterile plastic containers with their respective substratum. The appropriate phytoplankton species was added to the veligers in experiment 2 (Table 7.2), at a concentration of approximately 100 cells μl^{-1}. Only two animals were placed in each pot. They were filled with aquarium sea water, which was changed twice weekly. The pots were inspected daily to ensure dead animals were removed immediately. Their food was changed to *Zostera* gathered from the Fleet on 15 September 2003. Records were taken when the veligers metamorphosed, and when they died.

EXPERIMENT 1	CULTURE VESSEL NUMBER (EACH CONTAINED 20 VELIGERS)								
CONDITIONS	1	2	3	4	5	6	7	8	9
C. crispus	✓	×	×	×	×	×	×	×	×
N. helminthoides	×	✓	×	×	×	×	×	×	×
U. lactuca	×	×	✓	×	×	×	×	×	×
Rhinomonas	×	×	×	✓	×	×	×	×	×
Chaetoceros	×	×	×	×	✓	×	×	×	×
Tetraselmis	×	×	×	×	×	✓	×	×	×
Isochrysis	×	×	×	×	×	×	✓	×	×
*Biofilm	×	×	×	×	×	×	×	✓	×
Control	×	×	×	×	×	×	×	×	✓

Table 7.1. The combinations of conditions used in experiment 1. The first three are species of algae and the following four are species of phytoplankton. Only one condition was applied to each vessel, each vessel contained 20 *Akera bullata* veligers. *The biofilm was created on a microscope slide which had been left in an aquarium for two to three days containing flowing re-circulated sea water.

EXPERIMENT 2

	CULTURE VESSEL NUMBER (EACH CONTAINED 30 VELIGERS)											
CONDITIONS	1	2	3	4	5	6	7	8	9	10	11	12
Rhinomonas	✓	✓	✓	×	×	×	×	×	×	×	×	×
Chaetoceros	×	×	×	✓	✓	✓	×	×	×	×	×	×
Tetraselmis	×	×	×	×	×	×	✓	✓	✓	×	×	×
Isochrysis	×	×	×	×	×	×	×	×	×	✓	✓	✓
C. crispus	✓	×	×	✓	×	×	✓	×	×	✓	×	×
U. lactuca	×	✓	×	×	✓	×	×	✓	×	×	✓	×
*Biofilm	×	×	✓	×	×	✓	×	×	✓	×	×	✓

Table 7.2. The combinations of conditions used in experiment 2. Each vessel contained a substratum (the bottom three conditions) and one species of phytoplankton (the top four conditions). Each vessel contained 30 veligers. *The biofilm was created on a microscope slide which had been left in an aquarium for two to three days containing flowing re-circulated sea water.

Photographs of the juvenile *A. bullata* were taken using a Wild Heerbrugg Makroskop M420 connected to a JVC TK1270 digital camera using Image-Pro Plus version 4.1 for Windows 95/NT/98.

Unfortunately the experiments ended prematurely on 29 September 2003 when the constant temperature room was inadvertently switched off, and all the juveniles died.

A G-test was used to determine whether there was a difference in the numbers of veligers that metamorphosed under experimental conditions compared to control

conditions. Because the data were not normally distributed a Kruskal-Wallis test was performed to test whether a particular condition promoted settlement faster than others, and if the time taken for the juveniles to die was different under different conditions. Due to limitations encountered using the statistical software SPSS™ the exact significance could not be obtained, as a result the Monte Carlo method was therefore used. In the event of a significant Kruskal-Wallis test, all unplanned pairwise comparisons were made using the Mann-Whitney *U*-test, and corrected using the Dunn-Šidák method at experimentwise error rate α (Sokal & Rohlf, 1995). In order to investigate whether the conditions the juveniles were reared under was related to the numbers that died, an $R \times C$ test of independence was preformed (Sokal & Rohlf, 1995). All of the statistical tests assumed that settlement of one veliger did not promote others to settle, i.e. that each settlement event was independent.

7.3 *RESULTS*

7.3.1 *VELIGER DEVELOPMENT AND METAMORPHOSIS*

The ova of *Akera bullata* were 154.4 ± 4.0 μm diameter (Fig. 7.1A). After a period undetermined in this study, the veligers hatched with a Shell-type 2 (Thompson, 1967) (Fig. 7.1B-D). The shell length measured 255.1 ± 13.1 μm. The veligers have a Development-type 2 (Thompson, 1967); after hatching the larvae were competent to metamorphose almost immediately. The veligers were able to feed, and did so when phytoplankton was available, despite having yolk reserves (Fig. 7.1E). Metamorphosis followed a pattern similar to other opisthobranch molluscs. Most individuals had made the transition from swimming veliger to crawling juvenile in a few hours of metamorphosis. Metamorphosis in *A. bullata* is never an entirely stationary event, and crawling is frequently observed. Initially the velar cilia were absorbed (Fig. 7.1F), followed by the absorption of the velar lobes (Fig. 7.2A). During the absorption of the velar lobes, the bi-lobed anterior end to the cephalic shield was formed. The operculum was then lost and the juvenile assumed the adult form. A larval heart was not visible at any time during the veliger stage. Statocysts were present but were difficult to identify due to the density of the tissues around the shell aperture. Movement of the buccal mass was observed within 48 hours of metamorphosis, however I was unable to determine whether food was actually swallowed.

Fig. 7.2B shows an 11 day old juvenile *A. bullata* which had undergone a period of shell growth, at this stage the buccal mass was fully functional. They obtained their brown pigment between seven and 18 days post metamorphosis, depending on the substratum

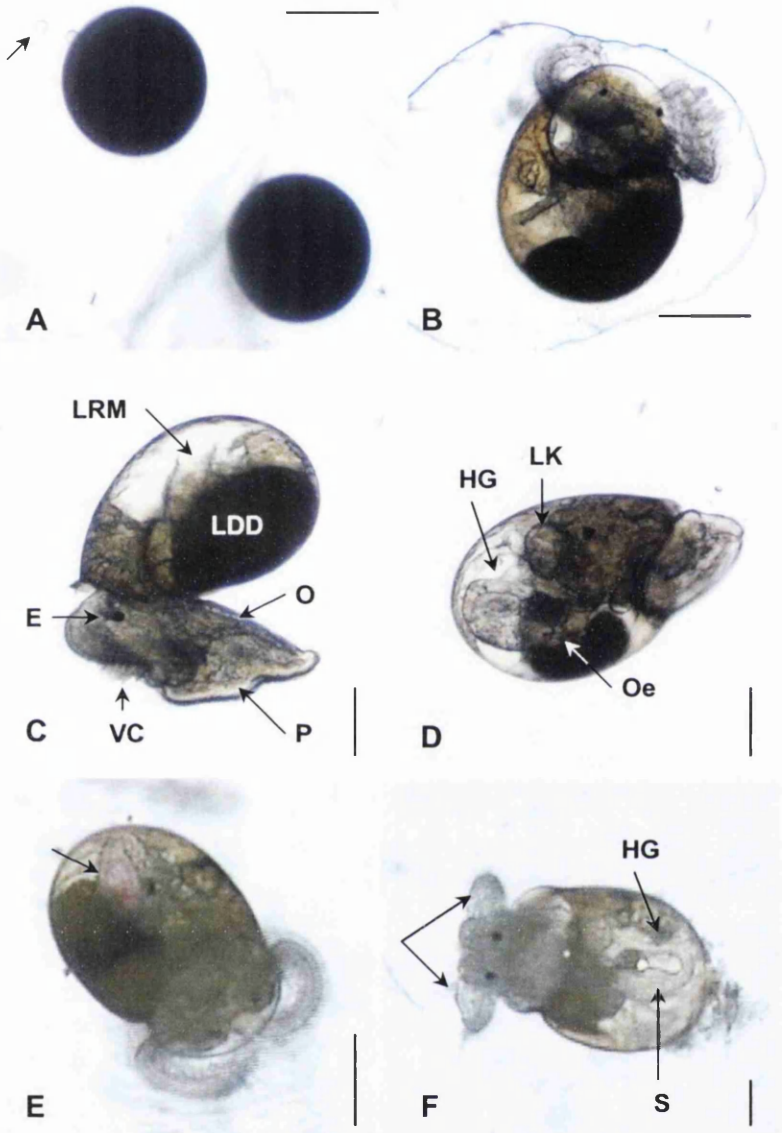

Figure 7.1. *Akera bullata*. **A.** Two ova from spawn gathered from Langton Hive Point, the Fleet, with polar bodies (arrow). **B.** A veliger prior to hatching. **C-D.** Newly hatched veliger with a Shell-type 2. **E.** A veliger showing evidence of planktotrophy, note the red pigment in the stomach (arrow). **F.** A veliger undergoing metamorphosis. Note the absorption of the velar cilia, and the formation of the bi-lobed anterior region of the cephalic shield (arrows). Scale bars: **A-F** = 100 μm. Abbreviations of labels given on page 178.

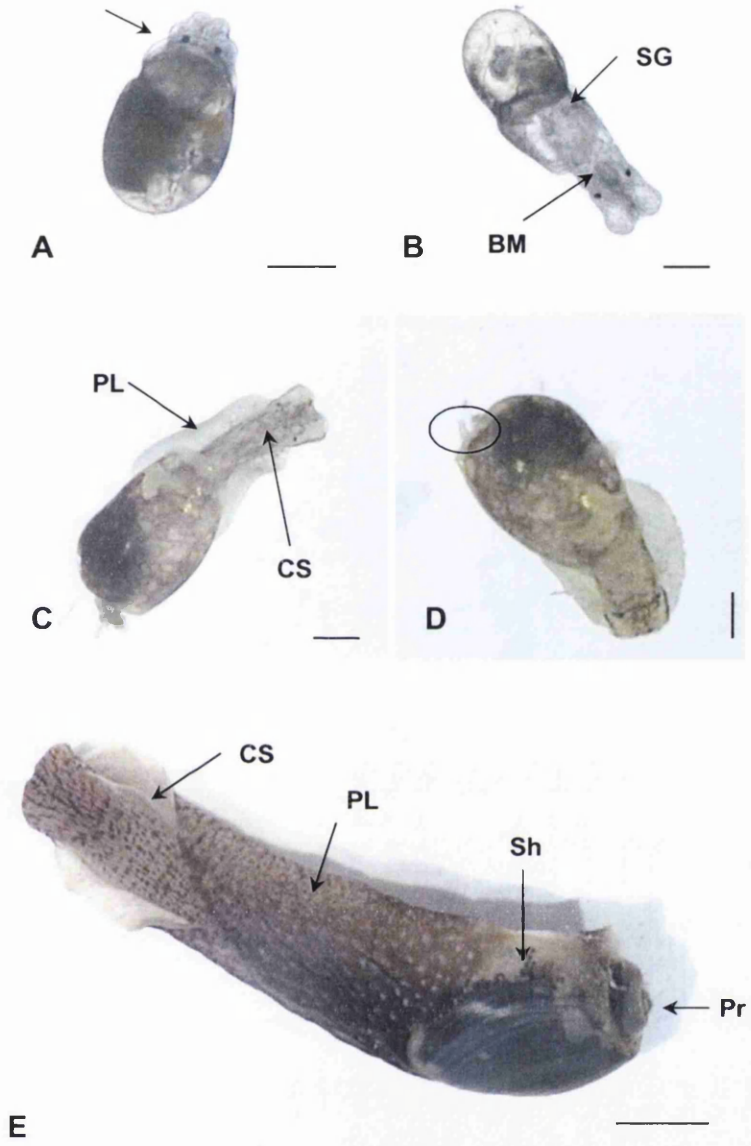

Figure 7.2. *Akera bullata.* **A.** A newly metamorphosed veliger having absorbed the velum, however the operculum (arrow) is still present. **B.** A juvenile 11 days after metamorphosis. **C.** An 18 day old *A. bullata*, the juvenile has acquired the dark pigmentation characteristic of the adult. **D.** A 21 day old showing a small filament possibly the pallial tentacle (circled). **E.** An adult gathered from the Fleet. Scale bars: **A-B** = 100 μm; **C-D** = 200 μm; **E** = 10 mm.

Figure 7.3. A juvenile shell showing similar markings to those described by Thompson & Seaward (1989). Scale bar: 500 μm.

Abbreviations to figures:

BM	Buccal mass
CS	Cephalic shield
E	Eye
HG	Hind gut
LDD	Left digestive diverticulum
LK	Larval kidney
LRM	Larval retractor muscle
O	Operculum
Oe	Oesophagus
P	Propodium
PL	Parapodial lobes
Pr	Protoconch
S	Stomach
SG	Shell growth
Sh	Shell
VC	Velar cilia

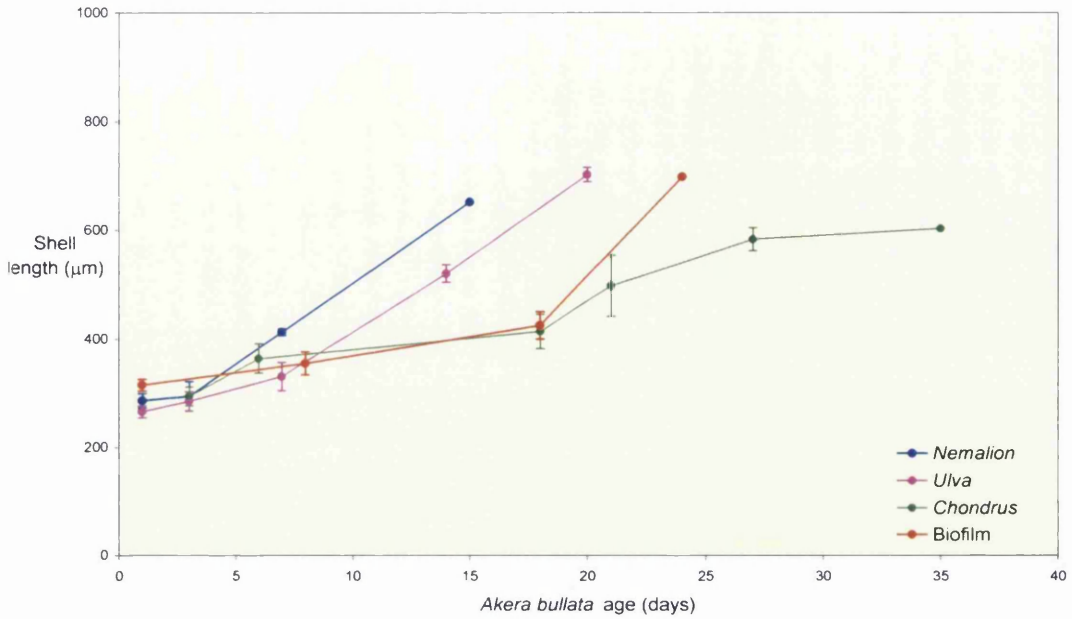

Figure 7.4. Shell growth of *Akera bullata* at 20°C from experiment 1 (*Nemalion* n= 7, *Ulva* n= 5, *Chondrus* n=10, *Biofilm* n=6). Data values represent the mean with standard deviation error bars.

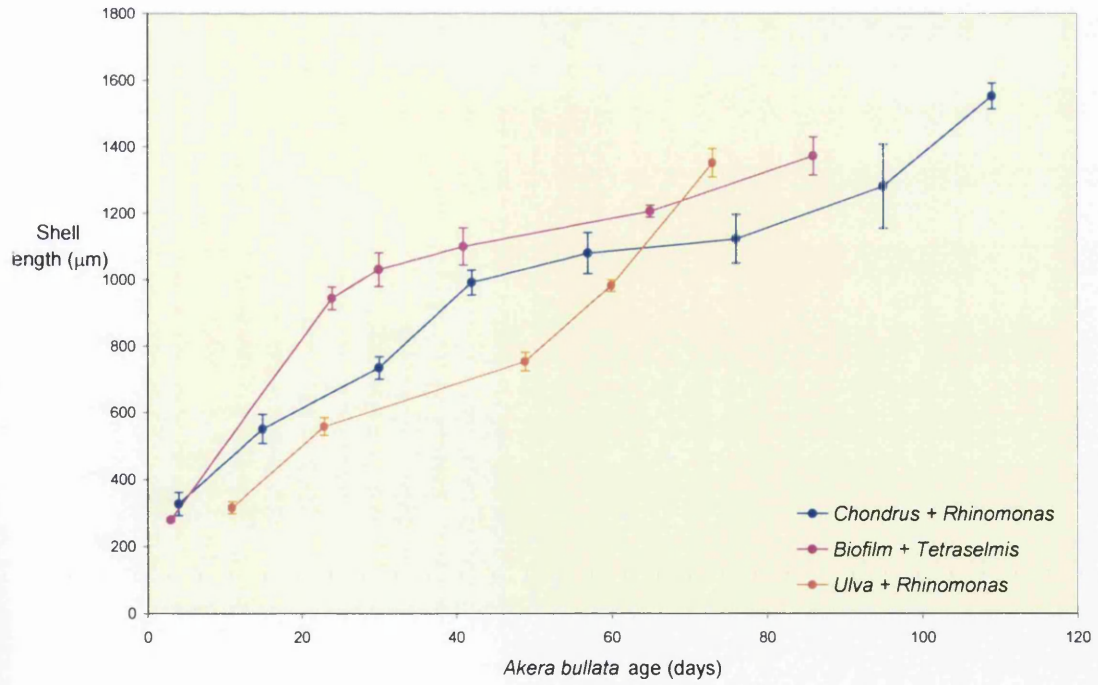

Figure 7.5. Shell growth of *Akera bullata* at 20°C from experiment 2 (*Chondrus* + *Rhinomonas* n=12, Biofilm + *Tetraselmis* n=.8, *Ulva* + *Rhinomonas* n=5). Data values represent the mean with standard deviation error bars.

they were reared upon (Fig. 7.2C). New shell growth initially formed as a collar extending from the original veliger shell. This progressively developed into whorls, which at sexual maturity contain the ovotestis. Although never reared through to sexual maturity, the juvenile *A. bullata* were miniature replicas of the adults found in the Fleet (Fig. 7.2E). The white pallial patterns of both juveniles and adults viewed through the shell were almost identical to that described by Thompson & Seaward (1989). These were due to the internal structures located within the shell rather than markings on the shell itself (Fig. 7.3). After death the shell was completely transparent.

7.3.2 *SHELL GROWTH*

Shell growth for individuals on different substrata was measured from metamorphosis until the individuals died. There was continuous growth under different experimental conditions right up until death. The veligers in experiment 2 survived longer than those in experiment 1 (Figs 7.4, 7.5).

7.3.3 *SETTLEMENT EXPERIMENT 1*

The settlement of *A. bullata* veligers was successful through metamorphosis to the juvenile stage on a variety of substrata and even in the absence of a substratum (control) (Appendix pg. 239). The number of veligers in experiment 1 group A which metamorphosed when provided with a substratum/species of phytoplankton (vessels 1-8) was significantly different to the number of veligers which metamorphosed under control conditions (in vessel 9) (*G*-test, G_{adj}= 136.714, $P<0.05$). A similar result was also produced in experiment 1 group B (*G*-test, G_{adj}= 163.756, $P<0.05$). The veligers that failed to metamorphose died between four to eight weeks post hatching; this was dependent on the availability of phytoplankton.

The number of veligers that underwent metamorphosis was highly variable between groups A and B. *Chondrus*, *Isochrysis*, and biofilm all induced a greater number of veligers to metamorphose than any of the other conditions (Figs 7.6, 7.7). The median time taken from hatching to the initiation of metamorphosis in groups A and B, was significantly different between all nine vessels (Kruskal-Wallis test, χ^2 = 73.518, $P<0.05$; and χ^2 = 68.603, $P<0.05$

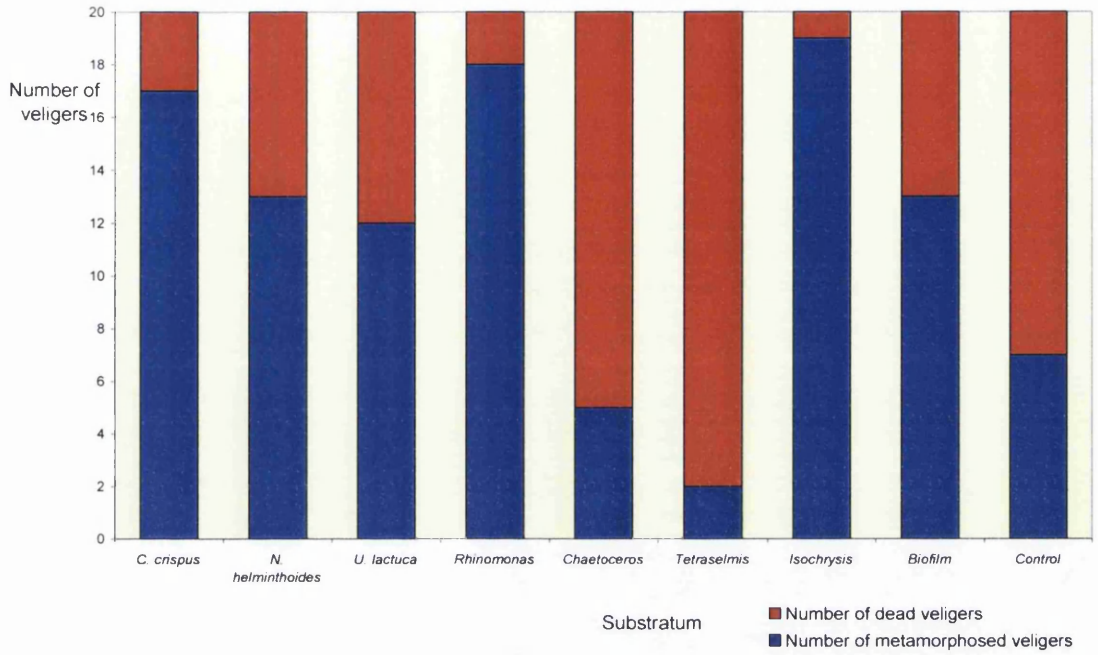

Figure 7.6. The success of metamorphosis in *Akera bullata* veligers from experiment 1 group A on different substrata.

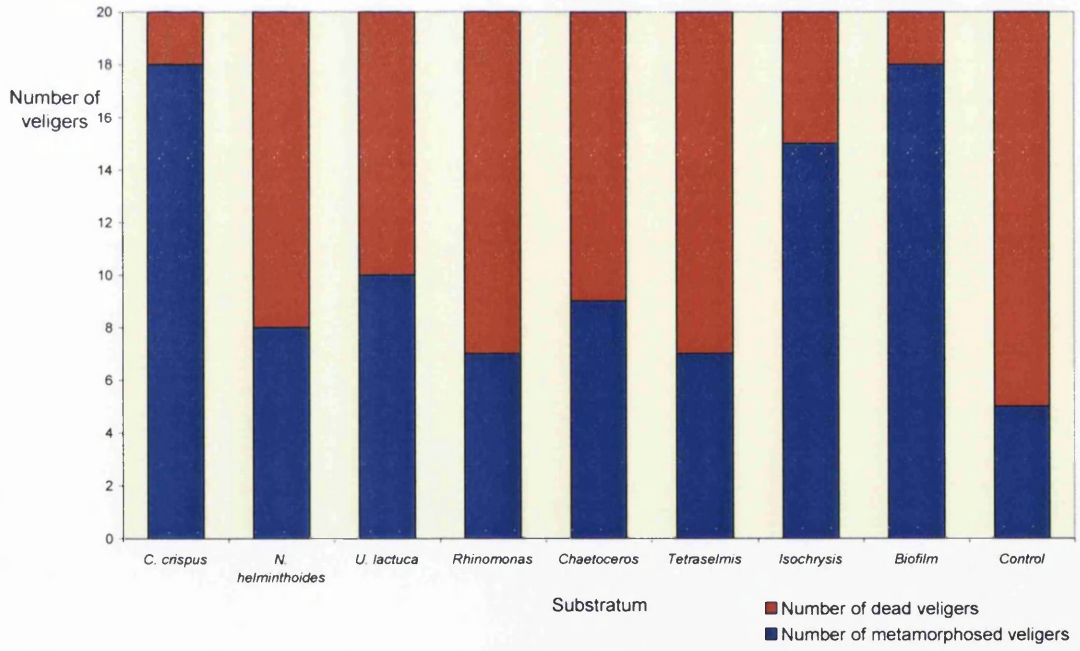

Figure 7.7. The success of metamorphosis in *Akera bullata* veligers from experiment 1 group B on different substrata.

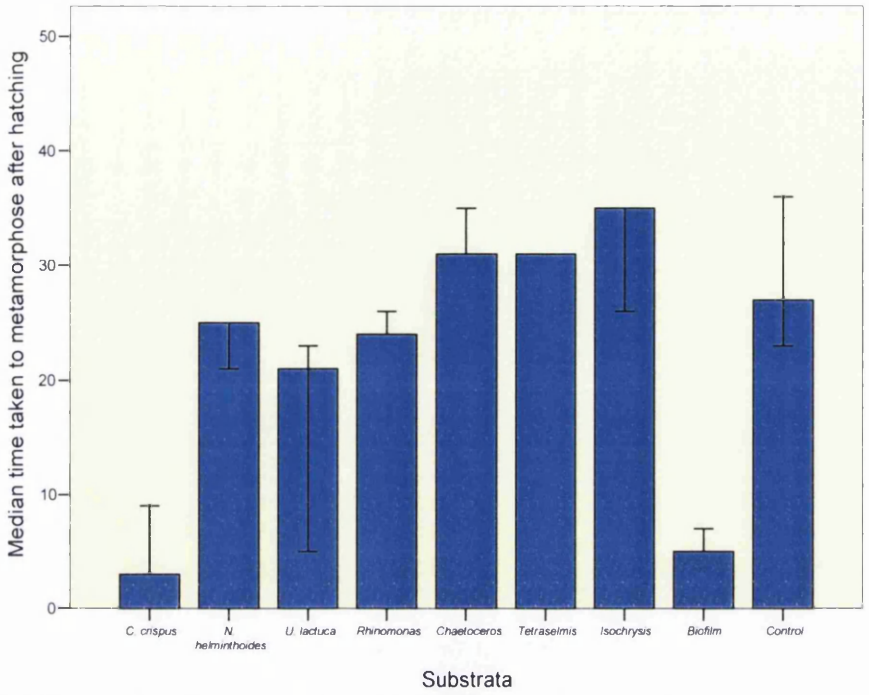

Figure 7.8. The median time taken for *Akera bullata* veligers to metamorphose from group A on different substrata. Error bars represent the 95% confidence interval.

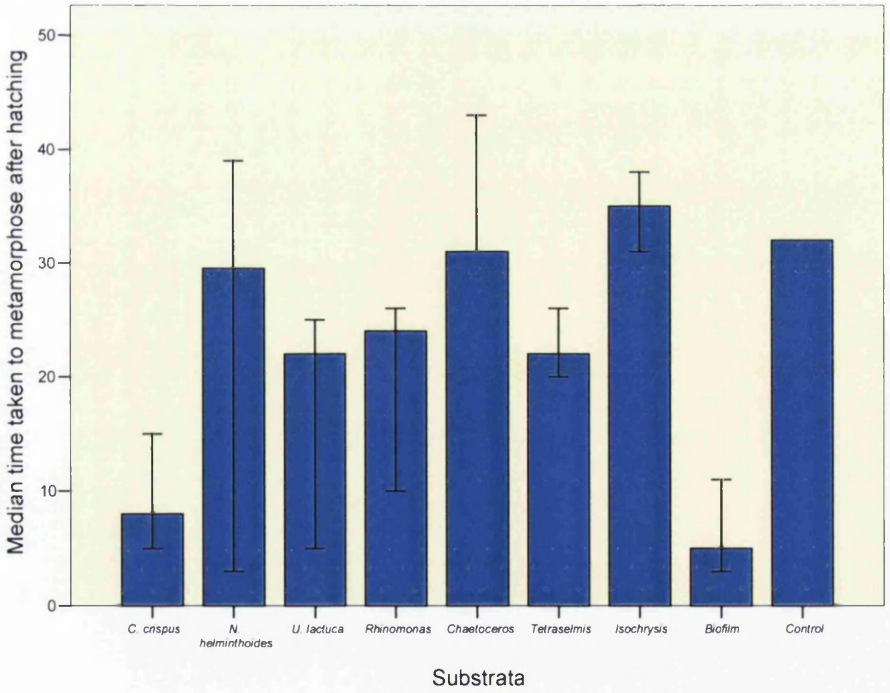

Figure 7.9. The median time taken for *Akera bullata* veligers to metamorphose from group B on different substrata. Error bars represent the 95% confidence interval.

Mann-Whitney *U*-test results group A

	N. helminthoides	*C. crispus*	*U. lactuca*	*Rhinomonas*	*Tetraselmis*	*Chaetoceros*	*Isochrysis*	Biofilm	Control
N. helminthoides	-	0.00	34.00	110.00	0.00	0.00	49.00	0.00	20.00
C. crispus	-	-	19.50	0.00	0.00	0.00	17.00	88.50	0.00
U. lactuca	-	-	-	35.00	0.00	0.00	32.00	24.00	3.50
Rhinomonas	-	-	-	-	5.00	9.50	93.50	0.00	38.00
Tetraselmis	-	-	-	-	-	3.00	15.00	0.00	4.00
Chaetoceros	-	-	-	-	-	-	46.50	0.00	8.00
Isochrysis	-	-	-	-	-	-	-	12.00	50.00
Biofilm	-	-	-	-	-	-	-	-	0.00
Control	-	-	-	-	-	-	-	-	-

Mann-Whitney *U*-test results group B

	N. helminthoides	*C. crispus*	*U. lactuca*	*Rhinomonas*	*Tetraselmis*	*Chaetoceros*	*Isochrysis*	Biofilm	Control
N. helminthoides	-	33.00	23.50	14.00	14.00	22.00	27.00	28.00	12.50
C. crispus	-	-	44.00	19.00	12.00	0.00	0.00	112.50	0.00
U. lactuca	-	-	-	28.00	32.00	5.00	4.00	25.50	2.50
Rhinomonas	-	-	-	-	24.00	0.00	1.00	12.00	0.00
Tetraselmis	-	-	-	-	-	0.00	0.50	0.00	0.00
Chaetoceros	-	-	-	-	-	-	55.50	0.00	20.00
Isochrysis	-	-	-	-	-	-	-	0.00	20.00
Biofilm	-	-	-	-	-	-	-	-	0.00
Control	-	-	-	-	-	-	-	-	-

Table 7.3. The results of the Mann-Whitney *U*-test of experiment one, corrected by the sequential Bonferroni test: k comparisons by the Dunn-Šidák method at a specified experimentwise error rate α (Sokal & Rohlf, 1995). The figures in red are the substrata which have a significant difference in the time taken for the veligers to settle ($P<0.05$).

respectively) (Figs 7.8, 7.9). See Table 7.3 for the corrected significant pairs. There is no consistency in the results between groups A and B in the substrata that resulted in significant differences.

In groups A and B, a higher proportion of veligers settled faster when provided with *Chondrus* and biofilm than those reared with the other substrata, which appeared to delay settlement for a period of ≈ 17-20 days (Fig. 7.10, 7.11).

7.3.4 *SETTLEMENT EXPERIMENT 2*

In experiment 2 all of the vessels (except 6 and 9) resulted in at least 50% of the veligers undergoing metamorphosis. Vessel 1 (*Chondrus* and *Rhinomonas*) resulted in the greatest metamorphic success, with 100% of the veligers undergoing metamorphosis (Fig. 7.12). The median time taken from hatching to the initiation of metamorphosis was significantly different in culture vessels 1 to 12 (Kruskal-Wallis test, $\chi^2 = 64.317$, $P<0.05$) (Fig. 7.13). Comparisons made in all four phytoplankton groups, showed that there were significant differences in the time taken to metamorphose (Kruskal-Wallis test, $\chi^2 = 14.359$, $P<0.05$ for *Rhinomonas*; $\chi^2 = 10.453$, $P<0.05$ for *Chaetoceros*; $\chi^2 = 20.670$, $P<0.05$ for *Tetraselmis*; and $\chi^2 = 6.742$, $P<0.05$ for *Isochrysis*). The substrata which resulted in significant differences in settlement time were identified using the corrected Mann-Whitney *U*-test (Table 7.4). *Chondrus* promoted faster settlement with *Rhinomonas*, *Tetraselmis* and *Chaetoceros*, although not with *Isochrysis*.

In vessels 1, 2, and 3 (*Rhinomonas*), the veligers provided with *Chondrus* settled faster. There was a delay of between 8 to 10 days before the majority of *Ulva* and biofilm veligers settled (Fig. 7.14). In vessels 4, 5, and 6 (*Chaetoceros*), *Chondrus* induced a large number of veligers to settle quickly. It was not until ten days had passed before the majority of *Ulva* and biofilm veligers settled (Fig. 7.15). Settlement was not as fast in vessels 7, 8, and 9 (*Tetraselmis*) however, after a delay of 7 days a large proportion of *Chondrus* veligers settled. More veligers settled in response to *Ulva* although settlement was delayed for ≈ 13 days (Fig. 7.16). In vessels 10, 11, and 12 (*Isochrysis*), a high proportion of veligers exposed to *Chondrus* initiated settlement quickly. There was a delay of ≈ 7 days before the majority of biofilm veligers settled. The veligers exposed to *Ulva* settled steadily over time (Fig. 7.17).

7.3.5 *JUVENILE MORTALITY*

The time taken for individual juveniles to die after metamorphosis was significantly different for juveniles reared under experiment 1 conditions for groups A and B (Kruskal-

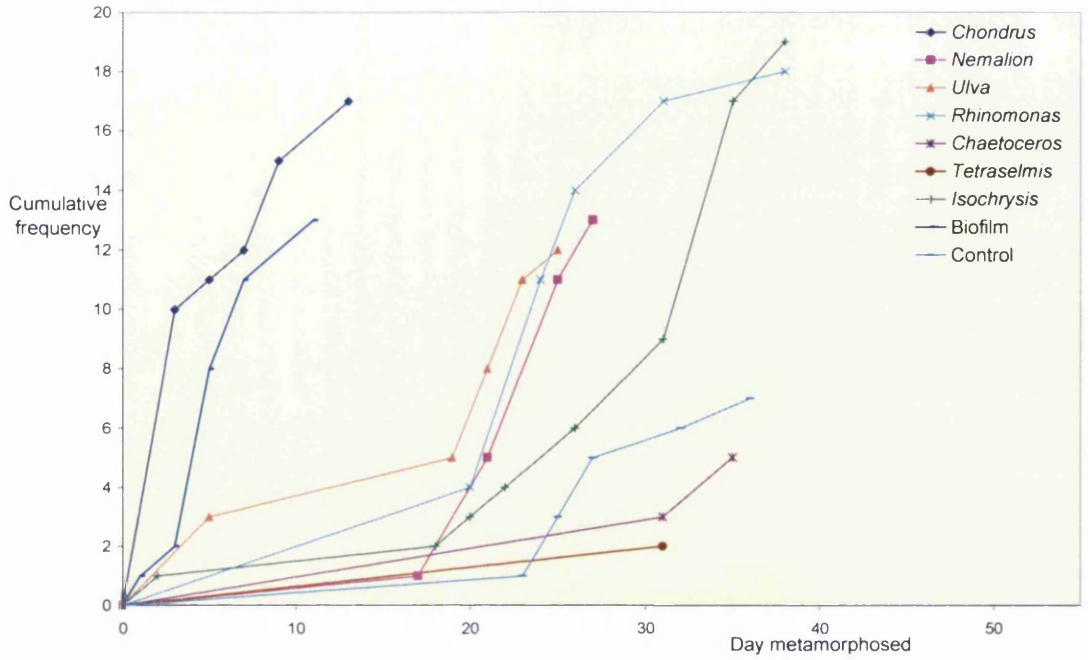

Figure 7.10. The cumulative frequency of *Akera bullata* veligers undergoing metamorphosis per day in experiment 1 group A, on different substrata.

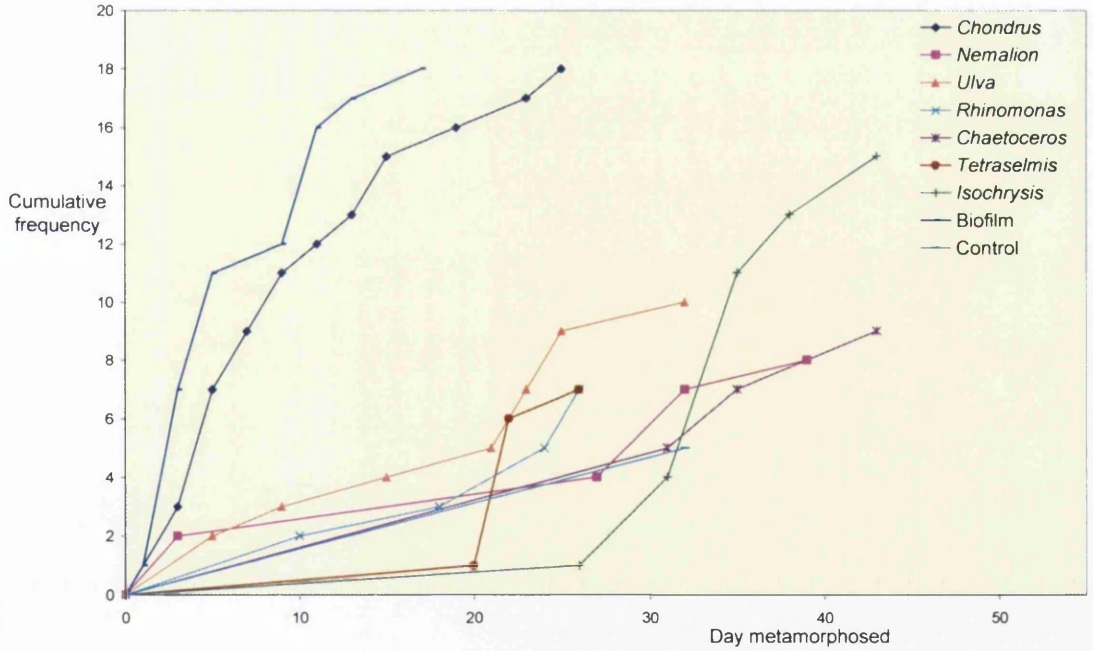

Figure 7.11. The cumulative frequency of *Akera bullata* veligers undergoing metamorphosis per day in experiment 1 group B, on different substrata.

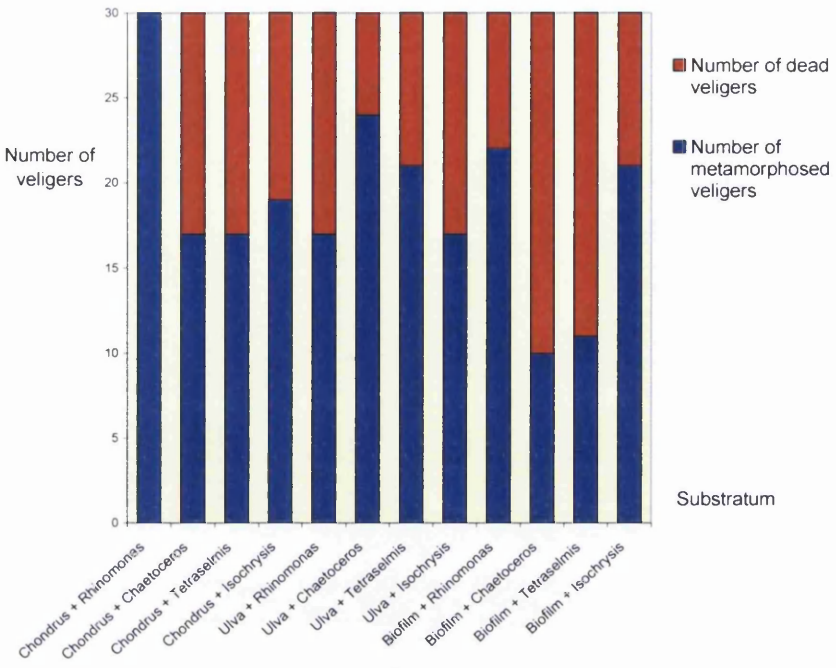

Figure 7.12. The success of metamorphosis of *Akera bullata* veligers on different substrata in experiment 2.

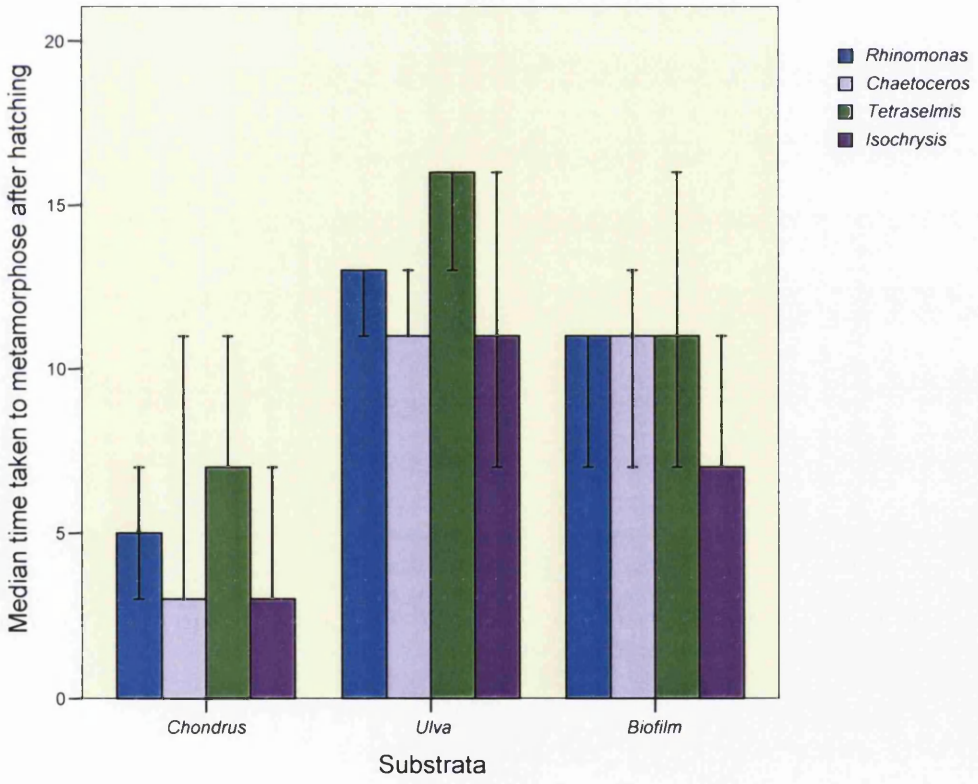

Figure 7.13. The median time taken for *Akera bullata* veligers to metamorphose in experiment two, on different substrata. Error bars represent the 95% confidence interval.

Mann-Whitney *U*-test Results:

Rhinomonas sp.	*Chondrus*	*Ulva*	Biofilm
Chondrus	-	101.00	220.50
Ulva	-	-	108.00
Biofilm	-	-	-

Chaetoceros sp.	*Chondrus*	*Ulva*	Biofilm
Chondrus	-	98.00	32.50
Ulva	-	-	114.50
Biofilm			

Tetraselmis sp.	*Chondrus*	*Ulva*	Biofilm
Chondrus	-	35.00	62.00
Ulva	-	-	48.00
Biofilm	-	-	-

Isochrysis sp.	*Chondrus*	*Ulva*	Biofilm
Chondrus	-	91.00	128.00
Ulva	-	-	149.00
Biofilm	-	-	-

Table 7.4. The results of the Mann-Whitney *U*-test of experiment two, corrected by the sequential Bonferroni test: *k* comparisons by the Dunn-Šidák method at a specified experimentwise error rate α (Sokal & Rohlf, 1995). The figures in red are the substrata which have a significant difference in the time taken for the veligers to settle ($P<0.05$).

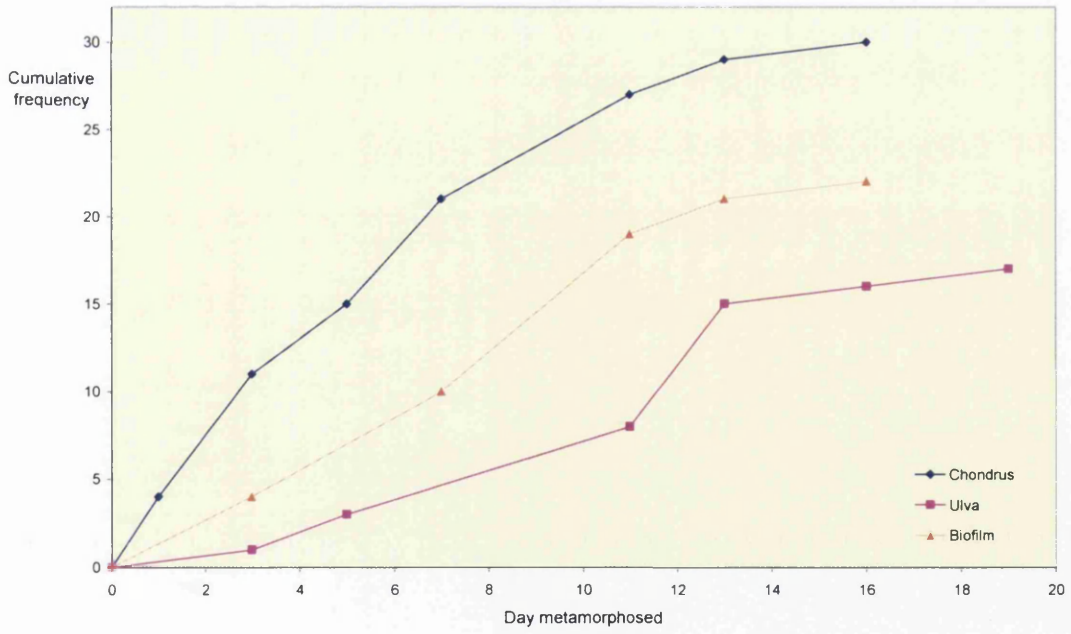

Figure 7.14. The cumulative frequency of *Akera bullata* veligers undergoing metamorphosis per day in experiment 2 on different substrata, when provided with *Rhinomonas* sp.

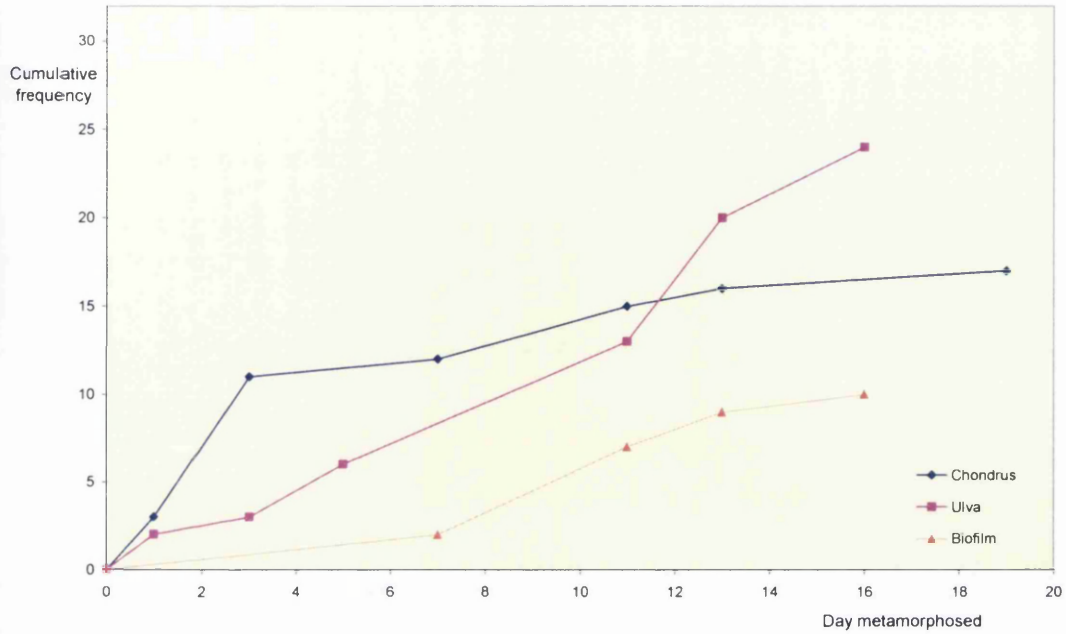

Figure 7.15. The cumulative frequency of *Akera bullata* veligers undergoing metamorphosis per day in experiment 2 on different substrata, when provided with *Chaetoceros* sp.

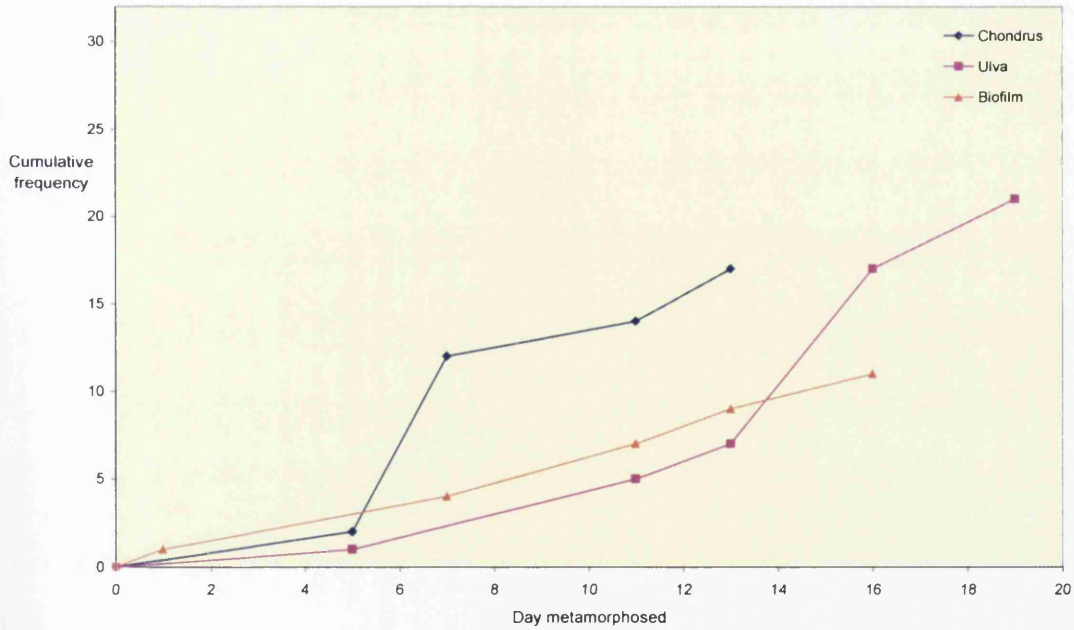

Figure 7.16. The cumulative frequency of *Akera bullata* veligers undergoing metamorphosis per day in experiment 2 on different substrata, when provided with *Tetraselmis* sp.

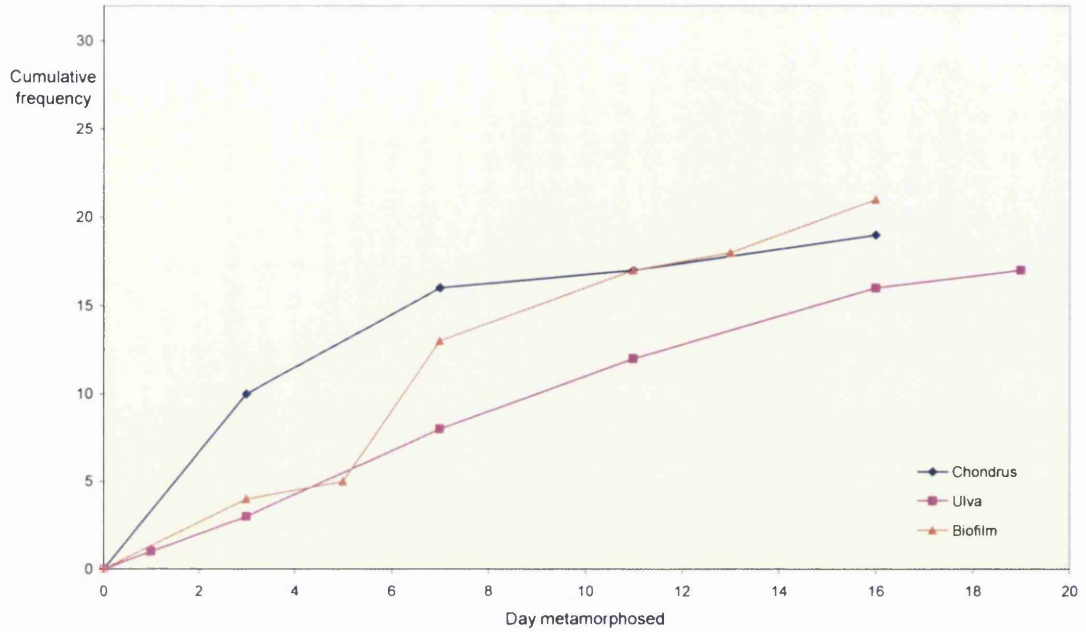

Figure 7.17. The cumulative frequency of *Akera bullata* veligers undergoing metamorphosis per day in experiment 2 on different substrata, when provided with *Isochrysis* sp.

Wallis test, $\chi^2 = 25.020$, $P<0.05$; and $\chi^2 = 28.883$, $P<0.05$ respectively) (Figs 7.18, 7.19).

The substrata that resulted in significant differences were not consistent between groups A and B (Table 7.5). Group B exhibited significant differences under the control group when compared with *Nemalion*, *Chaetoceros* and biofilm. In group A the only significant difference was between *Chondrus* and the control. The time taken for the juveniles to die after metamorphosis when reared under experiment 2 conditions was not significantly different (Kruskal-Wallis test, $\chi^2 = 15.056$, $P>0.05$).

To identify whether the number of juveniles which had died up until day 135 was dependent on the conditions provided within the vessels (not taking into account the change of food to *Zostera* on day 121), an $R \times C$ test of independence was performed (Sokal & Rohlf, 1995). In experiment 1 the number of veligers that died in groups A and B was independent of the substratum on which they were reared ($R \times C$ test of independence, $G_{adj} = 13.686$, $P>0.05$). In experiment 2, the number of juveniles that died when provided with *Rhinomonas*, *Chaetoceros*, *Tetraselmis* and *Isochrysis* was found to be independent of the substrata on which they were reared ($R \times C$ test of independence, $G_{adj} = 7.366$, $P>0.05$).

7.3.6 *OTHER OBSERVATIONS*

When rearing the juveniles, feeding was only observed on *Ulva*; the fronds of *Chondrus* and *Nemalion* remained untouched. However, the juveniles were observed grazing the biofilm from those fronds. When provided with just a biofilm the juveniles were observed consuming the bacterial film and diatoms from the cover glass. *Chondrus* and *Nemalion* have thick fronds compared to *U. lactuca*, and due to the small size of the juvenile *A. bullata* it is possible that physical limitations of the buccal mass prevented them from feeding directly on *Chondrus* and *Nemalion*.

There were two occasions when the newly metamorphosed juveniles produced purple ink. In both of these incidences, the release was as a result of rough handling. Both juveniles were reared in vessels containing *Rhinomonas*. Due to the rarity of these inking events, and the severity of the handling required to produce it, attempts to capture it on film failed. However, adult *A. bullata* gathered from the Fleet were filmed releasing ink after a mild electric shock (D.N. Price, personal communication).

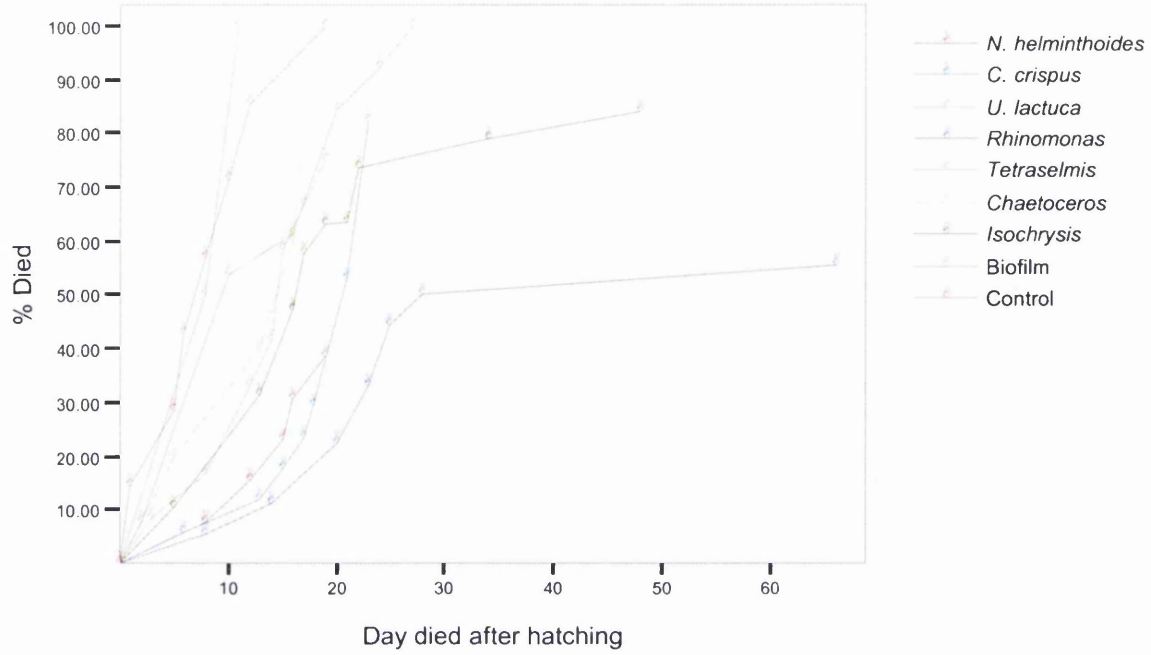

Figure 7.18. The mortality of *Akera bullata* in experiment 1, group A, excluding the mortality of all surviving individuals that occurred on day 135.

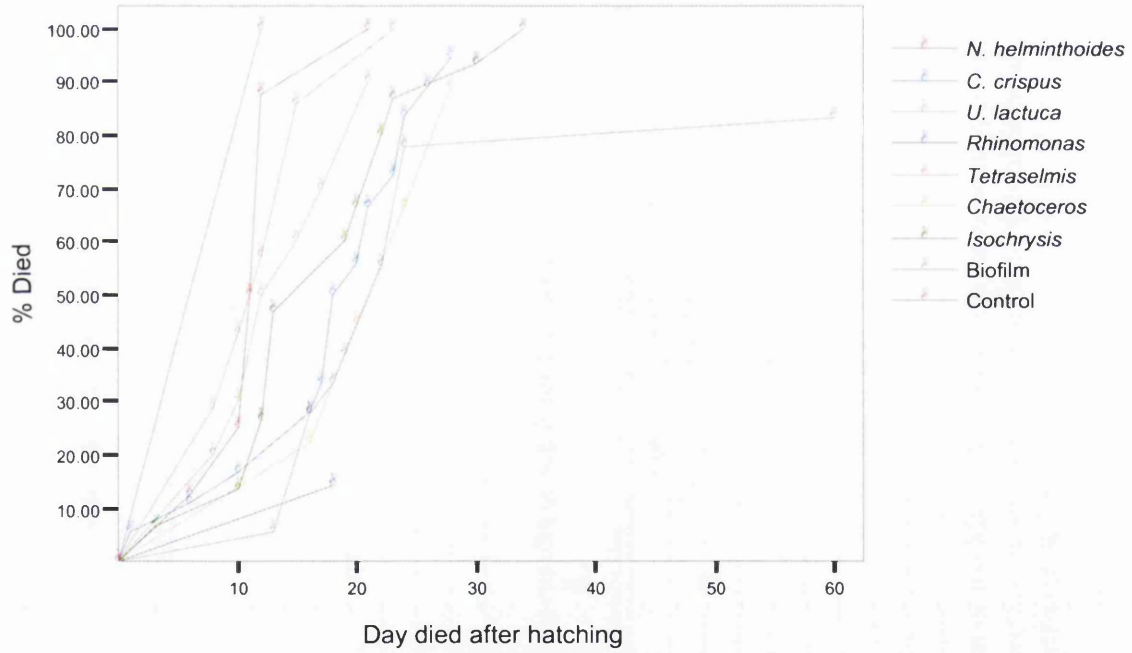

Figure 7.19. The mortality of *Akera bullata* in experiment 1, group B, excluding the mortality of all surviving individuals that occurred on day 135.

Mann-Whitney *U*-test results group A for mortality:

	N. helminthoides	*C. crispus*	*U. lactuca*	*Rhinomonas*	*Tetraselmis*	*Chaetoceros*	*Isochrysis*	Biofilm	Control
N. helminthoides	-	12.50	19.00	7.50	1.50	9.50	29.00	30.50	7.50
C. crispus	-	-	18.50	50.50	2.00	8.00	71.00	52.00	8.50
U. lactuca	-	-	-	12.00	3.50	16.50	45.50	53.00	17.00
Rhinomonas	-	-	-	-	1.50	5.00	42.00	31.00	4.50
Tetraselmis	-	-	-	-	-	2.00	4.00	8.00	5.50
Chaetocerous	-	-	-	-	-	-	22.50	23.50	8.50
Isochrysis	-	-	-	-	-	-	-	83.50	20.50
Biofilm	-	-	-	-	-	-	-	-	22.00
Control	-	-	-	-	-	-	-	-	-

Mann-Whitney *U*-test results group B for mortality:

	N. helminthoides	*C. crispus*	*U. lactuca*	*Rhinomonas*	*Tetraselmis*	*Chaetocerous*	*Isochrysis*	Biofilm	Control
N. helminthoides	-	30.00	28.50	1.00	25.00	4.00	25.50	6.00	12.50
C. crispus	-	-	43.00	7.50	28.00	48.00	116.50	97.50	15.00
U. lactuca	-	-	-	2.00	28.50	10.00	43.50	17.00	20.00
Rhinomonas	-	-	-	-	1.00	2.00	7.00	4.50	0.00
Tetraselmis	-	-	-	-	-	4.00	33.00	11.00	17.50
Chaetocerous	-	-	-	-	-	-	35.00	55.00	0.00
Isochrysis	-	-	-	-	-	-	-	68.50	15.00
Biofilm	-	-	-	-	-	-	-	-	0.00
Control	-	-	-	-	-	-	-	-	-

Table 7.5. The results of the Mann-Whitney *U*-test for mortality, corrected by the sequential Bonferroni test: k comparisons by the Dunn-Šidák method at a specified experimentwise error rate α (Sokal & Rohlf, 1995). The figures in red are the substrata which have a significant difference in survivorship ($P<0.05$).

7.4 *DISCUSSION*

7.4.1 *VELIGER DEVELOPMENT*

The *Akera bullata* veligers hatched from spawn gathered from LHP the Fleet, Dorset. The embryonic period was not determined in this study, although work carried out by Thorson (1946) (cited by Thompson (1976)) stated it to be 30 days at 15°C or 20 days at 20°C. The ova measured 154.4 ± 4.0 μm in diameter, and there was only one per capsule (Fig. 7.1A). This is in close agreement with Thompson (1976) who reported the ova were 156-170 μm. The veligers exhibit a Development-type 2, and a Shell-type 2 (Thompson, 1967). This report supports Thorson's (1946) observations of *A. bullata* possessing his Type C shell, and contradicts Thompson's (1976) statement that *A. bullata* has a Shell-type 1. It is interesting to note that Tardy (1991) states "Type 2 veligers occur only amongst the most highly-evolved opisthobranchs (i.e. the Nudibranchia)...". However, it has long been known that *Akera* is a primitive genus, even when they were classified incorrectly. In Medina & Walsh's (2000) reconstruction of the Anaspidea, they classify *Akera* as one of the earliest evolved members. This clearly contradicts Tardy (1991).

On hatching *A. bullata* had a shell length of 255.1 ± 13.1 μm and possessed eyes, a large yellow yolk store (within the left digestive diverticulum, which Thorson (1946) termed the liver), a stomach, a hind gut (terminating at the anus), a larval kidney, a metapodium, a larval retractor muscle, velar lobes with cilia, statocysts, and an operculum (Figs 7.2A-C). Other larval structures were difficult to identify due to the density of the yolk. The veligers from Fleet spawn are considerably smaller than those measured by Thorson (1946) from Denmark, which were reported to be 300-375 μm in length. A possible reason for this difference is that Thorson (1946) measured the whole veliger from shell to velum, although his description does imply that only the shell was measured. This is perhaps the only substantial evidence to support the existence of var. *nana* in the Fleet.

Despite possessing a Development-type 2, the veligers are fully competent plankton feeders (facultative planktotrophs). When provided with *Rhinomonas*, the veligers' stomachs became pigmented (Fig. 7.1E), thus feeding is possible and does occur when provided with phytoplankton. However, feeding is not necessary for metamorphosis. This has been recorded for other species of opisthobranchs. Kempf & Hadfield (1985) discovered that the Development-type 2 *Phestilla sibogae* veligers do not need to feed in order to undergo metamorphosis but will feed on phytoplankton if available. The longer the period between

hatching and settlement, the more important feeding becomes, and at some point it becomes obligate if they are to settle and metamorphose. Feeding on phytoplankton increased the veligers competent life span by up to 90% (Kempf & Hadfield, 1985). Thompson (1958) discovered that despite *Adalaria proxima* (Alder & Hancock) veligers ingesting phytoplankton, it is not a prerequisite for metamorphosis. Facultative planktotrophy by lecithotrophic veligers does not always occur. *Tritonia hombergi* Cuvier, veligers do not feed, and are capable of development in boiled filtered sea water. However, metamorphosis is dependent upon exposure to the anthozoan *Alcyonium digitatum* L. (Thompson, 1962). The significance of planktotrophy by lecithotrophic veligers was discussed by Kempf & Hadfield (1985) in their study of *P. sibogae.* This nudibranch lives in an environment where the adult prey item, the coral *Porites*, is abundant, and larval dispersal therefore is not necessary, but they discovered the distribution of *P. sibogae* was far beyond that attributable to a planktonic life span of a few days. Kempf & Hadfield (1985) postulated that the larvae survive longer in the natural environment, and therefore are aided by their planktotrophic ability. In adopting both lecithotrophy and planktotrophy strategies, the veligers are more likely to withstand adverse conditions. Crisp (1976) noted that planktotrophy may increase the amount of energy reserves the larva has, thereby increasing post-metamorphic survivorship.

The major difference in this study regarding discussions pertaining to facultative planktotrophy by lecithotrophic veligers is the enclosed environment in which *A. bullata* lives in the Fleet. Despite the large size of the Fleet, its enclosed system, shallow depth, and extreme tidal asymmetry (Robinson, Warren & Longbottom, 1983) are likely to impede larval dispersal. Wind and tidal effects are restricted due to the shallow depth in the north end of the Fleet. At LHP the tides follow a fortnightly regime with a fast flood but slow ebb (Robinson *et al.*, 1983). It is possible that veligers are transported into the sea at Smallmouth, from southerly populations in the Fleet, and that veligers are recruited into the Fleet this way; however this is unlikely to have any measurable effect on northern populations as the normal factors influencing larval dispersal (e.g. tidal currents, wind etc.) are likely to have minimal influence in the Fleet. Despite the physical constraints due to the hydrographics of the lagoon, larval behaviour is likely to play a role in dispersal. The lecithotrophic veligers of *A. proxima* settle in the vicinity of release; Todd *et al.* (1998) and Lambert *et al.* (2003) concluded that they were "behaviourally constrained (which prevents

them) from becoming pelagic". After hatching opisthobranch veligers are documented as being positively phototactic and/or negatively geotactic (Hurst, 1967; Franz, 1975; Harris, 1975). On hatching they swim upwards toward the surface, and on attaining competence they change their behaviour and swim towards the benthos. The larval dispersal of *A. bullata* is likely to be restricted due to the short time between hatching and settlement, and the effects of a possible behavioural constraint as postulated for *A. proxima* (Todd *et al.*, 1998; Lambert *et al.*, 2003).

It is interesting to note that in Thompson & Seaward's (1989) study, the population at LHP suffered a mass mortality event in February 1986 due to adverse weather conditions. The population failed to become established for at least one year despite spawn drifting in from nearby populations in the Fleet, and the presence of swimming juveniles in August and September 1986. It was only the following summer, after more spawn had drifted into the area adjacent to LHP, that the population became re-established. Thus in this example, it appears that larval dispersal played a very minor role in recruitment at LHP, and re-establishment was not effected by the arrival of swimming juveniles. Instead, the dispersal of spawn, most likely distributed via wind and or tidal currents, played a role in re-establishing the LHP population (Thompson & Seaward, 1989). It would appear that the ability of the larvae to undertake planktotrophy is of little significance in populations of the Fleet, due to its apparent short dispersal distances; however, in the dynamic habitat of the marine environment, facultative planktotrophy and larval behaviour is likely to play a significant role in larval ecology and population dynamics.

7.4.2 *SETTLEMENT AND METAMORPHOSIS*

The definitions of settlement and metamorphosis vary depending upon the author. In this report both terms will be defined according to Pawlik (1992). Settlement includes the whole transition from larva to benthic adult. Metamorphosis is a recognized stage of settlement, but so too is the initial substratum contact, the exploration phase, and orientation etc. Early settlement is reversible; if the benthos is unsuitable the larvae will return to swimming in the water column. Metamorphosis however is irreversible; it involves the transition into the adult body form and involves the reorganisation of the internal organs. In opisthobranchs this includes the process of detorsion. Chia (1989) sub-divided settlement and termed the processes "temporary settlement" and "permanent settlement". Permanent settlement is of particular importance to the success of the individual;

metamorphosis in an unsuitable environment may result in reduced fitness, lowered fecundity, or even death. Once the transition to the adult stage has occurred, opisthobranchs become immediately restricted in the distances they can travel, thus relocation into a favourable environment may be impossible, or at best a drain upon the individual's resources (reviews by Crisp (1974) and Pawlik (1992)). Another governing factor is gregariousness. Many opisthobranchs are semelparous; metamorphosis is followed by a period of rapid growth, copulation, spawning, and death over a short generation time. Spawning is dependent upon sexual encounters with other individuals. Typically populations are a direct result of recruitment of veligers from the previous generation. If settlement is specific to a particular prey item, this will increase the likelihood of encountering conspecifics. In turn this will increase the likelihood of recruitment to the next generation. Settlement is an important factor in governing the success of an individual, and is perhaps the most significant step in the life cycle of many benthic marine invertebrate species.

In *A. bullata* metamorphic competence is attained once the propodium has inflated which occurs soon after hatching. In agreement with Thorson's (1946) conclusion, shell growth did not occur during the time spent in the plankton. Settlement and consequently metamorphosis occurred within 43 days after hatching depending on the substratum and or phytoplankton provided. The veligers that failed to settle died within two months. *A. bullata* veligers are stimulated to settle when provided with a substratum; however, it appeared that a few will metamorphose in response to just a surface. Pawlik (1989) reported similar behaviour in *A. californica* veligers. In experiment 1 group A, a higher proportion of veligers metamorphosed when provided with *Chondrus* and a biofilm. For all the other conditions there was a delay of approximately 17 days before the majority of veligers settled (Fig. 7.8). The results were similar for group B (Fig. 7.9). For both groups there was a sharp increase in the numbers of veligers commencing metamorphosis after 20 days. It would appear that *Chondrus* and biofilm promoted faster settlement, and when exposed to other, perhaps not as favourable conditions, the veligers appeared to delay metamorphosis for several days. The ability to delay metamorphosis is not unusual in marine invertebrates. Kempf (1981) maintained *A. juliana* veligers in culture for 316 days, however only one in five larvae developed into a juvenile. Kempf & Todd (1989) discovered that the lecithotrophic (facultative planktotroph) *A. proxima* could survive 15 to 20 days, and these individuals were

still metamorphically competent after 18 days. Pechenik (1990) addressed the advantages and disadvantages of delaying metamorphosis in marine invertebrate larvae. She criticized Kempf's (1981) methodology by suggesting that the veligers which had apparently delayed metamorphosis may have reached competency after the other individuals used in the study, therefore the time spent in a delayed state would be shorter than otherwise estimated. Despite the ability to delay metamorphosis, the majority of marine invertebrates cannot do so indefinitely. A lowering of cue threshold sensitivity and/or a genetically determined maximal time period has been implicated in the ability of some larvae to undergo spontaneous metamorphosis (review by Pechenik (1990)). It is possible that the *A. bullata* veligers exposed to unfavourable conditions (i.e. *Nemalion*, *Rhinomonas*, *Chaetoceros*, *Tetraselmis*, *Isochrysis* and the control conditions) delayed metamorphosis until their threshold sensitivity decreased which then allowed them to spontaneously metamorphose; *in vivo* this delay would increase the veligers' chances of seeking out conditions that favour settlement and increase post-metamorphic survival. However, by delaying metamorphosis they increase the risk of pre-settlement mortality (Pechenik, 1990). This fails to explain why the biofilm initiated a faster settlement response as theoretically a biofilm should have been present on the surface of all of the substrata provided. The veligers that failed to metamorphose may never have done so due to inherent genetic defects, however this is speculation and would require further investigation.

There was little consistency between groups A and B when comparing settlement time. However, *Chaetoceros* and *Isochrysis* produced consistently slower settlement times, and a biofilm appeared to stimulate faster settlement (Table 7.3). This lack of consistency might have been due to small sample sizes in the vessels, or minor variations in conditions in replicate vessels. The conditions in each replicate vessel were made as close to identical as possible. The results were corrected to avoid making type I and type II errors. Other possible reasons for the discrepancies include the fact that the veligers were sampled from different spawn masses, therefore genetic factors intrinsic to the larvae could account for these differences.

In experiment 2, a similar pattern of settlement was identified to experiment 1. When the veligers were provided with *Chaetocerous*, *Tetraselmis*, and *Isochrysis*, *Chondrus* promoted faster settlement (although this was slightly delayed in *Tetraselmis*). *Rhinomonas* and *Chondrus* promoted the fastest and greatest number of veligers metamorphosing out of all the

conditions (Figs 7.12-7.15). From experiments 1 and 2, it would appear that *A. bullata* veligers are stimulated to metamorphose faster in response to *Chondrus* (irrespective of the species of phytoplankton present), when compared with *Nemalion*, *Ulva*, biofilm, or just phytoplankton alone. In experiment 2 all 30 veligers metamorphosed when provided with a combination of *Chondrus* and *Rhinomonas*, compared with only ten when provided with biofilm and *Chaetoceros* (Fig. 7.10). However, *Chondrus* appears to promote faster settlement of veligers, irrespective of whether a species of phytoplankton is present.

Kempf & Hadfield (1985) discovered that after 22 days of starvation, *P. sibogae* veligers lose the ability to metamorphose. When fed the veligers were capable of metamorphosis after 42 days in culture. *P. sibogae* adults have very specific dietary requirements, consequently the veligers will only metamorphose when exposed to extracts of *Porites*. Adult *A. bullata* are not as specific, they consume a variety of different substrata (Morton & Holme, 1955; Thompson, 1976; Thompson & Seaward, 1989). Despite preferring *Chondrus* to settle on (this study), if this was not available, after a delay, they underwent spontaneous metamorphosis and settled in response to the available substrata. Crisp (1974) documents a similar behaviour in barnacle cyprids. In the absence of a preferred "pit", the cyprids settlement threshold lowers over time, which causes settlement to be delayed. Thus, Crisp (1974) discovered that in the event of unsuitable "pits" the cyprids settle in higher numbers with increasing age.

In the closely related aplysiids, the preferred substratum for metamorphosis is fairly unspecific with several species of algae inducing metamorphosis, however post-metamorphic growth is restricted to only a few species. Switzer-Dunlap & Hadfield (1977) investigated the settlement preferences of several different aplysiids The veligers of *A. juliana* settled in response to the green algae *U. fasciata* and *U. reticulata*. *A. dactylomela* veligers were not as specific and settled in response to *Laurencia*, *Chondrococcus*, *Gelidium*, *Martensia*, *Polysiphonia* and *Spyridia*. However, *Laurencia* induced the greatest numbers of veligers to metamorphose. The larvae of *Dolabella auricularia* also metamorphosed in response to a variety of different algae: *Laurencia*, *Amansia*, *Spyridia*, *Sargassum* and an unidentified mat-forming blue-green alga. Despite the range of metamorphic inducers, the juveniles initially only consumed diatoms and blue-green algae. As they grew older their preferred diet changed to a mixture of *Spyridia*, *Acanthophora* and *Laurencia*. The aplysiid *Stylocheilus longicauda* metamorphosed in response to *Lyngbya majuscula*, *Acanthophora* and *Laurencia*,

although only *L. majuscula* induced post larval growth. The species which resulted in the greatest post metamorphic survivorship in Switzer-Dunlap & Hadfield's (1977) study were the species on which the juveniles were typically found in the field. They did note however, that for all species, in the absence of substrata no metamorphosis occurred. The veligers of *A. brasiliana* were induced to metamorphose by contact with *Callithamnion* and *Polysiphonia*, however the greatest metamorphic success occurred on the latter species (Strenth & Blankenship, 1978). Pawlik (1989) discovered that *A. californica* metamorphosed when exposed to any one of 18 different species of algae and in control dishes with no stimulus. Despite their indiscriminate settlement, they require a diet of either *Laurencia pacifica* or *Plocamium cartilagineum* for post-larval development (Pawlik, 1989). *A. oculifera* metamorphosed in response to six out of 12 macroalgal species tested by Plaut *et al.* (1995). As with Switzer-Dunlap & Hadfield (1977), Plaut *et al.* (1995) discovered that none of the veligers metamorphosed under control conditions.

Members of the Anaspidia have a broad prey range therefore it is likely that the veligers settle in response to a similarly broad range of species. However, in every member studied a few species seem to promote post larval growth. *A. bullata* is known to be herbivorous, Thompson (1989) found that it fed on *Enteromorpha* and possibly *Zostera* roots. Morton & Holme (1955) found it to be a grazer of *Ulva* and a deposit feeder, and in this study it was also found to graze red seaweeds. This study demonstrated that settlement occurs under a variety of different conditions, which can vary from a simple bacterial biofilm, to phytoplankton or a species of seaweed. However, *Chondrus* and the biofilm promoted settlement faster. In the vessels containing only a species of phytoplankton, it is possible that metamorphosis was not induced by the motile cells in suspension but by settlement of cells on the bottom of the vessels creating a biofilm. Metamorphosis without a stimulus does occur. For some individuals a surface alone was enough to stimulate settlement, although this was a rare event. As with the newly settled juveniles of *D. auricularia* (Switzer-Dunlap & Hadfield, 1977), *A. bullata* were initially only observed consuming the biofilm on the surface of the algae or the glass slide. As they grew larger they were then able to consume the thin fronds of *Ulva*, but never during this study were the juveniles observed feeding upon *Chondrus* or *Nemalion*. It is likely that they did not reach a physical size that enabled them to consume it. These juveniles probably consumed the biofilm and phytoplankton that had settled upon the sides of the culture vessels and the

alga. The adults gathered from LHP do feed upon red seaweeds when available, as they produce defensive ink (D.N. Price, personal communication)

The involvement of other mechanisms which may influence the attractiveness of the substratum and thereby influence the settlement of *A. bullata* e.g. surface texture and or a visual stimulus, cannot be ruled out. This has not been investigated with opisthobranch veligers so their effect, if any, is unknown.

Once the veligers are satisfied regarding the surface, the process of metamorphosis, "permanent settlement", begins. The shell does not grow whilst the *A. bullata* veliger is free-swimming, growth only occurs after metamorphosis when feeding commences. The morphological changes which occur during metamorphosis are similar to those described for other opisthobranch species, although the shell is not discarded. On the commencement of metamorphosis, the velar cilia are rapidly absorbed, and the metapodium forms the bi-lobed anterior end of the cephalic shield (Figs 7.1F, 7.2A). Following this, the velar lobes reduce in size and the operculum is discarded. This completes the veliger's transition into the benthic phase and it can no longer swim. This process is complete within 24 hours of the initiation of metamorphosis. Crawling often occured throughout the transition, which is unusual for documented accounts of anaspidean metamorphosis. Switzer-Dunlap & Hadfield (1977) noted that aplysiid veligers remain attached for a period of 1-2 days and only become active once the cephalic tentacles had begun to form. Kriegstein *et al.* (1974) also noted a stationary phase during the metamorphosis of *A. californica.* They remained attached with the velum and propodium retracted, and the operculum partially closed during the initial stages of metamorphosis. Strenth & Blankenship (1978) documented a period of time after initial attachment during which the juvenile *A. brasiliana* underwent limited crawling during metamorphosis.

The buccal mass of *A. bullata* was observed moving 48 hours after the initiation of metamorphosis; however it was unknown whether food was swallowed at this time. Once the juveniles are able to swallow metamorphosis is regarded as complete (Kriegstein *et al.*, 1974).

Growth of the shell began after the completion of metamorphosis, hence at the start of feeding (Fig. 7.2B). After metamorphosis the propodium formed a temporary pedal sole, which then developed into the parapodial lobes that fold around the cephalic shield. Adult pigmentation appeared seven to 18 days after metamorphosis. The juvenile shell markings

were very similar to that described by Thompson & Seaward (1989) in their adults gathered from LHP (Fig. 7.3). These marking were due to organs within the pallial cavity which were visible through the transparent shell. During juvenile development the animals undergo partial detorsion, the anus became located posteriorly close to the exhalant siphon. The entrance to the pallial cavity opened on the right hand side within the parapodium next to the head. Hancock's organ was located on the left hand side (Morton, 1972). The dorsal and ventral raphe, the pallial caecum, plicate gill, and purple gland can all be found within the pallial cavity. At sexual maturity the ovotestes develop on the left hand side of the cavity and are also distributed within the whorls of the shell.

7.4.3 *JUVENILE MORTALITY AND SHELL GROWTH*

In many aplysiid species, newly metamorphosed juveniles will only undergo further development on one or two species (Switzer-Dunlap & Hadfield, 1977; Strenth & Blankenship, 1978; Pawlik, 1989; Plaut *et al.*, 1995), they will die if they are not able to feed on the necessary food. In experiment 1, the time taken for the juveniles to die was dependent on the substratum they were reared upon (Table 7.5). The juveniles that survived the longest in group A were those reared with *Nemalion* or *Rhinomonas* (Fig. 7.16). Juveniles reared with *Chaetoceros*, *Ulva*, *Chondrus* or *Isochrysis* survived, although there were fewer of them. In group B the juveniles that survived the longest were reared with *Rhinomonas* or a biofilm (Fig. 7.17). Juveniles reared with *Chaetoceros*, *Ulva*, *Tetraselmis*, or *Chondrus* survived until the end of the experiment although there were fewer of them. For aplysiids, the substratum that stimulated metamorphosis was typically the species that promoted the greatest post larval survivorship (Switzer-Dunlap & Hadfield, 1977; Strenth & Blankenship, 1978; Pawlik, 1989); in this study *Chondrus* stimulated faster settlement, and therefore should have promoted the greatest post larval survivorship. It is possible that, had the experiment continued beyond day 135 the results would reflect this.

There were no differences in the time taken for the juveniles to die in experiment 2 (Figs 7.18-7.21). Despite this the juveniles that survived the longest (up until day 135) were provided with *Rhinomonas* or *Tetraselmis* on all three substrata. Those veligers in experiment 2 (provided with phytoplankton and a substratum) survived longer than those veligers provided with just a substratum in experiment 1. These results indicate increased survivorship after settlement which is most likely attributable to facultative planktotrophy. Crisp's (1976) conclusion that facultative planktotrophy provides greater post metamorphic

survival appears to apply to the *A. bullata* used in this study. Kempf & Hadfield (1985) discovered planktotrophy extended the life of the *P. sibogae* veligers, thereby allowing greater dispersal distances. Contrary to Kempf & Hadfield (1985), Kempf & Todd (1989) discovered that despite the ability of *Tritonia hombergi* Cuvier veligers to ingest phytoplankton, they failed to assimilate nutrients. This was attributed to the inability of the veligers to digest the phytoplankton cells. Even in the event of assimilation, e.g. in *A. proxima*, this does not necessarily incur a longer veliger stage (Kempf & Todd, 1989). The ability of *A. bulllata* veligers to actively digest and therefore assimilate the nutrients obtained from the phytoplankton is unknown; it requires further investigation beyond the scope of this project.

In the Fleet, evidence provided by Thompson & Seaward (1989) regarding the re-establishment of the LHP population after the 1986 mortality event, indicated that larval dispersal is restricted. As with *A. proxima* (Todd *et al.*, 1998; Lambert *et al.*, 2003), it seems likely that the duration of the veliger stage is short. However, further genetic evidence would be needed to substantiate this claim.

The inability of some juveniles to consume the substratum after metamorphosis did not appear to affect post metamorphic survivorship. It is therefore probable that the grazing of diatoms and other benthic microorganisms is nutritionally sufficient for early juvenile life. The food was switched to *Zostera* on day 121, because supplies of the algal substratum previously collected were exhausted and were no longer available at LHP. The effect this had on juvenile mortality is unknown because of the death of all *A. bullata* on day 135, due to the constant temperature room thermostat being inadvertently switched off.

Shell growth was initially slow in juveniles provided with only a substratum, but after five days their growth rates began to increase (Fig. 7.22). These juveniles died faster than those from experiment 2. In the latter the shell growth was steady from the start of the recordings (Fig. 7.23). This could have been due to the embryonic food reserves assimilated from the consumption of phytoplankton during the veliger phase; however whether the ingested phytoplankton cells were digested is unknown. The shell length between veligers in vessels 1, 3, and 9 were all relatively similar after 70 days (Fig. 7.23). These rates of growth are similar to that observed by Thorson (1946) who commented that after metamorphosis "hardly seven weeks later had attained a height of shell of 1080 μm".

7.4.4 *OTHER OBSERVATIONS*

The dwarf *Akera bullata* var. *nana* can be found in Fleet, Dorset at LHP. It was originally described as a subspecies by Jeffreys (1867) because it lacks certain characteristics of *A. bullata* sampled from other localities. These differences were further investigated by Thompson & Seaward (1989) who tried to establish whether the var. *nana* was a valid subspecies. Evidence reported here contradicts observations documented by Thompson & Seaward (1989). Thompson consistently stated that the purple gland is absent in *A. bullata* var. *nana* (Thompson, 1976; 1988; Thompson & Seaward, 1989). He attributed this to the precocious maturation of the adults, which die before reaching the stage when the gland becomes functional. In this study two very small (<2 mm shell length) juvenile *A. bullata* were observed to produce purple ink. Rough handling during transfer stimulated purple ink discharge. Attempts to capture the behaviour on film failed. Ink production has been observed in other *A. bullata* (Morton, 1972; Thompson, 1976), and even in the var. *nana* (Jeffreys, 1867). Closely related species with the ability to produce ink include members of the Aplysiidae, the mechanisms of which have been extensively studied. There are three pigments that constitute the ink of *Aplysia* spp: phycocyanobilin, phycourobilin and phycoerythrobilin, the details of which will not be discussed here. If *Aplysia* are fed a diet lacking in these pigments, they become "facultatively de-inked", and thus fail to produce aplysioviolin (Chapman & Fox, 1967). However, when fed a diet of algae containing phycoerythrobilin or even raw extracted R-phycoerythrin (in a 0.1 M sucrose solution), they regain their ability to secrete aplysioviolin (Chapman & Fox, 1967). It appears likely that those *A. bullata* gathered by Thompson & Seaward (1989) from LHP lacked the necessary pigments due to their diet of seagrass roots and *Enteromorpha*. The *A. bullata* spawn collected for this study at LHP were gathered from three dominant species of seaweed: *C. crispus*, *N. helminthoides* and *U. lactuca*. The former two species would have provided the phycoerythrobilin required for the production of aplysioviolin in *A. bullata*. It was only towards the end of the year that *Zostera* was found to be the dominant species at LHP.

The juveniles in this study that produced aplysioviolin had been fed a diet of the red Cryptophyceae *Rhinomonas reticulata* var. *reticulata* which contains phycobiliproteins, the precursor of the purple ink. Thus it appears *A. bullata* is similar to many aplysiids, whereby the ability to produce ink is dependent upon a diet containing phycobiliproteins.

The absence of swimming as adults is another characteristic of var. *nana*. Juveniles collected by Thompson & Seaward (1989) in late summer were able to swim. They suggested that dispersal is the likely reason behind swimming rather than predator avoidance. Morton & Holme (1955) observed both juvenile and adult *A. bullata* swimming, chiefly during the spring. They tentatively related this behaviour to the spawning season. Despite this, they noted that swimming was primarily a result of disturbance, although self-initiated swimming also occurred. During this study not one *A. bullata* was observed swimming. The very small juveniles lacked the parapodial lobes required for propulsion, but even those individuals with fully developed parapodial lobes were never observed to swim.

The pallial tentacle was another feature alleged to be absent in var. *nana* (Thompson & Seaward, 1989). In Jeffreys' (1867) original description of var. *nana* no mention is made of a pallial tentacle. In animals gathered from Plymouth, Morton & Holme (1955) describe a grooved white filament originating from the pallial margin that trailed behind the animal. No long pallial tentacle was found in any of the animals gathered from the Fleet at LHP, however there was a small white filament originating near the exhalant siphon. This structure could be analagous to the pallial tentacle, although it never reached the length reported by Thompson (1976) or that described by Morton & Holme (1955) (Fig. 7.2D). Thompson & Seaward (1989) failed to find evidence of a pallial tentacle in their animals gathered from LHP. However they did mention that specimens gathered from elsewhere in the Fleet "tended" to exhibit a pallial tentacle when over a shell length of 15-20 mm. It is therefore quite likely that the tentacle observed on animals in this study is rudimentary to the pallial tentacle, which would have developed into the "long white filament" described by Morton & Holme (1955) had the juveniles grown larger.

7.4.5 *CONCLUSIONS*

The veligers of *A. bullata* have a Shell-type 2 (Thompson, 1961a) and a Development-type 2 (Thompson, 1967). They hatch after a period of 20 to 30 days at 20°C or 15°C respectively (Thorson (1946) as cited by Thompson (1976)). Within one day (after the inflation of the propodium), they are competent to metamorphose. They are facultative planktotrophs and will ingest phytoplankton when available; however this is not a prerequisite for metamorphosis. Despite certain substrata promoting faster settlement, *A. bullata* will settle in response to a broad spectrum of substrata. In this study three species of algae (two red and one green), a biofilm and four species of phytoplankton all resulted in

successful settlement and metamorphosis. When provided with a combination of a substratum and phytoplankton there were no differences in the time taken to initiate metamorphosis. The number of juveniles that died following metamorphosis was independent of the substratum provided in experiment 1 and experiment 2. There was a significant difference in the time taken for the juveniles to die after metamorphosis in experiment 1; however there was no significant difference in the time taken for the juveniles to die in experiment 2. Shell growth was rapid at 20°C the juveniles reaching a maximum size of 1552 μm after 109 days.

On evidence gathered here and from other documented reports, notably those of Thompson & Seaward (1989) and Morton & Holme (1955); the validity of Jeffery's *Akera bullata* dwarf variety *nana* is doubtful. Thompson & Seaward (1989) are themselves sceptical about its existence in the Fleet, due to the presence of intermediates found near to the population of var. *nana* at LHP. The criteria Thompson & Seaward (1989) used to distinguish var. *nana* from other varieties, notably Norman's var. *farrani*, include the inability to produce purple ink, the absence of the pallial tentacle, and its reduced size. However, it has been proposed here that the animals Thompson & Seaward used lacked aplysioviolin as their diet did not contain a source of phycoerythin. The animals from LHP used in this study were gathered from a variety of algae, notably *Chondrus*, *Nemalion* and *Ulva*. The former two contain phycoerythin and therefore, individuals feeding on these species would have been able to produce the purple ink. The juveniles fed *Rhinomonas* were able to release purple ink when handled roughly, indicating that var. *nana* does have the ability to produce ink from a very early stage of development, contradicting Thompson & Seaward (1989).

A small finger-like projection was observed protruding from the juveniles which may be analogous to the pallial tentacle observed in *A. bullata* found elsewhere.

The only uncertainty raised which may support the validity of *A. bullata* var. *nana* was the difference in size between those newly hatched veligers from this study and those measured by Thorson (1946). To resolve the mystery of var. *nana* it is suggested that genetic analysis is undertaken to identify possible subspecies.

Chapter 8

Chapter 8

General Discussion

During the course of this thesis several new discoveries were made regarding the ecology of the Nudibranchia. However, many questions were raised which were beyond the scope of this project. In this discussion I will link in the findings of this thesis to what is already known for the Nudibranchia, and will address some of the questions raised which remain unresolved.

Despite the increasing use of electron microscopy in histology to assist in the phylogenetic analysis of the Nudibranchia, the process of gametogenesis largely remains unknown. Only two studies have been published regarding spermatogenesis and vitellogenesis by Eckelbarger & Eyster (1981) and Medina *et al.* (1988) respectively. In Chapter 2 I presented evidence that supports the hypothesis that vitellogenesis in *Thecacera pennigera* (Montagu), *Facelina auriculata* (Müller), *A. gibbosa* (Risso) and *Palio nothus* (Johnston) occurs in cycles whereby one batch of previtellogenic oocytes matures and then remains within the acini until spawning, after which vitellogenesis occurs again. This process avoids the wasteful degeneration of oocytes which may otherwise occur if vitellogenesis was a continuous process. Despite investigating vitellogenesis in *Hypselodoris tricolor* Cantraine and *Godiva banyulensis* Portmann & Schmekel, Medina *et al.* (1988) failed to identify the cellular processes involved in yolk synthesis. This is still unknown for all species of nudibranchs.

Spermatogenesis in *T. pennigera*, *F. auriculata* and *A. gibbosa* is similar to that described for other opisthobranch species (Beeman, 1970; Eckelbarger & Eyster, 1981 etc). In *P. nothus* however, the morphology of the ovotestes and the orientation of the developing spermatids differed from Rivest's (1984) description for *P. zosterae* O'Donoghue and *P. dubia* (Sars). In *P. nothus* the female acini were joined to the male acinus by a narrow sphincter, rather than the "short neck" structures Rivest (1984) described. The female acini were located immediately adjacent to the notum on the lateral and dorsal surfaces, and the male acini were arranged immediately between the female acini and the digestive gland,

rather than with the male acini at the periphery and the female acini between the digestive gland and the male acini as Rivest (1984) had described. It is not known why the ovotestes of *P. nothus* was arranged differently to *P. zosterae* and *P. dubia*, however it could be linked to the method of copulation; unfortunately within *P. nothus*, this is also unknown. It is possible that *P. nothus* copulates via hypodermic injection, for which it would be logical for the female acini to be located beneath the notum. This orientation would facilitate effective fertilization as the oocytes would have direct exposure to allosperm within the acini rather than the sperm first entering the male acini then swimming into the receptaculum seminis where it is stored for fertilization. Rivest (1984) described the receptaculum seminis of *P. dubia* and *P. zosterae* containing sperm after copulation, however the sperm were not associated with the epithelium of the receptaculum seminis as has been observed in most other nudibranch species. Within the male acini, cells undergoing spermatogenesis were orientated in long string-like clusters, rather than the typical spherical clusters seen in other species of nudibranchs. The string-like arrangement of the developing spermatozoa has never been described before within the Nudibranchia, and the reasons for this unusual arrangement are unknown. All three *Palio* spp. require further investigation, in particular regarding their copulatory behaviour and morphology. The genetic analysis of the Polyceridae would help to resolve their phylogeny and would determine whether *P. nothus* has been correctly classified.

In the 1950s attempts were made to rear nudibranch veligers to understand a stage of their life cycle which was unknown. Many nudibranch species can be found for only a few months, after which they seemingly disappear until a year later. The successful rearing of several species since the 1950s has revealed that, for planktotrophic species, the veliger stage constitutes a significant phase of their life. It governs the distances they travel, hence contributes to gene flow; it allows new populations to become established; it can alleviate competition if resources are scarce by ensuring larvae are carried away from the parent population; it facilitates recolonization if populations become extinct due to bottlenecks etc; and it prevents inbreeding. However, it also increases the veliger's risk of mortality and the likelihood of dispersal away from suitable habitats. In some species, settlement is specific to the prey of the adults; thus by rearing the veligers their ecology and larval dispersal can be better understood. In this study, *T. pennigera* was the only species to undergo mantle fold retraction after 10 days at 20°C. The veligers which reached this stage measured 177.2 ± 8.5

μm (shell length), however when reared at 12-15°C the maximum shell length reached was 148.2 ± 8.5 μm. *Onchidoris bilamellata* (L.) exhibited a maximum shell length of 440 μm when reared by Todd (1981) but only 320 μm when reared by Chia & Koss (1988). The latter authors failed to note the size difference or discuss possible reasons for it. It is unknown whether *T. pennigera* achieved competency as metamorphosis was unsuccessful; however there was no indication that veliger development was abnormal. Poor quality *Bugula* and/or insufficient development may have been the reasons for the failure to metamorphose. The combination of high mortality and an extended veliger stage (slow development at the culture temperature), was probably the cause for the slow/no development exhibited by *A. gibbosa*, *P. nothus* and *Cuthona gymnota* Couthouy. With the exception of *O. bilamellata*, neither *T. pennigera*, *C. gymnota*, *P. nothus* nor *A. gibbosa* have been reared successfully through metamorphosis, hence their veliger development and settlement stimuli remain unknown.

Investigations into nudibranch population genetics have been used to asses the extent of larval dispersal in both planktotrophic and lecithotrophic species (Todd, Havenhand & Thorpe, 1988; Todd, Lambert & Thorpe, 1998). This in turn has been used to explain differences in life history traits between populations with restricted larval dispersal (Jones, Todd & Lambert, 1996; Lambert, Todd & Thorpe, 2003). Contrary to expectations, it was discovered that populations of the lecithotrophic *Adalaria proxima* (Alder & Hancock) self-recruited despite having a planktonic veliger stage lasting 1-2 days (Thompson, 1958; Lambert & Todd, 1994). Behavioural constraints were implicated as the reason for the veligers settling soon after release (Todd *et al.*, 1998; Lambert *et al.*, 2003). *Goniodoris nodosa* (Montagu), which has planktotrophic veligers which spend between 10 to 13 weeks in the plankton (Todd *et al.*, 1998), were found to have genetically homogeneous populations over distances of 1600 km. Similarly, this study discovered three populations of the planktotrophic *T. pennigera* to be genetically homogeneous over a distance of 172 km. This homogeneity was attributed to a teleplanic larval stage, although the larval duration remains unknown. Since its initial description from Devon, several foreign sightings of *T. pennigera* have been reported. These sightings are sporadic, and only one population from Brazil (Marcus, 1957) has been recorded in numbers comparable to that of the Welsh populations. Increased transoceanic shipping was suggested by Thompson & Brown (1984) to have assisted in its world-wide dispersal. Further genetic analysis of foreign individuals would

provide a valuable insight into the dispersal of this species, and provide information on its evolution and possible speciation.

Thorough histological and morphological studies of the Goniodorididae are scarce, with the exception of *G. castanea* (Alder & Hancock) by Wägele & Willan (2001). The goniodorid *A. gibbosa* has been described as "an uncommon species" but also as having "one of the widest geographical ranges of any nudibranch of the North Atlantic" (Thompson & Brown, 1984). For the first time, the internal morphology and histology were examined in *A. gibbosa*. It is the first member of the Goniodorididae found to have a diaulic reproductive system. A vagina is absent; instead the bursa copulatrix and the receptaculum seminis are located proximally to the nidamental glands on the oviduct. When investigating the phylogeny of dorids, Thollesson (1999) discovered ambiguity within the Goniodorididae; in his phylogenetic reconstruction *Ancula* was positioned as a primitive member of the group. The histological and morphological results from this study support Thollesson's (1999) genetic data, which suggested that *Ancula* is a primitive member of the Goniodorididae. Further genetic and histological investigations are required to determine the validity of, and the evolutionary relationships between, the Goniodorididae genera.

Schrödl (1997; 2002) and Haumayr & Schrödl (2003) have contributed significantly to our understanding of *Ismaila* spp., and the effects of these parasites on South American nudibranchs. However, parasitism by *Splanchnotrophus* in north eastern Atlantic species of nudibranchs has yet to be properly investigated. Although there were a few discoveries in the late 1800s to early 1900s, the descriptions of the parasites themselves and the effects on their hosts are vague and sometimes their identification is ambiguous. A population of *A. gibbosa* discovered at Mumbles was found to be infected with the rare splanchnotrophid: *S. willemi* Canu. This is the first recorded discovery of *S. willemi* in UK coastal waters. The effects of *S. willemi* varied from no obvious internal damage to the complete disintegration of the digestive gland. The reason for the differences in internal damage is unknown, although the period of infection by *S. willemi* was implicated. The presence of allosperm within the receptaculum seminis indicated that all of the individuals infected were found to have copulated at least once in their lifetime. Whether the individuals were capable of copulating and whether they produced viable spawn after infection is unknown. The high prevalence of parasitism in *A. gibbosa* may be the cause of the sporadic occurrence of this

nudibranch around the UK. Until populations of infected *A. gibbosa* are discovered and studied, the behavioural effects and the effect on fecundity remain unsolved.

The anaspidean *Akera bullata* (Müller) was gathered from the Fleet, Dorset, at Langton Hive Point. The individuals collected from this location were identified by Thompson & Seaward (1989) as var. *nana*, the dwarf variety originally described by Jeffreys (1867). They spawn lecithotrophic veligers, but contrary to Thompson (1976), they were found to possess a Shell-type 2. There was a difference in shell length between the Danish veligers recorded in Thorson's (1946) study to those gathered from the Fleet for this study; this size difference could be due to the two studies having investigated different sub-species. Thorson (1946) did not specify which variety he investigated. The veligers studied in this report were capable of metamorphosis within 24 hours of release. Settlement was initiated faster in response to *Chondrus* and a biofilm; however, after a delay of several days settlement was also stimulated by *Nemalion*, *Ulva*, *Rhinomonas*, *Isochrysis*, *Tetraselmis*, *Chaetoceros* (and combinations of both phytoplankton and alga), and a control. It appears that in the absence of the preferred substratum the veliger settlement threshold lowers, thus the veligers are forced to settle. Contrary to one of Thompson & Seaward's (1989) classification criteria for var. *nana*, the discharge of purple ink was observed in two individuals reared on *Rhinomonas*. This study has demonstrated that the morphological conditions listed by Thompson & Seaward (1989) to differentiate between var. *nana* and var. *farrani*, are not steadfast and consequently the use of var. *nana* should be avoided. Genetic analysis of the populations within the Fleet and populations elsewhere (in particular where var. *farrani* has been reported), would provide valuable insight into larval dispersal and would resolve whether var. *nana* and var. *farrani* are genuine subspecies of *A. bullata*..

Bibliography

Bibliography

ALBERT HUBBARD, E.J. 1988. Larval growth and the induction of metamorphosis of a tropical sponge-eating nudibranch. *Journal of Molluscan Studies*, **54**: 259-269.

ALDER, J. & HANCOCK, A. 1845-1855. *A Monograph of the British Nudibranchiate Mollusca*, **Parts 1-7**. Ray Society, London.

AVILA, C. 1998. Competence and metamorphosis in the long term planktotrophic larvae of the nudibranch mollusc *Hermissenda crassicornis* (Eschscholtz, 1831). *Journal of Experimental Marine Biology and Ecology*, **231**: 81-117.

AVILA, C. & PAUL, V.J. 1997. Chemical ecology of the nudibranch *Glossodoris pallida*: is the location of diet derived metabolites important for defense? *Marine Ecology Progress Series*, **150**: 171-180.

AVILA, C., GRENIER, S., TAMSE, C.T. & KUZIRIAN, A.M. 1997. Biological factors affecting larval growth in the nudibranch mollusc *Hermissenda crassicornis* (Eschscholtz, 1831). *Journal of Experimental Marine Biology and Ecology*, **218**: 243-262.

BABA, K. 1960. The genera *Polycera*, *Palio*, *Greilada* and *Thecacera* from Japan (Nudibranchia-Polyceridae). *Publications of the Seto Marine Biology Laboratory*, **8**: 75-79.

BARNARD, K.H. 1933. Description of a new species of *Thecacera*. *Journal of Conchology, London*, **19**: 294-295.

BEEMAN, R.D. 1970. An autoradiographic and phase contrast study of spermatogenesis in the anaspidean opisthobranch *Phyllaplysia taylori* Dall, 1900. *Archives de Zoologie expérimentale et générale*, **111**: 5-22.

BELCIK, F.P. 1981. The male of *Ismaila monstrosa* Bergh, 1867 (Copepoda, Splanchnotrophidae). *Crustaceana*, **40**: 16-25.

BERGH, R. 1868. On *Phidiana lynceus* and *Ismaila monstrosa*. *Annals and Magazine of Natural History*, **(4)2**: 133-138.

BICKELL, L.R. & CHIA, F.-S. 1979. Organogenesis and histogenesis in the planktotrophic veliger of *Doridella steinbergae* (Opisthobranchia: Nudibranchia). *Marine Biology*, **52**: 291-313.

BICKELL, L.R. & KEMPF, S.C. 1983. Larval and metamorphic morphogenesis in the nudibranch *Melibe leonina* (Mollusca: Opisthobranchia). *Biological Bulletin*, **165**: 119-138.

BLOWER, S. & ROUGHGARDEN, J. 1989. Parasites detect host spatial pattern and density: a field experimental analysis. *Oecologia*, **78**: 138-141.

BONAR, D.B. 1978. Morphogenesis at metamorphosis in opisthobranch molluscs. In: *Settlement and metamorphosis of marine invertebrate larvae* (F.-S. Chia & M.E. Rice, eds.), 177-196. Elsevier, New York.

BONAR, D.B. & HADFIELD, M.G. 1974. Metamorphosis of the marine gastropod *Phestilla sibogae* Bergh (Nudibranchia: Aeolidacea). 1. Light and electron microscopic analysis of larval and metamorphic stages. *Journal of Experimental Marine Biology and Ecology*, **16**: 227-255.

BOYDEN, C.R., CROTHERS, J.H., LITTLE, C. & METTAM, C. 1977. The intertidal invertebrate fauna of the severn estuary. *Field Studies*, **4**: 477-554.

BRIDGES, C.B. 1975. Larval development of *Phyllaplysia taylori* Dall, with a discussion of the development in the Anaspidea (Opisthobranchiata: Anaspidea). *Ophelia*, **14**: 161-184.

CANU, E. 1891. Sur quelques Copépodes parasites, observés dans le Boulonnais. *Compte rendu hebdomadaire des Séances de l'Académie des Sciences, Paris*, **113**: 435-437.

CANU, E. 1898. Note sur les Copépodes et les Ostracodes marins des côtes de Normandie. In: *Bulletin de la Société des Amis des Sciences naturelles de Rouen* **33**, 389-422.

CANU, E. 1899. Sur *Lichomolgus trochi*, nov. sp., Copépode nouveau parasite d'un mollusque. *Travaux de la Station zoologique de Wimereux*, **7**: 73-79.

CASTEEL, D.B. 1904. The cell-lineage and early larval development of *Fiona marina*, a nudibranch mollusc. *Proceedings of the Academy of Natural Sciences of Philadelphia*: 325-419.

CARROLL, D.J. & KEMPF, S.C. 1990. Laboratory culture of the Aeolid nudibranch *Berghia verrucicornis* (Mollusca, Opisthobranchia): Some aspects of its development and life history. *Biological Bulletin*, **179**: 243-253.

CHAPMAN, D.J. & FOX, D.L. 1967. Bile pigment metabolism in the sea hare *Aplysia*. *Journal of Experimental Marine Biology and Ecology*, **4**: 71-78.

CHESTER, C.M. 1996. The effect of adult nutrition on the reproduction and development of the estuarine nudibranch *Tenellia adspersa* (Nordmann, 1845). *Journal of Experimental Marine Biology and Ecology*, **198**: 113-130.

CHIA, F.-S. 1989. Differential larval settlement of benthic marine invertebrates. In: *Reproduction, genetics and distributions of marine organisms* (J.S. Ryland & P.A. Tyler, eds.), 3-12. Olsen and Olsen, Fredensborg Denmark.

CHIA, F.-S. & KOSS, R. 1978. Development and metamorphosis of the planktotrophic larvae of *Rostanga pulchra* (Mollusca: Nudibranchia). *Marine Biology*, **46**: 109-119.

CHIA, F.-S. & KOSS, R. 1988. Induction of settlement and metamorphosis of the veliger larvae of the nudibranch, *Onchidoris bilamellata*. *International Journal of Invertebrate Reproduction and Development*, **14**: 53-70.

CHRISTENSEN, H. 1977. Feeding and reproduction in *Precuthona peachi* (Mollusca Nudibranchia). *Ophelia*, **16**: 131-142.

CLARK, K.B. 1975. Nudibranch life cycles in the Northwest Atlantic and their relationship to the ecology of fouling communities. *Helgoländer wiss. Meeresunters*, **27**: 28-69.

CRISP, D.J. 1974. Factors influencing the settlement of marine invertebrate larvae. In: *Chemoreception in marine invertebrate larvae* (P.T. Grant & A.M. Mackie, eds.), 177-265. Academic Press, New York.

CRISP, D.J. 1976. The role of the pelagic larva. In: *Perspectives in Environmental Biology* **1**, (P. Spencer-Davies, ed.) 145-155. Pergamon Press, Oxford.

CUÉNOT, L. 1927. Contribution á la faune du bassin d' Arcachon. III. Doridiens. *Travaux des Laboratories. Société Scientifique et Station Biologique d' Arcachon*, **7**: 1-22.

DEBOUTTEVILLE, C.D. 1951. Description du mâle du genre Splanchnotrophus Hancock et Norman (Crust. Copepoda). *Vie et Milieu*, **2**: 367-370.

DE JONG-BRINK, M., BOER, H.H. & JOOSSE, J. 1983. Mollusca. In: *Reproductive Biology of Invertebrates.* **1**, Oogenesis, oviposition, and oosorption (K.G. Adiyodi & R.G. Adiyodi, eds.), 297-355. John Wiley & Sons, Chichester.

DEKKER, R. 1986. Second record of *Thecacera pennigera* (Gastropoda: Opisthobranchia) from the Dutch coast. *Basteria*, **50**: 45-46.

DOHMEN, M.R. 1983. Gametogenesis. In: *The Mollusca* **3**, Development (K. Wilbur, ed.) 1-48. Academic Press, New York.

ECKELBARGER, K.J. & EYSTER, L.S. 1981. An ultrastructural study of spermatogenesis in the nudibranch mollusc *Spurilla neapolitana. Journal of Morphology*, **170**: 283-299.

EDMUNDS, M. 1968. Acid secretion in some species of Doridacea (Mollusca, Nudibranchia). *Proceedings of the Malacological Society London*, **38**: 121-133.

EDMUNDS, M. 1987. Color in Opisthobranchs. *American Malacological Bulletin*, **5**: 185-196.

EDMUNDS, M. & KRESS, A. 1969. On the European species of *Eubranchus* (Mollusca Opisthobranchia). *Journal of the Marine Biological Association of the United Kingdom*, **49**: 879-912.

EYSTER, L.S. 1979. Reproduction and developmental variability in the opisthobranch *Tenellia pallida. Marine Biology*, **51**: 133-140.

EYSTER, L.S. 1981. Observations on the growth, reproduction and feeding of the nudibranch *Armina tigrina. Journal of Molluscan Studies*, **47**: 171-181.

FAHEY, S.J. & HEALY, J.M. 2003. Sperm ultrastructure in the nudibranch Genus *Halgerda* with reference to other Discodorididae and to Chromodorididae (Mollusca: Opisthobranchia). *Journal of Morphology*, **257**: 9-21.

FAULKNER, D.J. & GHISELIN, M. 1983. Chemical defense and evolutionary ecology of dorid nudibranchs and some other opisthobranch gastropods. *Marine Ecology Progress Series*, **13**: 295-301.

FEATHERSTONE, N. & LAMBIE, P. 2005. *Reeds Oki Western Almanac*, A & C Black

FOLMER, O., BLACK, M., HOEH, W., LUTZ, R. & VRIJENHOEK, R. 1994. DNA primers for amplification of mitchondrial cytochrome C oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, **3**: 294-299.

FONTANA, A., CIAVATTA, M.L., D'SOUZA, L., MOLLO, E., NAIK, C.G., PARAMESWARAN, P.S., WAHIDULLA, S. & CIMINO, G. 2001. Selected

chemo-ecological studies of marine opisthobranch from Indian coasts. *Journal of the Indian Institute of Science*, **81**: 403-415.

FORREST, J.E. 1953. On the feeding habits and the morphology and mode of functioning of the alimentary canal in some littoral dorid nudibranchiate mollusca. *Proceedings of the Linnean Society London*, **164**: 225-235.

FRANZ, D.R. 1970. Zoogeography of northwest Atlantic opisthobranch molluscs. *Marine Biology*, **7**: 171-180.

FRANZ, D.R. 1971. Development and metamorphosis of the gastropod *Acteocina canaliculata* (Say). *Transactions of the American Microscopical Society*, **90**: 174-182.

FRANZ, D.R. 1975. Opisthobranch culture. In: *Culture of Marine Invertebrate Animals* (Smith & M.H. Chanley, eds.), 245-256. Plenum Press, London.

GARSTANG, W. 1889. Report on the nudibranchiate mollusca of Plymouth Sound. *Journal of the Marine Biological Association of the United Kingdom*, **1**: 173-198.

GARSTANG, W. 1890. A complete list of the Opisthobranchiate Mollusca found at Plymouth, with further observations on their morphology, colours and natural history. *Journal of the Marine Biological Association of the United Kingdom*, **1**: 399-457.

GHISELIN, M. 1965. Reproductive function and the phylogeny of opisthobranch gastropods. *Malacologia*, **3**: 327-379.

GOHAR, H.A.F. & ABUL-ELA, I.A. 1959. The biology and development of three nudibranchs (from the Red Sea). *Publications of the Marine Biological Station Al-Ghardaqa*, **10**: 41-62.

GOHAR, H.A.F. & SOLIMAN, G.N. 1963. The biology and development of Hexabranchus sanguineus (Rüpp. and Leuck.) (Gastropoda, Nudibranchiata). *Publications of the Marine Biological Station Al-Ghardaqa*, **10**: 219-248.

GOHAR, H.A.F. & SOLIMAN, G.N. 1967a. On two rare nudibranchs of the genus *Trippa* Bergh (of different developmental behaviour). *Publications of the Marine Biological Station Al-Ghardaqa*, **14**: 269-293.

GOHAR, H.A.F. & SOLIMAN, G.N. 1967b. The direct development of the nudibranch *Casella obsoleta* (Rüpp. and Leuck.) (with remarks on metamorphosis). *Publications of the Marine Biological Station Al-Ghardaqa*, **14**: 149-166.

GIBSON, G.D. & CHIA, F.-S. 1994. A metamorphic inducer in the opisthobranch *Haminaea callidegenita*: partial purification and biological activity. *Biological Bulletin*, **187**: 133-142.

GRANDE, C., TEMPLADO, J., CERVERA, J.L. & ZARDOYA, R. 2004. Phylogenetic relationships among Opisthobranchia (Mollusca: Gastropoda) based on mitchondrial *cox 1*, *trn V*, and *rrnL* genes. *Molecular Phylogenetics and Evolution*, **33**: 378-388.

GRANT, A. & WILLIAMSON, P. 1985. The settlement-timing hypothesis: a critique. *Marine Ecology Progress Series*, **23**: 193-196.

GRIMSTONE, A. & SKAER, R. 1972. *A guidebook to microscopical methods*, 1-134. Cambridge University Press, London.

GUILLARD, R.R.L. 1975. Culture of phytoplankton for feeding marine invertebrates. In: *Culture of marine invertebrates* (W.L. Smith & M.H. Chanley, eds.), 29-60. Plenum Press, London.

GUILLARD, R.R.L. & RYTHER, J.H. 1962. Studies of marine planktonic diatoms 1. *Cyclotella nana* Hustedt, and *Detonula confervacea* (Cleve) gran. *Canadian Journal of Microscopy*, **8**: 229-239.

HADFIELD, M.G. 1963. The biology of nudibranch larvae. *Oikos*, **14**: 85-95.

HADFIELD, M.G. 1978. Metamorphosis in marine molluscan larvae: An analysis of stimulus and response. In: *Settlement and metamorphosis of marine invertebrate larvae* (F.-S. Chia & M.E. Rice, eds.), 165-175. Elsevier, New York.

HADFIELD, M.G. & KARLSON, R. 1969. Externally induced metamorphosis in a marine gastropod. *American Zoologist*, **9**: 1122.

HADFIELD, M.G. & MILLER, S. 1987. On developmental patterns of opisthobranchs. *American Malacological Bulletin*, **5**: 197-214.

HADFIELD, M.G. & SCHEUER, D. 1985. Evidence for a soluble metamorphic inducer in *Phestilla*: ecological, chemical and biological data. *Bulletin of Marine Science*, **37**: 556-566.

HAMATANI, I. 1960a. Notes on veligers of Japanese opithobranchs (1). *Publications of the Seto Marine Biology Laboratory*, **8**: 59-70.

HAMATANI, I. 1960b. Notes on veligers of Japanese opisthobranchs (2). *Publications of the Seto Marine Biology Laboratory*, **8**: 85-93.

HAMATANI, I. 1967. Notes on veligers of Japanese opisthobranchs (7). *Publications of the Seto Marine Biology Laboratory*, **15**: 121-131.

HANCOCK, A. & EMBLETON, D. 1852. On the anatomy of *Doris*. *Philosophical Transactions of the Royal Society Series B*, **142**: 207-252.

HANCOCK, A. & NORMAN, A.M. 1863. On *Splanchnotrophus*, an undescribed genus of Crustacea, parasitic on nudibranchiate Mollusca. *Transactions of the Linnean Society of London, Zoology*, **24**: 49-60.

HARRIGAN, J.F. & ALKON, D.L. 1978. Larval rearing, metamorphosis, growth and reproduction of the Eolid nudibranch *Hermissenda crassicornis* (Eschscholtz, 1831) (Gastropoda: Opisthobranchia). *Biological Bulletin*, **154**: 430-439.

HARRIS, L.G. 1973. Nudibranch Associations. In: *Current topics in comparative pathobiology* **2**, (T.C. Cheng, ed.) 213-315. Academic Press, Baltimore.

HARRIS, L.G. 1975. Studies on the life history of two coral-eating nudibranchs of the genus *Phestilla*. *Biological Bulletin*, **149**: 539-550.

HARRIS, L.G., WRIGHT, L. & RIVEST, B. 1975. Observations on the occurence and biology of the aeolid nudibranch *Cuthona nana* in New England waters. *The Veliger*, **17**: 264-268.

HAUMAYR, U. & SCHRÖDL, M. 2003. Revision of the endoparasitic copepod genus *Ismaila* Berg, 1867, with description of eight new species. *Spixiana*, **26**: 1-33.

HAVENHAND, J.N. 1995. Evolutionary ecology of larval types. In: *Ecology of Marine Invertebrate Larvae* (L. McEdward, ed.) 79-122. CRC Press, London.

HEALY, J.M. & WILLAN, R.C. 1984. Ultrastructure and phylogenetic significance of Notaspidean spermatozoa (Mollusca, Gastropoda, Opisthobranchia). *Zoologica Scripta*, **13**: 107-120.

HECHT, E. 1893. Note sur un nouveau Copépode parasite des Nudibranches. *Archives de Zoologie expérimentale et générale (3)1, notes et revue*: xiii-xvi.

HO, J.-S. 1981. *Ismaila occulta*, a new species of poecilostomatoid copepod parasite in a dendronotid nudibranch from California. *Journal of Crustacean Biology*, **1**: 130-136.

HO, J.-S. 1987. Reconsideration of *Ismaila monstrosa* Bergh, 1867 (Copepoda, Splanchnotrophidae) from Oregon. *Crustaceana*, **52**: 109-111.

HODGSON, A.N., BERNARD, R.T.F. & LINDLEY, D.S. 1991. Comparative spermatology of four sympatric species of *Siphonaria* (Pulmonata: Basommatophora). *Journal of Molluscan Studies*, **57**: 309-322.

HOLLEMAN, J.J. 1972. Observations on growth, feeding, reproduction and development in the opisthobranch, *Fiona pinnata* (Eschscholtz). *The Veliger*, **15**: 142-146.

HOLMAN, J.A. 1972. The ultrastructure and activity of sperm in *Acanthodoris pilosa*, a nudibranch mollusk. *Marine Biology*, **12**: 179-183.

HUBBARD, E.J.A. 1988. Larval growth and the induction of metamorphosis of a tropical sponge-eating nudibranch. *Journal of Molluscan Studies*, **54**: 259-269.

HUNHAM, P. & BROWN, G. 1975. Sublittoral nudibranch mollusca (sea slugs) in Pembrokeshire waters. *Field Studies*, **4**: 131-159.

HURST, A. 1967. The egg masses and veligers of thirty northeast pacific opisthobranchs. *The Veliger*, **9**: 255-288.

HUYS, R. 2001. Splanchnotrophid systematics: a case of polyphyly and taxanomic myopia. *Journal of Crustacean Biology*, **21**: 106-156.

HYMAN, L. 1967. *The Invertebrates: Mollusca I*, **VI**, 1-792. McGraw-Hill, New York.

JEFFREYS, J.G. 1867. Genus III A'cera. In: *British Conchology* **4**, 429-432. Van Coorst, London.

JENSEN, K.R. 1987. *Ismaila monstrosa* Bergh (Copepoda: Splanchnotrophidae) found parasitizing in *Ercolania funerea* (Costa) (Gastropoda: Ascoglossa). *Ophelia*, **28**: 75-84.

JENSEN, K.R. 1990. *Splanchnotrophus elysiae* n. sp. (Copepoda; Splanchnotrophidae) found parasitizing in the sacoglossan opisthobranch *Elysia australis* (Quoy and Gaimard, 1932) Proceedings of the Third International Marine Biology Workshop: The Marine Flora and Fauna of Albany, Western Australia, Western Australian Museum, Perth, pp 291-296.

JOHANNESSON, K. 1988. The paradox of Rockall: why is a brooding gastropod (*Littorina saxatilis*) more widespread than one having a planktonic larval dispersal stage (*L. littorea*)? *Marine Biology*, **99**: 507-513.

JONES, H., TODD, C. & LAMBERT, W. 1996. Intraspecific variation in embryonic and larval traits of the dorid nudibranch mollusc *Adalaria proxima* (Alder and Hancock) around the northern coasts of the British Isles. *Journal of Experimental Marine Biology and Ecology*, **202**: 29-47.

KEMPF, S.C. 1981. Long-lived larvae of the gastropod *Aplysia juliana*: do they disperse and metamorphose or just slowly fade away? *Marine Ecology Progress Series*, **6**: 61-64.

KEMPF, S.C. & HADFIELD, M.G. 1985. Planktotrophy by the lecithotrophic larvae of a nudibranch, *Phestilla sibogae* (Gastropoda). *Biological Bulletin*, **169**: 119-130.

KEMPF, S.C. & TODD, C. 1989. Feeding potential in the lecithotrophic larvae of *Adalaria proxima* and *Tritonia hombergi*: an evolutionary perspective. *Journal of the Marine Biological Association of the United Kingdom*, **69**: 659-682.

KEMPF, S.C. & WILLOWS, A.O.D. 1977. Laboratory culture of the nudibranch *Tritonia diomedea* Bergh (Tritoniidae: Opisthobranchia) and some apsects of its behavioural development. *Journal of Experimental Marine Biology and Ecology*, **30**: 261-276.

KLUSSMANN-KOLB, A. 2001. The reproductive systems of the Nudibranchia (Gasteropod, Opisthobranchia): comparative histology and ultrastructure of the nidamental glands with aspects of functional morphology. *Zoologischer Anzeiger*, **240**: 119-136.

KLUSSMANN-KOLB, A. 2003. Phylogeny of the Aplysiidae (Gastropoda, Opisthobranchia) with new aspects of the evolution of seahares. *Zoologica Scripta*, **33**: 439-462.

KOLB, A. 1998. Morphology, anatomy and histology of four species of *Armina* Rafinesque, 1814 (Nudibranchia, Arminoidea, Arminidae) from the Mediterranean sea and the Atlantic ocean. *Journal of Molluscan Studies*, **64**: 355-386.

KRESS, A. 1970. A new record of *Trapania pallida* (Opisthobranchia, Gasteropoda) with a description of its reproductive system and a comparion with *T. fusca*. *Proceedings of the Malacological Society London*, **39**: 111-116.

KRESS, A. 1981. A scanning electron microscope study of notum structures in some dorid nudibranchs (Gastropoda: Opisthobranchia). *Journal of the Marine Biological Association of the United Kingdom*, **61**: 177-191.

KRESS, A. 1986. Ultrastructural study of oogenesis and yolk formation in an opisthobranch mollusc, *Runcina*. *Tissue and cell*, **18**: 915-935.

KRIEGSTEIN, A.R. 1977. Stages in the post-hatching development of *Aplysia californica*. *Journal of Experimental Zoology*, **199**: 275-288.

KRIEGSTEIN, A.R., CASTELLUCCI, V. & KANDEL, E.R. 1974. Metamorphosis of *Aplysia californica* in laboratory culture. *Proceedings of the National Academy of Sciences USA*, **71**: 3654-3658.

KUMAR, S., TAMURA, K. & NEI, M. 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics*, **5**: 150-163.

LAMBERT, W. & TODD, C.D. 1994. Evidence for a water-borne cue inducing metamorphosis in the dorid nudibranch mollusc *Adalaria proxima* (Gasteropoda: Nudibranchia). *Marine Biology*, **120**: 265-271.

LAMBERT, W., TODD, C. & THORPE, J. 2000. Variation in growth rate and reproductive output in British populations of the dorid nudibranch *Adalaria proxima*: consquences of restricted larval dispersal? *Marine Biology*, **137**: 149-159.

LAMBERT, W., TODD, C. & THORPE, J. 2003. Genetic population structure of two intertidal nudibranch molluscs with contrasting larval types: temporal variation and transplant experiments. *Marine Biology*, **142**: 461-471.

LEE, T. & Ó FOIGHIL, D. 2004. Hidden Floridian biodiversity: mitchondrial and nucleur gene trees reveal four cryptic species within the scorched mussel, *Branchidontes exustus*, species complex. *Molecular Ecology*, **13**: 3527-3542.

LEMCHE, H. 1929. Gastropoda Opisthobranchiata. *Zoology of the Faroes*, **53**: 1-35.

MACNAE, W. 1957. The families Polyceridea and Goniodorididae (Mollusca, Nudibranchiata) in southern Africa. *Transactions of the Royal Society of South Africa*, **35**: 341-372.

MARCUS, E. 1957. On Opisthobranchia from Brazil. *Journal of the Linnean Society Zoology*, **43**: 390-486.

MAUCH, S. & ELLIOTT, J. 1997. Protection of the nudibranch *Aeolidia papillosa* from nematocyst discharge of the sea anemone *Anthopleura elegantissima*. *The Veliger*, **40**: 148-151.

MCFARLAND, F.K. & MULLER-PARKER, G. 1993. Photosynthesis and retention of zooxanthellae and zoochlorellae within the aeolid nudibranch *Aeolidia papillosa*. *Biological Bulletin*, **184**: 223-229.

MEDINA, A., GARCIA, J., MORENO, F. & LOPEZ-CAMPOS, J. 1986. Comparative studies on the histology of the ovotestis in *Hypseldoris tricolor* and *Godiva banyulensis* (Gastropoda, Opisthobranchia), with special reference to yolk formation. *Journal of Morphology*, **188**: 105-118.

MEDINA, A., GRIFFOND, B., GARCIA-GOMEZ, J. & GARCIA, F. 1988. Ultrastructure of the gametolytic gland and seminal receptacle in *Hypseldoris messinensis* (Gastropoda, Opisthobranchia). *Journal of Morphology*, **195**: 95-102.

MEDINA, M. & WALSH, P.J. 2000. Molecular systematics of the Order Anaspidea based on mitchondrial DNA sequence (12S, 16S, and CO1). *Molecular Phylogenetics and Evolution*, **15**: 41-58.

MONOD, T.H. & DOLLFUS, R.-P. 1932. Les Copépodes parasites de mollusques. *Annales de Parasitologie humaine et comparée*, **10**: 129-204.

MORTON, J.E. 1972. The form and functioning of the pallial organs in the Opisthobranch *Akera bullata* with a discussion on the nature of the gill of Notaspidea and other Techibranchs. *The Veliger*, **14**: 337-349.

MORSE, M.P. 1971. Biology and life history of the nudibranch mollusc, *Coryphella stimpsoni* (Verrill, 1879). *Biological Bulletin*, **140**: 84-94.

MORTON, J.E. & HOLME, N.A. 1955. The occurence at Plymouth of the opisthobranch *Akera bullata*, with notes on its habits and relationships. *Journal of the Marine Biological Association of the United Kingdom*, **34**: 101-112.

NADEAU, L., PAIGE, J.A., STARCZAK, V., CAPO, T., LAFLER, J. & BIDWELL, J.P. 1989. Metamorphic competence in *Aplysia californica* Cooper. *Journal of Experimental Marine Biology and Ecology*, **131**: 171-193.

NASS, C.A.G. 1959. The X^2 test for small expectations in contigency tables, with special reference to accidents and absenteesim. *Biometrika*, **46**: 365-385.

NOVARINO, G. 1991. Observations on *Rhinomonas reticulata* comb. nov. and *R. reticulata* var. *eleniana* var. nov. (Cryptophyceae), with comments on the genera Pyrenomonas and Rhodomonas. *Nordic Journal of Botany*, **11**: 243-252.

PAIGE, J.A. 1986. The laboratory culture of two aplysiids, *Aplysia brasiliana* Rang, 1828, and *Bursatella leachii plei* (Rang, 1828) (Gastropoda: Opisthobranchia) in artifical seawater. *The Veliger*, **29**: 64-69.

PAIGE, J.A. 1988. Biology, metamorphosis and postlarval development of *Bursatella leachii plei* Rang (Gastropoda: Opisthobranchia). *Bulletin of Marine Science*, **42**: 65-75.

PALUMBI, S.R. 1995. Using genetics as an indirect measure of larval dispersal. In: *Ecology of Marine Invertebrate Larvae* (L. McEdward, ed.) 369-387. CRC Press, London.

PAWLIK, J.R. 1989. Larvae of the sea hare *Aplysia californica* settle and metamorphose on an assortment of macroalgal species. *Marine Ecology Progress Series*, **51**: 195-199.

PAWLIK, J.R. 1992. Chemical ecology of the settlement of benthic marine invertebrates. *Oceanography and Marine Biology Annual Review*, **30**: 273-335.

PECHENIK, J. 1990. Delayed metamorphosis by larvae of benthic marine invertebrates: does it occur? Is there a price to pay? *Ophelia*, **32**: 63-94.

PELSENEER, P. 1894. *Recherches sur divers Opisthobranches. - Mémoires couronnés et Mémoires des Savants étrangers, l'Académie Royale des Sciences, des Lettres et des Beaux-Arts de Belgique*, **i-iii**, 157.

PERRON, F.E. & TURNER, R.D. 1977. Development, metamorphosis, and natural history of the nudibranch *Doridella obscura* Verrill (Corambidae: Opisthobranchia). *Journal of Experimental Marine Biology and Ecology*, **27**: 171-185.

PICTON, B. & MORROW, C. 1994. *A field guide to the nudibranchs of the British Isles*, 1-143. Immel Publishing, London.

PLAUT, I., BORUT, A. & SPIRA, M.E. 1995. Growth and metamorphosis of *Aplysia oculifera* larvae in laboratory culture. *Marine Biology*, **122**: 425-430.

POLA, M., CERVERA, J.L. & GOSLINER, T.M. 2005. Review of the systematics of the genus *Roboastra* Bergh, 1877 (Nudibranchia, Polyceridae, Nembrothinae) with the description of a new species from the Galápagos Islands. *Zoological Journal of the Linnean Society*, **144**: 167-189.

PORTER, J.S., RYLAND, J.S. & CARVALHO, G.R. 2002. Micro-and macrogeographic genetic structure in bryozoans with different larval strategies. *Journal of Experimental Marine Biology and Ecology*, **272**: 119-130.

PRUVOT-FOL, A. 1954. *Fanue de France Mollusques Opisthobranches*, **58**, 1-460. Lechevalier, Paris.

QUESADA, H., BEYNON, C. & SKIBINSKI, D.O.F. 1995. A mitchondrial DNA discontinuity in the mussel *Mytilus galloprovincialis* Lmk: pleistocene vicariance biogeography and secondary intergration. *Molecular Biology and Evolution*, **12**: 251-254.

RAO, K.V. 1961. Development and life history of a Nudibranchiate gastropod *Cuthona adyarensis* Rao. *Journal of the Marine Biological Association of India*, **3**: 186-197.

RAO, K.V. & ALAGARSWAMI, K. 1960. An account of the structure and early development of a new species of a Nudibranchiate gastropod *Eolidina* (*Eolidina*) *mannarensis*. *Journal of the Marine Biological Association of India*, **2**: 6-16.

RASMUSSEN, E. 1944. Faunistic and biological notes on marine invertebrates I. *Videnskabelige meddelelser Fra Dansk Naturhistorisk Forening Kobenhavn*, **107**: 207-333.

RISSO, A. 1818. Sur quelques Gastéropodes nouveaux, Nudibranches et Tectibranches observés dans la mer de Nice (1). *Journal de physique, de chimie et d'histoire naturalle*, **87**: 368-377.

RIVEST, B.R. 1978. Development of the eolid nudibranch *Cuthona nana* (Alder and Hancock, 1842), and its relationship with a hydroid and hermit crab. *Biological Bulletin*, **154**: 157-175.

RIVEST, B.R. 1984. Copulation by hypodermic injection in the nudibranchs *Palio zosterae* and *P. dubia* (Gastropoda, Opisthobranchia). *Biological Bulletin*, **167**: 543-554.

ROBINSON, I.S., WARREN, L. & LONGBOTTOM, J.F. 1983. Sea-level fluctuations in the Fleet, an English tidal lagoon. *Estuarine, Coastal and Shelf Science*, **16**: 651-668.

SCHELTEMA, R.S. 1971. Larval dispersal as a means of genetic exchange between geographically separated populations of shallow-water benthic marine invertebrates. *Biological Bulletin*, **140**: 284-322.

SCHMEKEL, L. & PORTMANN, A. 1982. *Opisthobranchia des Mittelmeeres, Nudibranchia und Saccoglossa*, 1-410. Springer-Verlag, Berlin.

SCHNEIDER, S., ROESSLI, D. & EXCOFFIER, L. (2001) Arlequin version 2.001 a software for population genetics analysis

SCHRÖDL, M. 1997. Aspects of Chilean nudibranch biology: Effects of splanchnotrophid copepod parasitism on *Flabellina* sp. 1 (Mollusca, Nudibranchia). *Opisthobranch Newsletter.*

SCHRÖDL, M. 2002. Heavy infestation by endoparasitic copepod crustaceans (Poecilostomatoida: Splanchnotrophidae) in Chilean opisthobranch gastropods, with aspects of splanchnotrophid evolution. *Organisms Diversity and Evolution*, **2**: 19-26.

SCHULTE, G.R. & SCHEUER, P.J. 1982. Defense allomones of some marine mollusks. *Tetrahedron*, **38**: 1857-1863.

SCHULZE, A. & WÄGELE, H. 1998. Morphology, anatomy and histology of *Flabellina affinis* (Gmelin, 1791) (Nudibranchia, Aeolidoidea, Flabellinidae) and its relation to other Mediterranean *Flabellina* species. *Journal of Molluscan Studies*, **64**: 195-214.

SMITH, D.A. & SEBENS, K.P. 1983. The physiological ecology of growth and reproduction in *Onchidoris aspera* (Alder & Hancock) (Gastropoda: Nudibranchia). *Journal of Experimental Marine Biology and Ecology*, **72**: 287-304.

SOKAL, R.R. & ROHLF, F.J. 1995. *Biometry*, 1-887. W. H. Freeman and Company, New York.

SOKOLOV, E. 2000. An improved method for DNA isolation from mucopolysaccharide-rich molluscan tissues. *Journal of Molluscan Studies*, **66**: 573-575.

SOLIMAN, G. 1977. A discussion of the systems of classification of dorid nudibranch veliger shells and their taxanomic significance. *Journal of Molluscan Studies*, **43**: 12-17.

STRATHMANN, R.R. 1986. What controls the type of larval development? Summary statement for the evolution session. *Bulletin of Marine Science*, **39**: 616-622.

STRENTH, N.E. & BLANKENSHIP, J.E. 1978. Laboratory culture, metamorphosis and development of *Aplysia brasiliana* Rang, 1828 (Gasteropoda: Opisthobranchia). *The Veliger*, **21**: 99-103.

SWENNEN, C. 1961. Data on distribution, reproduction and ecology of the nudibranchiate molluscs occuring in the Netherlands. *Netherlands Journal of Sea Research*, **1**: 191-240.

SWITZER-DUNLAP, M. 1978. Larval biology and metamorphosis of Aplysiid gastropods. In: *Settlement and metamorphosis of marine invertebrate larvae* (F.-S. Chia & M.E. Rice, eds.), 197-206. Elsevier, New York.

SWITZER-DUNLAP, M. & HADFIELD, M.G. 1977. Observations on development, larval growth and metamorphosis of four species of Aplysiidae (Gastropoda: Opisthobrancia) in laboratory culture. *Journal of Experimental Marine Biology and Ecology*, **29**: 245-261.

SWITZER-DUNLAP, M. & HADFIELD, M.G. 1981. Laboratory culture of Aplysia. In: *Laboratory Animal Management, Marine Invertebrates. Committee on Marine Invertebrates.* 199-216. National Academy Press, Washington DC.

TARDY, J. 1962a. Cycle biologique et métamorphose d'*Eolidina alderi* (Gastéropode, Nudibranche). *Académie des Sciences*, **255**: 3250-3252.

TARDY, J. 1962b. Observations et expériences sur la métamorphose et la croissance de *Capellinia exigua* (Ald. et H.) (Mollusque Nudibranche). *Académie des Sciences*, **254**: 2242-2244.

TARDY, J. 1970. Contribution a l'étude des métamorphoses chez les nudibranches. *Annales des Sciences Naturelles, Zoologie, Paris*, **12**: 299-370.

TARDY, J. 1991. Types of opisthobranch veligers: their notum formation and torsion. *Journal of Molluscan Studies*, **57**: 103-112.

THIRIOT-QUIEVREUX, C. 1977. Véligère planctotrophe du doridien *Aegires punctilucens* (D'Orbigny) (Mollusca: Nudibranchia: Notodorididae): description et métamorphose. *Journal of Experimental Marine Biology and Ecology*, **26**: 177-190.

THOLLESSON, M. 1999. Phylogenetic analysis of dorid nudibranchs (Gastropoda: Doridacea) using the mitchondrial 16S rRNA gene. *Journal of Molluscan Studies*, **65**: 335-353.

THOLLESSON, M. 2000. Increasing fidelity in parsimony analysis of dorid nudibranchs by differential weighting, or a tale of two genes. *Molecular Phylogenetics and Evolution*, **16**: 161-172.

THOMPSON, J.D., GIBSON, T.J., PLEWNIAK, F., JEANMOUGIN, F. & HIGGINS, D.G. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, **24**: 4876-4882.

THOMPSON, J.E., WALKER, R.P., WRATTEN, S.J. & FAULKNER, D.J. 1982. A chemical defense mechanism for the nudibranch *Cadlina luteomarginata*. *Tetrahedron*, **38**: 1865-1873.

THOMPSON, T.E. 1958. The natural history, embryology, larval biology and post-larval development of *Adalaria proxima* (Alder and Hancock) (Gastropoda, Opisthobranchia). *Philosophical Transactions of the Royal Society Series B*, **242**: 1-58.

THOMPSON, T.E. 1960a. Defensive acid-secretion in marine gastropods. *Journal of the Marine Biological Association of the United Kingdom*, **39**: 115-122.

THOMPSON, T.E. 1960b. Defensive adaptations in opisthobranchs. *Journal of the Marine Biological Association of the United Kingdom*, **39**: 123-134.

THOMPSON, T.E. 1961a. The importance of the larval shell in the classification of the Sacoglossa and the Acoela (Gastropoda Opisthobranchia). *Proceedings of the Malacological Society London*, **34**: 233-238.

THOMPSON, T.E. 1961b. The structure and mode of functioning of the reproductive organs of *Tritonia hombergi* (Gastropoda Opisthobranchia). *Quarterly Journal of Microscopical Science*, **102**: 1-14.

THOMPSON, T.E. 1962. Studies on the ontogeny of *Tritonia hombergi* Cuvier (Gastropoda, Opisthobranchia). *Philosophical Transactions of the Royal Society Series B*, **245**: 171-218.

THOMPSON, T.E. 1966. Studies on the reproduction of *Archidoris pseudoargus* (Rapp) (Gastropoda Opisthobranchia). *Philosophical Transactions of the Royal Society Series B*, **250**: 343-375.

THOMPSON, T.E. 1967. Direct development in the nudibranch *Cadlina laevis*, with a discussion of developmental processes in Opisthobranchia. *Journal of the Marine Biological Association of the United Kingdom*, **47**: 1-22.

THOMPSON, T.E. 1969. Acid secretion in Pacific ocean gastropods. *Australian Journal of Zoology*, **17**: 755-764.

THOMPSON, T.E. 1973. Euthyneuran and other molluscan spermatozoa. *Malacologia*, **14**: 167-206.

THOMPSON, T.E. 1976. *Biology of Opisthobranch Molluscs*, **1**, 207. The Ray Society, London.

THOMPSON, T.E. 1988. *Molluscs: Benthic Opisthobranchs (Mollusca: Gastropoda). Second edition*, **8**, (D.M. Kermack & R.S.K. Barnes, eds.), 356. E. J. Brill, Leiden.

THOMPSON, T.E. & BEBBINGTON, A. 1969. Structure and function of the reproductive organs of three species of *Aplysia* (Gastropoda: Opisthobranchia). *Malacologia*, **7**: 347-380.

THOMPSON, T.E. & BEBBINGTON, A. 1970. A new interpretation of the structure of the aplysiid spermatozoon (Gastropoda Opisthobranchia). *Archives de Zoologie expérimentale et générale*, **111**: 213-216.

THOMPSON, T.E. & BROWN, G.H. 1984. *Biology of Opisthobranch Molluscs*, **2**, 1-229. The Ray Society, Bristol.

THOMPSON, T.E. & SEAWARD, D.R. 1989. Ecology and taxanomic status of the Aplysiomorph *Akera bullata* in the British Isles. *Journal of Molluscan Studies*, **55**: 489-496.

THORSON, G. 1946. *Reproduction and larval development of Danish marine bottom invertebrates, with special reference to the planktonic larvae in the sound (Øresund)*, **4**, 1-523. C. A. Reitzels forlag, København.

TODD, C.D. 1978. Gonad development of *Onchidoris muricata* (Müller) in relation to size, age and spawning (Gastropoda: Opisthobranchia). *Journal of Molluscan Studies*, **44**: 190-199.

TODD, C.D. 1979. The population ecology of *Onchidoris bilamellata* (L.) (Gastropoda: Nudibranchia). *Journal of Experimental Marine Biology and Ecology*, **41**: 213-255.

TODD, C.D. 1981. The ecology of nudibranch molluscs. *Oceanography and Marine Biology Annual Review*, **19**: 141-234.

TODD, C.D. 1985. Settlement-timing hypothesis: reply to Grant and Williamson. *Marine Ecology Progress Series*, **23**: 197-202.

TODD, C.D. 1998. Larval supply and recruitment of benthic invertebrates: do larvae always disperse as much as we believe? *Hydrobiologia*, **375/376**: 1-21.

TODD, C.D. & DOYLE, R.W. 1981. Reproductive strategies of marine benthic invertebrates: a settlement timing hypothesis. *Marine Ecology Progress Series*, **4**: 75-83.

TODD, C.D. & HAVENHAND, J.N. 1985. Preliminary observations on the embryonic and larval development of three dorid nudibranchs. *Journal of Molluscan Studies*, **51**: 226-228.

TODD, C.D., BENTLEY, M. & HAVENHAND, J. 1991. Larval metamorphosis of the opisthobranch mollusc *Adalaria proxima* (Gastropoda: Nudibranchia): the effects of choline and elevated potassium ion concentration. *Journal of the Marine Biological Association of the United Kingdom*, **71**: 53-72.

TODD, C.D., HAVENHAND, J. & THORPE, J. 1988. Genetic differentation, pelagic larval transport and gene flow between local populations of the intertidal marine mollusc *Adalaria proxima* (Alder & Hancock). *Functional Ecology*, **2**: 441-451.

TODD, C.D., LAMBERT, W. & THORPE, J. 1998. The genetic structure of intertidal populations of two species of nudibranch molluscs with planktotrophic and pelagic lecithotrophic larval stages: are pelagic larvae "for" dispersal? *Journal of Experimental Marine Biology and Ecology*, **228**: 1-28.

USUKI, I. 1967. The direct development and the single cup-shaped larval shell of a nudibranch *Glossodoris sibogae* (Bergh). *Scientific Reports of the Niigata University Series D (Biology)*, **4**: 75-85.

VALDÉS, Á. 2002. A phylogenetic analysis and systematic revision of the cryptobranch dorids (Mollusca, Nudibranchia Anthobranchia). *Zoological Journal of the Linnean Society*, **136**: 535-636.

VONNEMANN, V., SCHRÖDL, M., KLUSSMANN-KOLB, A. & WÄGELE, H. 2005. Reconstruction of the phylogeny of the Opisthobranchia (Mollusca: Gastroposa) by means of 18S and 28S rRNA sequences. *Journal of Molluscan Studies*, **71**: 113-125.

WÄGELE, H. 1989. A revision of the Antarctic species of *Bathydoris* Bergh, 1884 and comparison with other known Bathydorids (Opisthobranchia, Nudibranchia). *Journal of Molluscan Studies*, **55**: 343-364.

WÄGELE, H. 1990. Revision of the Antarctic genus *Notaeolidia* (Gastropoda, Nudibranchia), with descriptions of a new species. *Zoologica Scripta*, **19**: 309-330.

WÄGELE, H., BRODIE, G. & KLUSSMANN-KOLB, A. 1999. Histological investigations on *Dendrodoris nigra* (Stimpson, 1855) (Gastropoda, Nudibranchia, Dendrodorididae). *Molluscan Research*, **20**: 79-94.

WÄGELE, H. & CERVERA, J.L. 2001. Histological study of *Goniodoris castanea* Alder and Hancock, 1845 (Nudibranchia, Doridoidea, Goniodorididae). *Journal of Morphology*, **250**: 61-69.

WÄGELE, H. & JOHNSEN, G. 2001. Observations on this histology and photosynthetic performance of "solar powered" opisthobranchs (Mollusca, Gastropoda, Opisthobranchia) containing symbiotic chloroplasts or zooxanthellae. *Organisms Diversity and Evolution*, **1**: 193-210.

WÄGELE, H. & WILLAN, R. 2000. Phylogeny of the Nudibranchia. *Zoological Journal of the Linnean Society*, **130**: 83-181.

WILLAN, R. 1976. The opisthobranch *Thecacera pennigera* (Montagu) in New Zealand, with a discussion of the genus. *The Veliger*, **18**: 247-352.

WILLIAMS, L.G. 1980. Development and feeding of larvae of the nudibranch gastropods *Hermissenda crassicornis* and *Aeolidia papillosa*. *Malacologia*, **20**: 99-116

WILSON, N.G. & HEALY, J.M. 2002. Comparative sperm ultrastructure in five genera of the nudibranch family Chromodorididae (Gastropoda: Opisthobranchia). *Journal of Molluscan Studies*, **68**: 133-145.

WOLLSCHEID-LENGELING, E., BOORE, J., BROWN, W. & WÄGELE, H. 2001. The phylogeny of Nudibranchia (Opisthobranchia, Gastropoda, Mollusca) reconstructed by three molecular markers. *Organisms Diversity and Evolution*, **1**: 241-256.

WOLLSCHEID, E. & WÄGELE, H. 1999. Initial results on the molecular phylogeny of the Nudibranchia (Gastropoda, Opisthobranchia) based on 18S rDNA data. *Molecular Phylogenetics and Evolution*, **13**: 215-226.

YONOW, N. 1996. Gametogenesis, egg production and development in *Acteon tornatilis* (Opisthobranchia: Cephalaspidea). *Malacological Review Suppliment*, **6**: 31-52.

Appendix

Appendix

Appendix Chapter 2

Stains:

STAIN	CHEMICAL FORMULA	QUANTITY
Cole's haematoxylin	$AlH_4NO_8S_2$	700 ml saturated
	Haematoxylin (C.I. 75290)	1.5 g
	Iodine in 95% alcohol	1 %
Scott's solution	$NaHCO_3$	2 g
	$MgSO_4$	20 g
	H_2O	1000 ml
Eosin	Eosin Y (C.I. 45380)	0.1 g
	70% ethanol	100 ml
	Glacial acetic acid	0.5 ml
Mallory's	Phosphotungstic acid	1 g
	Orange G	2 g
	Aniline Blue	1 g
	Acid fuchsin	3 g
	H_2O	200 ml

Data of the median/mean diameter of oocytes: test for normality in *Ancula*

Ancula Oogonia	Variance	Max variance ratio	DofF	Fmax	P	Distribution	Levene	Distribution
9a mm	3.121424	3.764788122	8	9.03	*P*>0.05	Normal	0.026	Not normal
17 mm	11.54542							
14 mm	5.851131							
11 mm	11.46612							
15 mm	9.612218							
9b mm	11.7515							
Previtel								
9a mm	101.2783	1.942938849	49	2.91	*P*>0.05	Normal	0.235	Normal
17 mm	126.7186							
14 mm	148.1305							
11 mm	155.2195							
15 mm	134.8172							
9b mm	79.88903							
Mature								
9a mm	62.58851	3.258131564	46	2.91	*P*<0.05	Not normal	0.00	Not normal
17 mm	72.49068							
14 mm	109.3564							
11 mm	79.20588							
15 mm	81.06198							
9b mm	203.9216							

Ancula oogonia

Ranks

	Size	N	Mean Rank
Width	9 mm	9	170.44
	14 mm	51	111.56
	17 mm	26	132.04
	11 mm	41	91.06
	15 mm	60	98.81
	9 mm	31	114.60
	Total	218	

Test Statistics [b,c]

			Width
Chi-Square			17.207
df			5
Asymp. Sig.			.004
Monte Carlo Sig.	Sig.		.003[a]
	99% Confidence Interval	Lower Bound	.003
		Upper Bound	.004

a. Based on 100000 sampled tables with starting seed 1314643744.

b. Kruskal Wallis Test

c. Grouping Variable: Size

Ancula previt

ANOVA

Width

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6636.437	5	1327.287	9.913	.000
Within Groups	72169.093	539	133.894		
Total	78805.531	544			

Ancula mature

Ranks

	Size	N	Mean Rank
Width	9 mm	115	303.81
	14 mm	83	309.75
	17 mm	174	280.46
	11 mm	45	190.79
	15 mm	89	329.25
	9 mm	48	133.31
	Total	554	

Test Statistics [b,c]

			Width
Chi-Square			67.990
df			5
Asymp. Sig.			.000
Monte Carlo Sig.	Sig.		.000[a]
	99% Confidence Interval	Lower Bound	.000
		Upper Bound	.000

a. Based on 100000 sampled tables with starting seed 624387341.

b. Kruskal Wallis Test

c. Grouping Variable: Size

Data of the median/mean diameter of oocytes: test for normality in *Thecacera*

Thecacera Oogonia	Variance	Max variance ratio	DofF	Fmax	*P*	Distribution	Levene	Distribution
10a mm	17.98377	2.1128	23	3.54	*P*>0.05	Normal	0.32	Normal
15 mm	8.511726							
13 mm	9.499312							
18 mm	17.68776							
10b mm	9.53385							
Previtellogenic								
10a mm	100.9804	2.69833	96	2.04/1.00	*P*<0.05	Not normal	0.00	Not normal
15 mm	88.84379							
13 mm	125.0261							
18 mm	85.72715							
10b mm	46.33462							
Mature								
10a mm	176.6143	1.50478	135	1.00	*P*<0.05	Not normal	0.026	Not normal
15 mm	182.7509							
13 mm	202.64							
18 mm	188.3705							
10b mm	265.7658							

Thecacera oogonia

ANOVA

Size

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	368.849	4	92.212	7.331	.000
Within Groups	2377.167	189	12.578		
Total	2746.016	193			

Thecacera previt

Ranks

	Length	N	Mean Rank
Size	10a mm	163	487.95
	15 mm	158	445.39
	13 mm	151	418.03
	18 mm	221	360.43
	10b mm	98	209.70
	Total	791	

Test Statistics [b,c]

			Size
Chi-Square			105.693
df			4
Asymp. Sig.			.000
Monte Carlo Sig.	Sig.		.000[a]
	99% Confidence Interval	Lower Bound	.000
		Upper Bound	.000

a. Based on 100000 sampled tables with starting seed 957002199.

b. Kruskal Wallis Test

c. Grouping Variable: Length

Thecacera mature

Ranks

	Length	N	Mean Rank
Size	10a mm	136	380.31
	15 mm	128	346.55
	13 mm	107	328.06
	18 mm	190	420.89
	10b mm	158	301.83
	Total	719	

Test Statistics[b,c]

			Size
Chi-Square			33.092
df			4
Asymp. Sig.			.000
Monte Carlo Sig.	Sig.		.000[a]
	99% Confidence Interval	Lower Bound	.000
		Upper Bound	.000

a. Based on 100000 sampled tables with starting seed 112562564.

b. Kruskal Wallis Test

c. Grouping Variable: Length

Data of the median/mean diameter of oocytes: test for normality in *Facelina*

Facelina Oogonia	Variance	Max Variance ratio	DofF	Fmax	P	Distribution	Levene	Distribution
16a mm	17.4807	17.7723	6	13.70	P<0.05	Not normal	0.07	Normal
18 mm	38.38108							
17 mm	9.146835							
15 mm	2.159588							
20 mm	27.2094							
16b mm	3.715657							
Previt								
16a mm	85.56708	1.941	163	1.00	P<0.05	Not normal	0.00	Not normal
18 mm	108.6476							
17 mm	58.57184							
15 mm	113.685							
20 mm	75.70061							
16b mm	65.59655							
Mature								
16a mm	208.2129	3.0356	48	2.11	P<0.05	Not normal	0.067	Normal
18 mm	206.3168							
17 mm	166.2706							
15 mm	97.46502							
20 mm	295.8602							
16b mm	212.6599							

Facelina oogonia

Ranks

	Length	N	Mean Rank
Size	16a mm	81	104.42
	18 mm	55	106.74
	17 mm	19	115.24
	15 mm	7	44.86
	20 mm	17	56.59
	16b mm	11	31.91
	Total	190	

Test Statistics[b,c]

			Size
Chi-Square			36.032
df			5
Asymp. Sig.			.000
Monte Carlo Sig.	Sig.		.000[a]
	99% Confidence Interval	Lower Bound	.000
		Upper Bound	.000

a. Based on 100000 sampled tables with starting seed 334431365.

b. Kruskal Wallis Test

c. Grouping Variable: Length

Facelina previt

Ranks

	Length	N	Mean Rank
Size	16a mm	148	587.26
	18 mm	205	520.21
	17 mm	165	508.92
	15 mm	269	547.13
	20 mm	174	511.67
	16b mm	95	466.92
	Total	1056	

Test Statistics[b,c]

			Size
Chi-Square			11.732
df			5
Asymp. Sig.			.039
Monte Carlo Sig.	Sig.		.038[a]
	99% Confidence Interval	Lower Bound	.037
		Upper Bound	.040

a. Based on 100000 sampled tables with starting seed 1502173562.

b. Kruskal Wallis Test

c. Grouping Variable: Length

Facelina mature

Ranks

	Length	N	Mean Rank
Size	16a mm	123	425.82
	18 mm	108	398.60
	17 mm	131	241.70
	15 mm	50	270.18
	20 mm	154	395.08
	16b mm	163	396.61
	Total	729	

Test Statistics[b,c]

			Size
Chi-Square			74.868
df			5
Asymp. Sig.			.000
Monte Carlo Sig.	Sig.		.000[a]
	99% Confidence Interval	Lower Bound	.000
		Upper Bound	.000

a. Based on 100000 sampled tables with starting seed 743671174.

b. Kruskal Wallis Test

c. Grouping Variable: Length

Data of the median/mean diameter of oocytes: test for normality in *Palio*

Palio Oogonia	Variance	Max variance ratio	DofF	Fmax	P	Distribution	Levene	Distribution
15 mm	6.487214	5.285324332	25	2.40	*P*<0.05	Not normal	0.00	Not normal
9 mm	16.52814							
10 mm	34.28703							
Previtel								
15 mm	164.2356	1.399148936	63	1.85	*P*>0.05	Normal	0.322	Normal
9 mm	117.3825							
10 mm	123.814							
Mature								
15 mm	127.1833	1.140829291	132	1.00	*P*<0.05	Not normal	0.58	Normal
9 mm	111.4832							
10 mm	114.4289							

Palio oogonia

Ranks

	Size	N	Mean Rank
Diameter	15 mm	37	36.59
	9 mm	10	22.70
	10 mm	27	44.22
	Total	74	

Test Statistics[b,c]

			Diameter
Chi-Square			7.440
df			2
Asymp. Sig.			.024
Monte Carlo Sig.	Sig.		.022[a]
	99% Confidence Interval	Lower Bound	.021
		Upper Bound	.023

a. Based on 100000 sampled tables with starting seed 926214481.

b. Kruskal Wallis Test

c. Grouping Variable: Size

Palio previt

ANOVA

Width

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7842.959	2	3921.479	30.661	.000
Within Groups	32485.907	254	127.897		
Total	40328.865	256			

Palio mature

Ranks

	Size	N	Mean Rank
Width	15 mm	172	303.56
	9 mm	133	211.81
	10 mm	127	103.50
	Total	432	

Test Statistics[b,c]

			Width
Chi-Square			187.842
df			2
Asymp. Sig.			.000
Monte Carlo Sig.	Sig.		.000[a]
	99% Confidence Interval	Lower Bound	.000
		Upper Bound	.000

a. Based on 100000 sampled tables with starting seed 2000000.

b. Kruskal Wallis Test

c. Grouping Variable: Size

Data of the median/mean diameter of nucleolus: test for normality

	Ancula		Thecacera		FAcelina		Palio	
	Previt	Mature	Previt	Mature	Previt	Mature	Previt	Mature
Variance	0.520428508	0.329609	0.518473138	0.302956	1.674887892	0.588164	0.418953805	0.247497
Mean	4.833246896	6.757311	4.443986136	6.573365	5.946907717	8.781718	4.906728461	6.423456
Standard deviation	0.721407311	0.574116	0.720050788	0.550415	1.294174599	0.766919	0.64726641	0.49749
	Fmax	1.672	Fmax	2.475	Fmax	2.722	Fmax	2.404
	DofF	334	DofF	270	DofF	301	DofF	279
	Critical value	1.00	Critical value	1.00	Critical value	1.00	Critical value	1.00
	P	P<0.05	P	P<0.05	P	P<0.05	P	P<0.05
	Not normal		Not normal		Not normal		Not normal	
	Levene	0.00	Levene	0.00	Levene	0.00	Levene	0.00
	P	P<0.05	P	P<0.05	P	P<0.05	P	P<0.05

Ancula

Ranks

	Nucleolus	N	Mean Rank	Sum of Ranks
Diameter	Mature	334	555.76	185623.00
	Previt	393	201.03	79005.00
	Total	727		

Test Statistics [a]

	Diameter
Mann-Whitney U	1584.000
Wilcoxon W	79005.000
Z	-22.696
Asymp. Sig. (2-tailed)	.000
Exact Sig. (2-tailed)	.000
Exact Sig. (1-tailed)	.000
Point Probability	.000

a. Grouping Variable: Nucleolus

Thecacera

Ranks

	Nucleolus	N	Mean Rank	Sum of Ranks
Diameter	Mature	320	427.97	136949.00
	Previt	270	138.50	37396.00
	Total	590		

Test Statistics [a]

	Diameter
Mann-Whitney U	811.000
Wilcoxon W	37396.000
Z	-20.549
Asymp. Sig. (2-tailed)	.000
Exact Sig. (2-tailed)	.000
Exact Sig. (1-tailed)	.000
Point Probability	.000

a. Grouping Variable: Nucleolus

Facelina

Ranks

	Nucleolus	N	Mean Rank	Sum of Ranks
Diameter	Mature	334	459.04	153587.50
	Previt	299	157.44	47073.50
	Total	633		

Test Statistics [a]

	Diameter
Mann-Whitney U	2223.500
Wilcoxon W	47073.500
Z	-20.770
Asymp. Sig. (2-tailed)	.000
Exact Sig. (2-tailed)	.000
Exact Sig. (1-tailed)	.000
Point Probability	.000

a. Grouping Variable: Nucleolus

Palio

Ranks

	Nucleolus	N	Mean Rank	Sum of Ranks
Diameter	Mature	327	434.75	142164.50
	Previtellogenic	279	149.66	41756.50
	Total	606		

Test Statistics [a]

	Diameter
Mann-Whitney U	2696.500
Wilcoxon W	41756.500
Z	-19.979
Asymp. Sig. (2-tailed)	.000
Exact Sig. (2-tailed)	.000
Exact Sig. (1-tailed)	.000
Point Probability	.000

a. Grouping Variable: Nucleolus

Appendix Chapter 3

f/2 composition:

NUTRIENTS	CHEMICAL FORMULA	CONCENTRATION REQUIRED
Major nutrients	$NaNO_3$	75.0 mg l^{-1}
	$NaH_2PO4.H_2O$	5.0 mg l^{-1}
	$*Na_2SiO_3.9H_2O$	15-30 mg l^{-1}
Trace elements	$Na_2.EDTA+$	4.36 mg l^{-1}
	$FeCl_3.6H_2O+$	3.15 mg l^{-1}
	$CuSO_4.5H_2O$	0.01 mg l^{-1}
	$ZnSO_4.7H_2O$	0.022 mg l^{-1}
	$CoCl_2.6H_2O$	0.01 mg l^{-1}
	$MnCl_2.4H_2O$	0.18 mg l^{-1}
	$Na_2MoO_4.2H_2O$	0.006 mg l^{-1}
Vitamins	Thiamine.HCl	0.1 mg l^{-1}
	Biotin	0.5 µg l^{-1}
	B_{12}	0.5 µg l^{-1}

Silicate was only added for *Chaetoceros* sp.

Shell length data:

Palio Day	Mean	Standard deviation
1	126.329	5.759729425
6	140.1047	7.595156669
7	141.1755	3.767539255
10	146.4782	1.326361819
11	142.4978	0.99908742
12	168.7869	8.049324043
14	164.6518	4.683423057
22	176.3338	11.19784904
28	158.6971	7.14601366

Onchidoris Day	Mean	Standard deviation
1	165.7234	9.463451818
3	173.0098	10.5584776
5	178.8817	#DIV/0!
7	176.668	12.47937445
10	191.5666	#DIV/0!
11	207.0454	2.978541785
18	258.0869	16.34519751
24	260.5344	#DIV/0!
26	260.9484	0.156270599

Thecacera 20 Day	Mean	Standard deviation
1	124.6134	11.14582642
3	120.6692	9.80458974
10	145.6595	13.33248919
16	148.1926	8.465086299

Cuthona Day	Mean	Standard deviation
1	246.5468	13.23827199
5	252.1778	17.10533681
9	261.4037	6.95117324

Ancula Day	Mean	Standard deviation
1	105.5882	2.51057735
5	122.5315	8.437098835
7	116.4708	9.508688401
8	125.8426	7.830499508
10	123.5307	19.37140192
13	122.589	6.416523086

Thecacera 12-15 Day	Mean	Standard deviation
1	124.6134	11.14582642
7	145.9438	5.845434127
8	132.0284	4.119933969
13	140.6146	9.05270761
14	146.5778	4.598941175
22	177.159	8.544655676

Appendix Chapter 4

mtDNA sequences from CO1 for Porthcawl (P), Mumbles (M), and Pembroke Dock (PD).

>P13
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGGCATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>P20
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTTGGGCATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>M1
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>PD
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>PD18
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>M15
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>M21
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>P12
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>P39
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>M19
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>M8
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>PD23
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>P18
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>PD3
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>PD1
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTACTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGGCCAGTAGGACATAGTGGCTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>M3
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGCTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGGCCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>P29
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>P32
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>P37
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>PD28
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>PD5
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>P24
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>P33

ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>PD4
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>P5
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>M16
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>M10
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>P19
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>P23
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>PD2
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>M9
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>P22
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>M5
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>P17
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>P11
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>P30
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>PD22
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>M17
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>PD20
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>P26
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>PD27
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>M7
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>M2
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>M18
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>PD10
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTCTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>P35
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTTTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>PD9

ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGCGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>P10
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>P31
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTCTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>P27
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTGATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTAGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>PD6
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTGATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTTGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>P25
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTGATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTTGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>P38
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTGATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTTGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>M6
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTAATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTTGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>PD26
ATTGTTACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGCTTTGGTAATTGAATGATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTTGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>PD25
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGCTTTGGTAATTGAATGATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTTGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>PD7
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGCTTTGGTAATTGAATGATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTTGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>PD8
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGCTTTGGTAATTGAATGATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTTGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>P36
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTTGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>M12
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGGATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTTGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>M20
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTTGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>P40
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTTGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>PD17
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTTGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>P28
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTTGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>P16
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTTGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>P21
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTTGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>PD24
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTTGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>P45
ATTGTCACGGCACATGCTTTTGTTATAATTTTTTTTATAGTTATACCAGTAACTATAGGAGGTTTTGGTAATTGAATAATTCCTTTATTAATTGGGGCCCCGGACAT
GAGTTTCCCTCGAATAAATAATATGAGTTTTTGATTCTTGCCTCCTTCTTTTATTTTACTTTTATGCTCGACACTTATAGAAGGAGGGGCTGGGACAGGGTGAACT
GTCTATCCTCCTTTGTCTGGACCAGTTGGACATAGTGGTTCTTCTGTAGATCTTGCTATTTTTTCTTTACATTTGGCAGGTGCTTCTTCA
>Clustal Consensus
**** *** *********** ****** ************************** ********** ** ************************
********** *************************** ************** **** ** **** ** **

Frequency of haplotypes between the three populations:

Haplotype	Porthcawl	Mumbles	Pembroke Dock	Totals
1	17	13	11	**41**
2	1	0	0	**1**
3	1	0	0	**1**
4	1	0	0	**1**
5	2	0	1	**3**
6	6	1	2	**9**
7	0	1	0	**1**
8	0	1	0	**1**
9	0	0	1	**1**
10	0	0	3	**3**
11	1	0	0	**1**
12	0	0	1	**1**
13	1	0	0	**1**
14	0	0	1	**1**
15	0	0	1	**1**
16	0	1	0	**1**
Totals	**30**	**17**	**21**	**68**

Analysis of MOlecular VAriance (AMOVA) output:

```
////////////////////////////////////////////////////////////////////////////
RUN NUMBER 1 (12/08/05 at 12:14:30)
////////////////////////////////////////////////////////////////////////////
 #[WARNING # 1] : data already defined
 #[WARNING # 2] : data already defined
 #[WARNING # 3] : data already defined
 #[WARNING # 4] : data already defined
Project information:
--------------------
            NbSamples      = 3
            DataType       = DNA
            GenotypicData  = 0
============================
Settings used for Calculations
============================
 General settings:
 -----------------
       Deletion Weight                = 1
       Transition Weight Weight       = 1
       Tranversion Weight Weight      = 1
       Epsilon Value                  = 1e-07
       Significant digits for output  = 5
       Use original haplotype definition
       Alllowed level of missing data = 0.05
 Active Tasks:
 -------------
    Analysis of Molecular Variance:
    -------------------------------
       No. of Permutations = 1000
      Distance matrix:
           Compute distance matrix
           Molecular distance : Pairwise difference
           Gamma a value      = 0
============================================================================
== GENETIC STRUCTURE ANALYSIS
============================================================================
Number of usable loci for distance computation : 306
Allowed level of missing data                  : 0.05
List of usable loci :
---------------------
   1    2    3    4    5    6    7    8    9   10   11   12   13   14   15
  16   17   18   19   20   21   22   23   24   25   26   27   28   29   30
  31   32   33   34   35   36   37   38   39   40   41   42   43   44   45
  46   47   48   49   50   51   52   53   54   55   56   57   58   59   60
  61   62   63   64   65   66   67   68   69   70   71   72   73   74   75
  76   77   78   79   80   81   82   83   84   85   86   87   88   89   90
  91   92   93   94   95   96   97   98   99  100  101  102  103  104  105
 106  107  108  109  110  111  112  113  114  115  116  117  118  119  120
 121  122  123  124  125  126  127  128  129  130  131  132  133  134  135
 136  137  138  139  140  141  142  143  144  145  146  147  148  149  150
 151  152  153  154  155  156  157  158  159  160  161  162  163  164  165
 166  167  168  169  170  171  172  173  174  175  176  177  178  179  180
 181  182  183  184  185  186  187  188  189  190  191  192  193  194  195
 196  197  198  199  200  201  202  203  204  205  206  207  208  209  210
 211  212  213  214  215  216  217  218  219  220  221  222  223  224  225
 226  227  228  229  230  231  232  233  234  235  236  237  238  239  240
 241  242  243  244  245  246  247  248  249  250  251  252  253  254  255
 256  257  258  259  260  261  262  263  264  265  266  267  268  269  270
 271  272  273  274  275  276  277  278  279  280  281  282  283  284  285
 286  287  288  289  290  291  292  293  294  295  296  297  298  299  300
 301  302  303  304  305  306
List of loci with too much missing data :
-----------------------------------------
NONE
============================================================
  AMOVA ANALYSIS
============================================================
```

```
---------------------------
Genetic structure to test :
---------------------------
No. of Groups = 1
            [[Structure]]
                        StructureName = "An example of structure with 3 geographic regions"
                        NbGroups = 1
                        IndividualLevel = 0
                        DistMatLabel = ""
                        Group={
                        "Mumbles"
                        "Pembroke"
                        "Porthcawl"
                        }
---------------------------
Distance method: Pairwise difference
---------------------------
AMOVA design and results :
---------------------------
Reference: Weir, B.S. and Cockerham, C.C. 1984.
           Excoffier, L., Smouse, P., and Quattro, J. 1992.
           Weir, B. S., 1996.
-----------------------------------------------------------------------
 Source of                  Sum of      Variance         Percentage
 variation       d.f.       squares     components       of variation
-----------------------------------------------------------------------
 Among
 populations      2          1.817       0.01552 Va           2.66
 Within
 populations     65         36.845       0.56684 Vb          97.34
-----------------------------------------------------------------------
 Total           67         38.662       0.58236
-----------------------------------------------------------------------
 Fixation Index      FST :      0.02665
-----------------------------------------------------------------------
Significance tests (1023 permutations)
--------------------
Va and FST : P(rand. value > obs. value)  =  0.11535
             P(rand. value = obs. value)  =  0.00000
             P(rand. value >= obs. value) =  0.11535+-0.00722
//////////////////////////////////////////////////////////////////////
END OF RUN NUMBER 1 (12/08/05 at 12:14:30))
Total computing time for this run : 0h 0m 0s 235 ms
//////////////////////////////////////////////////////////////////////
```

Appendix Chapter 7

Raw numbers undergoing metamorphosis:

	Nemalion helminthoides		Chondrus crispus		Ulva lactuca		Control	
	1	2	1	2	1	2	1	2
Number of deaths	7	12	3	2	8	10	13	15
Number that metamorphosed	13	8	17	18	12	10	7	5
Total	20	20	20	20	20	20	20	20

	Rhinomonas reticulata		Tetraselmis sp.		Chaetoceros sp.		Isochrysis sp.		Biofilm	
	1	2	1	2	1	2	1	2	1	2
Number of deaths	2	13	18	13	15	11	1	5	7	2
Number that metamorphosed	18	7	2	7	5	9	19	15	13	18
Total	20	20	20	20	20	20	20	20	20	20

	Tetraselmis sp.			Chaetoceros sp.			Isochrysis sp.			Rhinomonas reticulata		
	Biofilm	Chondrus	Ulva	Biofilm	Chondrus	Ulva	Biofilm	Chondrus	Ulva	Biofilm	Chondrus	Ulva
Number of deaths	19	13	9	20	13	6	9	11	13	8	0	13
Number that metamorphosed	11	17	21	10	17	24	21	19	17	22	30	17
Total	30	30	30	30	30	30	30	30	30	30	30	30

Comparisons to the control conditions:

G-test Statistic using Intrinsic Hypotheses Group A

	Observed	Expected	Ln(Observed/Expected)	Observed*{Ln(Observed/Expected)}
N. helminthoides	13.000	7.000	0.619039208	8.047509709
C. crispus	17.000	7.000	0.887303195	15.08415432
U. lactuca	12.000	7.000	0.538996501	6.467958009
Rhinomonas	18.000	7.000	0.944461609	17.00030896
Tetraselmis	2.000	7.000	-1.252762968	-2.505525937
Chaetoceros	5.000	7.000	-0.336472237	-1.682361183
Isochrysis	19.000	7.000	0.99852883	18.97204777
Biofilm	13.000	7.000	0.619039208	8.047509709
Control	7.000	7.000	0	0
	106.000			
			Sum of above	69.43160135
			Sum of above *2	138.8632027
Williams correction factor	1.01572327			
Gadj	**136.7136176**			
DofF	**8**			
X2	**15.51**			
P<0.05				

G-test Statistic using Intrinsic Hypotheses Group B

	Observed	Expected	Ln(Observed/Expected)	Observed*{Ln(Observed/Expected)}
N. helminthoides	8.000	5.000	0.470003629	3.760029034
C. crispus	18.000	5.000	1.280933845	23.05680922
U. lactuca	10.000	5.000	0.693147181	6.931471806
Rhinomonas	7.000	5.000	0.336472237	2.355305656
Tetraselmis	7.000	5.000	0.336472237	2.355305656
Chaetoceros	9.000	5.000	0.587786665	5.290079984
Isochrysis	15.000	5.000	1.098612289	16.47918433
Biofilm	18.000	5.000	1.280933845	23.05680922
Control	5.000	5.000	0	0
	97.000			
			Sum of above	83.2849949
			Sum of above *2	166.5699898
Williams correction factor	1.017182131			
Gadj	**163.7563075**			
DofF	**8**			
X2	**15.51**			
P<0.05				

Settlement KW test: group A and group B

Ranks

	Metamorphosis substr	N	Mean Rank
Time taken to metamorphose after hatching	C. crispus	17	16.35
	N. helminthoides	13	60.15
	U. lactuca	12	41.58
	Rhinomonas	18	64.56
	Chaetoceros	5	90.60
	Tetraselmis	2	86.00
	Isochrysis	19	80.32
	Biofilm	13	19.96
	Control	7	77.07
	Total	106	

Test Statistics [b,c]

			Time taken to metamorphose after hatching
Chi-Square			73.518
df			8
Asymp. Sig.			.000
Monte Carlo Sig.	Sig.		.000[a]
	95% Confidence Interval	Lower Bound	.000
		Upper Bound	.000

a. Based on 1000000 sampled tables with starting seed 2000000.

b. Kruskal Wallis Test

c. Grouping Variable: Metamorphosis substrate

Ranks

	Metamorphosis subst	N	Mean Rank
Time taken to metamorphose after hatching	C. crispus	18	27.25
	F. lumbricalis	8	58.13
	U. lactuca	10	44.05
	Rhinomonas	7	47.29
	Chaetoceros	9	78.39
	Tetraselmis	7	49.21
	Isochrysis	15	82.80
	Biofilm	18	19.39
	Control	5	77.00
	Total	97	

Test Statistics [b,c]

			Time taken to metamorphose after hatching
Chi-Square			68.603
df			8
Asymp. Sig.			.000
Monte Carlo Sig.	Sig.		.000[a]
	95% Confidence Interval	Lower Bound	.000
		Upper Bound	.000

a. Based on 1000000 sampled tables with starting seed 299883525.

b. Kruskal Wallis Test

c. Grouping Variable: Metamorphosis substrate

Corrected data:

Sequential Bonferroni test:
***k* comparisons by the Dunn-Sidak method at a specified experimentwise error rate α**

Group 1	Group 2	Probability *Pi*	Associated α	*k*	Significant?
N. helminthoides	*C. crispus*	0.000000	0.001423799	36	Significant
N. helminthoides	Biofilm	0.000000	0.001464449	35	Significant
C. crispus	*Rhinomonas*	0.000000	0.001507489	34	Significant
C. crispus	*Isochrysis*	0.000000	0.001553135	33	Significant
Rhinomonas	Biofilm	0.000000	0.001601631	32	Significant
Isochrysis	Biofilm	0.000001	0.001653254	31	Significant
C. crispus	Control	0.000003	0.001708316	30	Significant
Biofilm	Control	0.000013	0.001767171	29	Significant
C. crispus	*Chaetocerous*	0.000038	0.001830226	28	Significant
C. crispus	*U. lactuca*	0.000061	0.001897948	27	Significant
Chaetocerous	Biofilm	0.000117	0.001970874	26	Significant
N. helminthoides	*Chaetocerous*	0.000233	0.002049628	25	Significant
U. lactuca	*Chaetocerous*	0.000323	0.002134938	24	Significant
U. lactuca	Control	0.000357	0.002227658	23	Significant
U. lactuca	*Isochrysis*	0.000413	0.002328798	22	Significant
U. lactuca	*Rhinomonas*	0.001179	0.002439557	21	Significant
U. lactuca	Biofilm	0.001633	0.002561379	20	Significant
N. helminthoides	*Isochrysis*	0.002937	0.002696006	19	Nonsignificant
Rhinomonas	*Chaetocerous*	0.005557	0.002845571	18	Nonsignificant
C. crispus	*Tetraselmis*	0.005848	0.003012705	17	Nonsignificant
N. helminthoides	*Tetraselmis*	0.009524	0.003200698	16	Nonsignificant
U. lactuca	*Tetraselmis*	0.010989	0.003413713	15	Nonsignificant
N. helminthoides	*U. lactuca*	0.012730	0.003657103	14	Nonsignificant
Rhinomonas	*Isochrysis*	0.016147	0.003937864	13	Nonsignificant
N. helminthoides	Control	0.034907	0.004265319	12	Nonsignificant
Rhinomonas	*Tetraselmis*	0.110526	0.004652172	11	Nonsignificant
Chaetocerous	Control	0.123737	0.005116197	10	Nonsignificant
Rhinomonas	Control	0.127680	0.005683045	9	Nonsignificant
Tetraselmis	Biofilm	0.190480	0.006391151	8	Nonsignificant
Isochrysis	Control	0.346253	0.007300832	7	Nonsignificant
C. crispus	Biofilm	0.352716	0.008512445	6	Nonsignificant
Tetraselmis	Control	0.472222	0.010206218	5	Nonsignificant
Tetraselmis	*Chaetocerous*	0.523810	0.012741455	4	Nonsignificant
Tetraselmis	*Isochrysis*	0.619048	0.016952428	3	Nonsignificant
N. helminthoides	*Rhinomonas*	0.788128	0.025320566	2	Nonsignificant
Chaetocerous	*Isochrysis*	0.960733	0.05	1	Nonsignificant

Group 1	Group 2	Probability *Pi*	Associated α	*k*	Significant?
Isochrysis	Biofilm	0.000000	0.001423799	36	Significant
C. crispus	*Chaetocerous*	0.000000	0.001464449	35	Significant
C. crispus	*Isochrysis*	0.000000	0.001507489	34	Significant
Chaetocerous	Biofilm	0.000000	0.001553135	33	Significant
Tetraselmis	Biofilm	0.000004	0.001601631	32	Significant
U. lactuca	*Isochrysis*	0.000006	0.001653254	31	Significant
Tetraselmis	*Isochrysis*	0.000012	0.001708316	30	Significant
Rhinomonas	*Isochrysis*	0.000018	0.001767171	29	Significant
Biofilm	Control	0.000030	0.001830226	28	Significant
C. crispus	Control	0.000030	0.001897948	27	Significant
Tetraselmis	*Chaetocerous*	0.000087	0.001970874	26	Significant
Rhinomonas	*Chaetocerous*	0.000087	0.002049628	25	Significant
U. lactuca	*Chaetocerous*	0.000184	0.002134938	24	Significant
Rhinomonas	Biofilm	0.000763	0.002227658	23	Significant
C. crispus	*Tetraselmis*	0.000909	0.002328798	22	Significant
U. lactuca	Biofilm	0.001020	0.002439557	21	Significant
Tetraselmis	Control	0.001263	0.002561379	20	Significant
Rhinomonas	Control	0.002525	0.002696006	19	Significant
U. lactuca	Control	0.003663	0.002845571	18	Nonsignificant
C. crispus	*Rhinomonas*	0.005665	0.003012705	17	Nonsignificant
N. helminthoides	Biofilm	0.010915	0.003200698	16	Nonsignificant
C. crispus	*U. lactuca*	0.025212	0.003413713	15	Nonsignificant
N. helminthoides	*C. crispus*	0.027965	0.003657103	14	Nonsignificant
N. helminthoides	*Isochrysis*	0.029526	0.003937864	13	Nonsignificant
N. helminthoides	*Tetraselmis*	0.107537	0.004265319	12	Nonsignificant
C. crispus	Biofilm	0.114426	0.004652172	11	Nonsignificant
N. helminthoides	*Rhinomonas*	0.115307	0.005116197	10	Nonsignificant
Isochrysis	Control	0.134417	0.005683045	9	Nonsignificant
N. helminthoides	*U. lactuca*	0.149755	0.006391151	8	Nonsignificant
N. helminthoides	*Chaetocerous*	0.186055	0.007300832	7	Nonsignificant
N. helminthoides	Control	0.191919	0.008512445	6	Nonsignificant
Chaetocerous	*Isochrysis*	0.460539	0.010206218	5	Nonsignificant
U. lactuca	*Rhinomonas*	0.517894	0.012741455	4	Nonsignificant
Chaetocerous	Control	0.720280	0.016952428	3	Nonsignificant
U. lactuca	*Tetraselmis*	0.790981	0.025320566	2	Nonsignificant
Rhinomonas	*Tetraselmis*	0.965035	0.05	1	Nonsignificant

Time taken to metamorphose after hatching KW test, comparisons over all groups:

Ranks

	Substrate and	N	Mean Rank
Time taken to metamorphose after hatching	Rhinomonas + Chondrus	32	76.19
	Rhinomonas + Ulva	17	147.53
	Rhinomonas + Biofilm	22	111.00
	Chaetoceros + Chondrus	17	68.65
	Chaetoceros + Ulva	24	135.29
	Chaetoceros + Biofilm	10	142.20
	Tetraselmis + Chondrus	17	105.41
	Tetraselmis + Ulva	21	187.19
	Tetraselmis + Biofilm	11	131.64
	Isochrysis + Chondrus	19	72.79
	Isochrysis + Ulva	17	123.76
	Isochrysis + Biofilm	21	105.90
	Total	228	

Test Statistics [b,c]

			Time taken to metamorphose after hatching
Chi-Square			64.317
df			11
Asymp. Sig.			.000
Monte Carlo Sig.	Sig.		.000[a]
	95% Confidence Interval	Lower Bound	.000
		Upper Bound	.000

a. Based on 1000000 sampled tables with starting seed 926214481.

b. Kruskal Wallis Test

c. Grouping Variable: Substrate and phytoplankton available for metamorphosis

Time taken to metamorphose after hatching KW test, within phytoplankton groups:

Ranks

	Substrate and	N	Mean Rank
Time taken to metamorphose after hatching	Rhinomonas + Chondru	30	26.22
	Rhinomonas + Ulva	17	48.71
	Rhinomonas + Biofilm	22	36.39
	Total	69	

Test Statistics [b,c]

			Time taken to metamorphose after hatching
Chi-Square			14.359
df			2
Asymp. Sig.			.001
Monte Carlo Sig.	Sig.		.001[a]
	99% Confidence Interval	Lower Bound	.000
		Upper Bound	.001

a. Based on 1000000 sampled tables with starting seed 2000000.

b. Kruskal Wallis Test

c. Grouping Variable: Substrate and phytoplankton available for metamorphosis

Ranks

	Substrate and	N	Mean Rank
Time taken to metamorphose after hatching	Chaetoceros + Chondrus	17	16.68
	Chaetoceros + Ulva	24	30.65
	Chaetoceros + Biofilm	10	30.70
	Total	51	

Test Statistics [b,c]

			Time taken to metamorphose after hatching
Chi-Square			10.453
df			2
Asymp. Sig.			.005
Monte Carlo Sig.	Sig.		.004[a]
	99% Confidence Interval	Lower Bound	.004
		Upper Bound	.004

a. Based on 1000000 sampled tables with starting seed 299883525.

b. Kruskal Wallis Test

c. Grouping Variable: Substrate and phytoplankton available for metamorphosis

Ranks

	Substrate and	N	Mean Rank
Time taken to metamorphose after hatching	Tetraselmis + Chondrus	17	14.71
	Tetraselmis + Ulva	21	35.05
	Tetraselmis + Biofilm	11	21.73
	Total	49	

Test Statistics [b,c]

			Time taken to metamorphose after hatching
Chi-Square			20.670
df			2
Asymp. Sig.			.000
Monte Carlo Sig.	Sig.		.000[a]
	99% Confidence Interval	Lower Bound	.000
		Upper Bound	.000

a. Based on 1000000 sampled tables with starting seed 926214481.

b. Kruskal Wallis Test

c. Grouping Variable: Substrate and phytoplankton available for metamorphosis

Ranks

	Substrate and	N	Mean Rank
Time taken to metamorphose after hatching	Isochrysis + Chondrus	19	21.53
	Isochrysis + Ulva	17	34.88
	Isochrysis + Biofilm	21	31.00
	Total	57	

Test Statistics [b,c]

			Time taken to metamorphose after hatching
Chi-Square			6.742
df			2
Asymp. Sig.			.034
Monte Carlo Sig.	Sig.		.032[a]
	99% Confidence Interval	Lower Bound	.031
		Upper Bound	.032

a. Based on 1000000 sampled tables with starting seed 1314643744.

b. Kruskal Wallis Test

c. Grouping Variable: Substrate and phytoplankton available for metamorphosis

Corrected data

Sequential Bonferroni test:
***k* comparisons by the Dunn-Sidak method at a specified experimentwise error rate** α

	Group 1	Group 2	Probability *Pi*	Associated α	*k*	Significant?
Rhinomonas	*Chondrus*	*Ulva*	0.000800	0.016952428	3	Significant
	Biofilm	*Ulva*	0.020150	0.025320566	2	Nonsignificant
	Chondrus	Biofilm	0.038100	0.05	1	Nonsignificant
Chaetoceros	*Chondrus*	Biofilm	0.005590	0.016952428	3	Significant
	Chondrus	*Ulva*	0.038100	0.025320566	2	Nonsignificant
	Biofilm	*Ulva*	0.838850	0.05	1	Nonsignificant
Tetraselmis	*Chondrus*	*Ulva*	0.000010	0.016952428	3	Significant
	Biofilm	*Ulva*	0.004790	0.025320566	2	Significant
	Chondrus	Biofilm	0.124780	0.05	1	Nonsignificant
Isochrysis	*Chondrus*	*Ulva*	0.020210	0.016952428	3	Nonsignificant
	Chondrus	Biofilm	0.043440	0.025320566	2	Nonsignificant
	Biofilm	*Ulva*	0.378390	0.05	1	Nonsignificant

Mortality K-W test experiment 1 groups A and B:

Ranks

	Substrate upon	N	Mean Rank
Day died	Chondrus A	14	54.79
	Furcellaria A	5	33.40
	Ulva A	9	30.00
	Rhinomonas A	10	60.10
	Chaetoceros A	4	30.88
	Tetraselmis A	2	17.00
	Isochrysis A	16	42.75
	Biofilm A	13	36.27
	Control A	7	17.43
	Total	80	

Test Statistics [b,c]

			Day died
Chi-Square			25.020
df			8
Asymp. Sig.			.002
Monte Carlo Sig.	Sig.		.000[a]
	99% Confidence Interval	Lower Bound	.000
		Upper Bound	.000

a. Based on 1000000 sampled tables with starting seed 2000000.

b. Kruskal Wallis Test

c. Grouping Variable: Substrate upon metamorphosis

Ranks

	Substrate upon	N	Mean Rank
Day died	Chondrus B	17	48.44
	Furcellaria B	8	21.06
	Ulva B	9	29.28
	Rhinomonas B	1	47.00
	Chaetoceros B	8	61.75
	Tetraselmis B	7	26.00
	Isochrysis B	15	44.40
	Biofilm B	15	60.03
	Control B	5	22.00
	Total	85	

Test Statistics [b,c]

			Day died
Chi-Square			28.883
df			8
Asymp. Sig.			.000
Monte Carlo Sig.	Sig.		.000[a]
	99% Confidence Interval	Lower Bound	.000
		Upper Bound	.000

a. Based on 1000000 sampled tables with starting seed 299883525.

b. Kruskal Wallis Test

c. Grouping Variable: Substrate upon metamorphosis

Corrected data:

Sequential Bonferroni test:
***k* comparisons by the Dunn-Sidak method at a specified experimentwise error rate α**

Group 1	Group 2	Probability *Pi*	Associated α	*k*	Significant?
C. crispus	Control	0.000980	0.001423799	36	Significant
Rhinomonas	Control	0.001487	0.001464449	35	Nonsignificant
C. crispus	*U. lactuca*	0.003342	0.001507489	34	Nonsignificant
U. lactuca	*Rhinomonas*	0.004817	0.001553135	33	Nonsignificant
Isochrysis	Control	0.015069	0.001601631	32	Nonsignificant
N. helminthoides	*Rhinomonas*	0.029515	0.001653254	31	Nonsignificant
Rhinomonas	*Chaetocerous*	0.029933	0.001708316	30	Nonsignificant
Rhinomonas	Biofilm	0.031769	0.001767171	29	Nonsignificant
C. crispus	*Chaetocerous*	0.032471	0.001830226	28	Nonsignificant
C. crispus	*Tetraselmis*	0.033414	0.001897948	27	Nonsignificant
N. helminthoides	*C. crispus*	0.034120	0.001970874	26	Nonsignificant
Rhinomonas	*Isochrysis*	0.044335	0.002049628	25	Nonsignificant
C. crispus	Biofilm	0.056767	0.002134938	24	Nonsignificant
Biofilm	Control	0.060916	0.002227658	23	Nonsignificant
C. crispus	*Isochrysis*	0.088056	0.002328798	22	Nonsignificant
Rhinomonas	*Tetraselmis*	0.090877	0.002439557	21	Nonsignificant
Tetraselmis	*Isochrysis*	0.091780	0.002561379	20	Nonsignificant
N. helminthoides	Control	0.122616	0.002696006	19	Nonsignificant
U. lactuca	Control	0.133254	0.002845571	18	Nonsignificant
U. lactuca	*Isochrysis*	0.137775	0.003012705	17	Nonsignificant
N. helminthoides	*Tetraselmis*	0.238054	0.003200698	16	Nonsignificant
C. crispus	*Rhinomonas*	0.254832	0.003413713	15	Nonsignificant
U. lactuca	*Tetraselmis*	0.254851	0.003657103	14	Nonsignificant
Chaetocerous	Control	0.339415	0.003937864	13	Nonsignificant
Isochrysis	Biofilm	0.376273	0.004265319	12	Nonsignificant
N. helminthoides	*Isochrysis*	0.381842	0.004652172	11	Nonsignificant
Chaetocerous	*Isochrysis*	0.398456	0.005116197	10	Nonsignificant
Tetraselmis	Biofilm	0.485011	0.005683045	9	Nonsignificant
Tetraselmis	*Chaetocerous*	0.533643	0.006391151	8	Nonsignificant
N. helminthoides	*U. lactuca*	0.677413	0.007300832	7	Nonsignificant
Tetraselmis	Control	0.694610	0.008512445	6	Nonsignificant
U. lactuca	Biofilm	0.728814	0.010206218	5	Nonsignificant
Chaetocerous	Biofilm	0.809332	0.012741455	4	Nonsignificant
U. lactuca	*Chaetocerous*	0.849297	0.016952428	3	Nonsignificant
N. helminthoides	Biofilm	0.863973	0.025320566	2	Nonsignificant
N. helminthoides	*Chaetocerous*	0.960668	0.05	1	Nonsignificant

Group 1	Group 2	Probability *Pi*	Associated α	*k*	Significant?
Biofilm	Control	0.000066	0.001423799	36	Significant
N. helminthoides	Biofilm	0.000101	0.001464449	35	Significant
Chaetocerous	Control	0.000783	0.001507489	34	Significant
N. helminthoides	*Chaetocerous*	0.001212	0.001553135	33	Significant
U. lactuca	Biofilm	0.001514	0.001601631	32	Significant
Tetraselmis	Biofilm	0.001695	0.001653254	31	Nonsignificant
Tetraselmis	*Chaetocerous*	0.003110	0.001708316	30	Nonsignificant
U. lactuca	*Chaetocerous*	0.009536	0.001767171	29	Nonsignificant
N. helminthoides	*Isochrysis*	0.023702	0.001830226	28	Nonsignificant
N. helminthoides	*C. crispus*	0.025061	0.001897948	27	Nonsignificant
C. crispus	Control	0.029187	0.001970874	26	Nonsignificant
C. crispus	*Tetraselmis*	0.045301	0.002049628	25	Nonsignificant
Isochrysis	Control	0.045551	0.002134938	24	Nonsignificant
Isochrysis	Biofilm	0.068079	0.002227658	23	Nonsignificant
C. crispus	*U. lactuca*	0.071655	0.002328798	22	Nonsignificant
Chaetocerous	*Isochrysis*	0.109467	0.002439557	21	Nonsignificant
U. lactuca	*Isochrysis*	0.158558	0.002561379	20	Nonsignificant
Rhinomonas	Control	0.166784	0.002696006	19	Nonsignificant
Tetraselmis	*Isochrysis*	0.177219	0.002845571	18	Nonsignificant
N. helminthoides	Control	0.192064	0.003012705	17	Nonsignificant
C. crispus	*Chaetocerous*	0.252926	0.003200698	16	Nonsignificant
C. crispus	Biofilm	0.262220	0.003413713	15	Nonsignificant
N. helminthoides	*Rhinomonas*	0.443968	0.003657103	14	Nonsignificant
N. helminthoides	*U. lactuca*	0.493783	0.003937864	13	Nonsignificant
Rhinomonas	*Tetraselmis*	0.500707	0.004265319	12	Nonsignificant
Rhinomonas	*Chaetocerous*	0.555474	0.004652172	11	Nonsignificant
U. lactuca	*Rhinomonas*	0.599700	0.005116197	10	Nonsignificant
C. crispus	*Isochrysis*	0.688478	0.005683045	9	Nonsignificant
Rhinomonas	Biofilm	0.749698	0.006391151	8	Nonsignificant
N. helminthoides	*Tetraselmis*	0.757514	0.007300832	7	Nonsignificant
Chaetocerous	Biofilm	0.763415	0.008512445	6	Nonsignificant
U. lactuca	*Tetraselmis*	0.772527	0.010206218	5	Nonsignificant
U. lactuca	Control	0.792314	0.012741455	4	Nonsignificant
C. crispus	*Rhinomonas*	1.000000	0.016952428	3	Nonsignificant
Rhinomonas	*Isochrysis*	1.000000	0.025320566	2	Nonsignificant
Tetraselmis	Control	1.000000	0.05	1	Nonsignificant

Time taken to die after hatching for both phytoplankton and substrate:

Ranks

	Metamorphoses	N	Mean Rank
Time taken to die after hatching (days)	Tetraselmis + Chondrus	14	75.61
	Tetraselmis + Ulva	12	60.38
	Tetraselmis + Biofilm	7	103.93
	Rhinomonas + Chondrus	25	104.94
	Rhinomonas + Ulva	14	114.39
	Rhinomonas + Biofilm	14	110.75
	Chaetoceros + Chondrus	17	103.94
	Chaetoceros + Ulva	23	88.13
	Chaetoceros + Biofilm	11	94.45
	Isochrysis + Chondrus	19	96.26
	Isochrysis + Ulva	17	82.44
	Isochrysis + Biofilm	20	118.58
	Total	193	

Test Statistics [b,c]

			Time taken to die after hatching (days)
Chi-Square			15.056
df			11
Asymp. Sig.			.180
Monte Carlo Sig.	Sig.		.176[a]
	99% Confidence Interval	Lower Bound	.175
		Upper Bound	.177

a. Based on 1000000 sampled tables with starting seed 926214481.

b. Kruskal Wallis Test

c. Grouping Variable: Metamorphoses Substrate & Phytoplankton

G-test for longetivity of Akera bullata

	Nemalion helminthoides		Chondrus crispus		Ulva lactuca		Control	
	1	2	1	2	1	2	1	2
Number of deaths	5	8	14	17	9	9	7	5
Number that survived	8	0	3	1	3	1	0	0
Total	13	8	17	18	12	10	7	5

	Rhinomonas reticulata		Tetraselmis sp.		Chaetoceros sp.		Isochrysis sp.		Biofilm	
	1	2	1	2	1	2	1	2	1	2
Number of deaths	10	1	2	7	4	8	16	15	13	15
Number that survived	8	6	0	0	1	1	3	0	0	3
Total	18	7	2	7	5	9	19	15	13	18

	Tetraselmis sp.			Chaetoceros sp.			Isochrysis sp.			Rhinomonas reticulata		
	Biofilm	Chondrus	Ulva	Biofilm	Chondrus	Ulva	Biofilm	Chondrus	Ulva	Biofilm	Chondrus	Ulva
Number of deaths	7	14	12	10	17	23	20	19	17	17	25	14
Number that survived	4	3	9	0	0	1	1	0	0	5	5	3
Total	11	17	21	10	17	24	21	19	17	22	30	17

***R* x *C* test of independance table**

	Dead A	Dead B	Total	$\Sigma\Sigma f \text{Ln} f$	$\Sigma\Sigma f \text{Ln} f$	$\Sigma(\Sigma f) \text{Ln}(\Sigma f)$	$\Sigma(\Sigma f) \text{Ln}(\Sigma f)$	$n \text{Ln} n$	G=	Williams correction factor	Gadj	*df*	*P*
N. helminthoides	5.00	8.00	13.00	16.63553233	8.047189562	33.34434165							
C. crispus	14.00	17.00	31.00	48.16462685	36.94680261	106.4536033							
U. lactuca	9.00	9.00	18.00	19.7750212	19.7750212	52.02669164							
Rhinomonas	10.00	1.00	11.00	0	23.02585093	26.376848							
Tetraselmis	2.00	7.00	9.00	13.62137104	1.386294361	19.7750212							
Chaetoceros	4.00	8.00	12.00	16.63553233	5.545177444	29.8188798							
Isochrysis	16.00	15.00	31.00	40.62075302	44.36141956	106.4536033							
Biofilm	13.00	15.00	28.00	40.62075302	33.34434165	93.30172628							
Control	7.00	5.00	12.00	8.047189562	13.62137104	29.8188798							
Totals	80.00	85.00	165.00	**390.1742477**		**497.369595**	728.1874876	842.48100	14.196336	1.037254	13.68646	8	*P*>0.05

***R* x *C* test of independance table (pg 738)**

	Dead *Rhino*	Dead *Tetra*	Dead *Chaet*	Dead *Isoc*	Totals	$\Sigma\Sigma f \text{Ln} f$	$\Sigma\Sigma f \text{Ln} f$	$\Sigma\Sigma f \text{Ln} f$	$\Sigma\Sigma f \text{Ln} f$	$\Sigma(\Sigma f) Ln(\Sigma f)$	$\Sigma(\Sigma f) Ln(\Sigma f)$	$n \text{Ln} n$	G=	Williams correction factor	Gadj	*df*	*P*
Chondrus	25.00	14.00	17.00	19.00	75.00	80.47190	36.94680	48.16463	55.94434	323.8116085							
Ulva	14.00	12.00	23.00	17.00	66.00	36.94680	29.81888	72.11637	48.16463	276.517213							
Biofilm	17.00	7.00	11.00	20.00	55.00	48.16463	13.62137	26.37685	59.91465	220.4033252							
Totals	56.00	33.00	51.00	56.00	196.00			556.65		820.7321467	766.75	1034.51	7.3658	1.0182221	7.234008482	6	*P*>0.05