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Prevalence and histopathology of the parasitic barnacle *Sacculina carcini* in shore crabs, *Carcinus maenas*

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Abstract

Sacculina carcini is a common parasite of the European shore crab, *Carcinus maenas*. Following successful penetration of the host, numerous rootlets are formed that permeate through the hosts' tissues. Ultimately, these form an externa that houses the developing nauplii larvae of the parasite. Most studies have quantified levels of infection by counting the presence of reproductive externae and their breakdown structures, called scars. However, the diagnosis of the disease based only on external features may lead to underreporting the prevalence of the parasite. In the current study, we examined the presence and severity of *S. carcini* in *C. maenas* (*n* = 221) in the Prince of Wales Dock, South Wales, U.K. using a range of diagnostic approaches to give an accurate representation of temporal dynamics of infection. Parasitized crabs were found with a mean prevalence of 24% as determined by histological examination of the hepatopancreas. However, the prevalence of *S. carcini* based on the presence of externae and scars was only 6.3% and 1.8%, respectively. Overall, parasitism was associated with smaller crabs, crabs later in the moulting cycle that were orange in colour (as opposed to green or yellow), and those with a higher number of bacteria in the haemolymph. Interestingly, only 7.5% of infected crabs showed evidence of distinct host (cellular) response to the presence of rootlets in the hepatopancreas.

Keywords: Parasitic sterilization; *Sacculina carcini*, *Carcinus maenas*, innate immunity; gonadogenesis; immune evasion; infection prevalence

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1. Introduction

Members of the barnacle family Sacculinidae are parasites of brachyuran crabs. One of the most studied members of this family is *Sacculina carcini*, a rhizocephalan parasite of the European shore crab, *Carcinus maenas*. Like most sacculinids, *S. carcini* has a complex life history that commences when the female kentrogen invades the host resulting in the formation of numerous finger-like rootlets (interna) that permeate through tissues including the ventral ganglionic mass, gonad, muscle and hepatopancreas (Høeg 1995; Høeg and Lützen, 1995). The rootlets absorb nutrients from the haemolymph and continue to grow (Powell and Rowley, 2008). This internal phase of the parasite's life cycle can last between 2 and 36 months in *C. maenas* (Lützen, 1984; Høeg and Lützen, 1995) with this variability likely depending on environmental factors. The next phase of the life cycle commences with the formation of an externa that emerges from the infected crab. This reproductive stage of the parasite is easily visible as a cream – yellow sac on the crab's ventral surface providing an easy way of determining which crabs are parasitized by *S. carcini*. The immature (virgin) externa contains developing eggs from the parasite that are fertilized when male cyprids attach to the mantle opening, leading to mature externa containing developing nauplii (Jensen et al., 2019). Once these nauplii are released, the externa degenerates resulting in the formation of a melanised scar. If rootlets survive in the tissues of such crabs, further externae may be produced but these hosts are perhaps more prone to disease than their uninfected cohorts (Høeg and Lützen, 1995), and therefore, less likely to survive to maintain another round of parasite development.

Often, *S. carcini* is described as a parasitic castrator or more accurately, a parasitic steriliser, as it hijacks the reproductive systems of its host. In the case of the male host, spermatogenesis is arrested, but the gonadal tissue does not always

degenerate entirely (Høeg, 1995). Parasitized male crabs show behavioural and external morphological changes, such as widening of the abdomen, giving them a more feminine appearance (Kristensen et al., 2012). In females, egg production is also largely arrested and the affected host does not carry its own eggs but instead bears an externa (Høeg, 1995). Crabs with externae show behavioural characteristics similar to those crabs bearing eggs (Høeg and Lützen, 1995).

There is extensive literature on the infection dynamics and prevalence of *S. carcini* in *C. maenas* across a variety of locations in Europe (e.g., Foxon, 1939; Lützen, 1984; Werner, 2001; Stentiford and Feist, 2005; Costa et al., 2013; Bojko et al., 2018; Lützen et al., 2018; Mouritsen et al., 2018), and North America (Bojko et al., 2018). Many of these use the presence of externae or scars alone as indicators of infection levels (e.g., Foxon, 1939; Werner, 2001; Costa et al., 2013). For instance, Werner (2001) reported a low (2.9%) prevalence of infection of *C. maenas* in the west coast of Sweden based on the presence of externae alone. More recently, Mouritsen et al. (2018) conducted a large study of *ca*. 60,000 crabs at several sites in Denmark and found a higher level of parasitization in females (12.6%) than in males (7.9%) but again these data were based on the presence of externae and scars. A smaller infection survey with a molecular screen using *S. carcini* specific primers (Rees and Glenner, 2014) was also carried out as part of this study. They found 12 out of 37 adult crabs – with no evidence of externae – were positive via PCR and concluded that this equates to a rate of 'hidden' infections of over 30% (Mouritsen et al., 2018). Hence, estimates of infection prevalence based on the presence of externae alone may grossly underestimate levels of infection of crabs by sacculinid parasites in general.

Several approaches to quantifying levels of infections by sacculinid parasites exist. As already mentioned, many studies have utilised the presence of externae and

scars alone, while some have explored using a morphometric approach to changes in the shape of crabs post-infection (Werner, 2001; Kristensen et al., 2012), but these are only applicable to male crabs at late stages of interna development. Molecular screens using tissues such as externae, muscle and hepatopancreas (Gurney et al., 2006; Rees and Glenner, 2014) are less widely used, as they require more resources and crabs sampled are not conducive for later experimentation. Dissection and microscope-based examination of rootlets *in situ* has also been employed (Zetlmeisl et al., 2011). Finally, histological examination of the level of rootlet penetration of tissues has the advantage of giving information on tissue interactions (e.g. Powell and Rowley, 2008) but is a lengthy process not particularly suited to population studies screening large numbers of crabs.

In this study, we used a combination of histopathology and visual examination of externae and scars in order to examine the temporal dynamics of infection of *C. maenas* by *S. carcini* at one site in South Wales, U.K. We also examined the effect of parasitization on the host's gonadogenesis and sought evidence of interaction between *S. carcini* rootlets, externae and scars and the host's cellular immune defences. This study forms part of a larger project on disease presence/diversity in European shore crabs (Davies et al., 2019) and disease connectivity in decapods (Edwards et al., 2019).

2. Materials and Methods

2.1. Survey site and sample collection

The prevalence and severity of *S. carcini* infections in shore crabs, *C. maenas* was determined at the Prince of Wales Dock, Swansea, U.K. (51°37'8.76" N, 3°55'36.84" W). Further details of this site are given in Davies et al. (2019). The shore crab

115 population was surveyed monthly, at both locations, for a 12-month period from
116 November 2017 to October 2018. Briefly, strings of fish-baited traps were deployed
117 and immersed for 24 h, retrieved and 50 crabs were chosen randomly, bagged
118 individually, and transported back to the laboratory on ice.
119
120 *2.2. Laboratory regime*
121 All crabs were processed on the day of collection. The following biometric data for
122 each crab were taken - carapace width, sex, moult stage, fouling (presence of
123 epibionts), limb loss and carapace colour (green, yellow or orange/red). The presence
124 of scars and externae of *S. carcini* was also recorded and routinely photographed. All
125 crabs were bled aseptically from a limb articulation and haemolymph was fixed 1:1
126 with 25 µL 5% formaldehyde (*v/v*) in 3% NaCl (*w/v*) solution. Total haemocyte counts
127 were recorded using an improved Neubauer haemocytometer. Additionally, bacterial
128 colony forming units (CFUs) were determined by spreading 200 µL 1:1
129 haemolymph:sterile 3% NaCl solution onto tryptone soya agar (TSA) plates (100
130 µl/plate) supplemented with 2% NaCl (two technical replicates were performed per
131 biological replicate). Plates were incubated at 25°C for 48 h and CFUs counted. The
132 bacterial load of the haemolymph is expressed as CFUs per mL haemolymph.
133
134 *2.3. Histopathology*
135 Histology was used as the primary method to assess the presence and severity of
136 rootlet invasion in tissues and to determine if any host response was present to these
137 parasites. Three pairs of gills and three portions (*ca.* 0.5 cm^3) of the
138 hepatopancreas/gonad were excised and fixed in Davidson's seawater fixative for 24h
139 (Smith et al., 2015) prior to storage in 70% ethanol. Samples were dehydrated in a
140 graded series of ethanol, transferred to Histo-Clear™ (National Diagnostics, USA) or

Histochoice® (Merck, Gillingham, U.K.) and infiltrated with molten wax using a Shandon automated tissue processor prior to embedding. Blocks were cut at 5-7 µm thickness using a Leica RM2245 microtome. Sections were mounted on glass slides using dilute (*ca*. 0.5%) glycerine albumin and stained with Cole's haematoxylin and eosin. Stained slides were viewed and imaged using an Olympus BX41 microscope. Because of the large number of individual tissue blocks produced from this site (>1,000), it was not feasible to cut, stain and examine all of these. Instead, a smaller sub-sample of blocks were examined from 221 crabs. These were selected on the presence and absence of other known diseases including *Hematodinium* as reported by Davies et al. (2019) across the population and reflected size range and sex distributions of the full population. The severity of invasion of *S. carcini* in tissues was determined by counting the numbers of rootlets in several fields of view of the hepatopancreas using the x10 objective of a compound microscope. These were expressed as a severity index of 0 - 3 where '0' refers to an absence of rootlets, '1' a small number of rootlets (1-3) per field of view to '3' where the intertubular spaces were swollen and replete with rootlets (See Supplementary Information #1 for examples of severity ratings).

2.4. DNA extraction, PCR and sequencing

Total genomic DNA was extracted from 100 µl of thawed haemolymph and ~25 mg externa using Qiagen Blood and Tissue Kits (Qiagen, Hilden, Germany) following the manufacturer's instructions. Extracted DNA was quantified using a Qubit® dsDNA High Sensitivity Assay Kit and Qubit® Fluorometer (Invitrogen, California, USA). *Sacculina* primers developed by Rees and Glenner (2014) 12SF_Sacc (5'-TGAATTCAGATTAGGTGCAAAGA-3') and COIR_Sacc (5'-

CCCCCACTAAACCTGATCATA-3') were used to target partial *S. carcini* 12S and COI genes resulting in a sequence of ~795-803 bp and verify the presence of any *Sacculina* in the extracted DNA. PCR reactions were carried out in 25 µl total reaction volumes using 2× Master Mix (New England Biolabs Inc., Ipswitch, USA), oligonucleotide primers synthesized by Eurofins (Ebersberg, Germany), 1 µl of genomic DNA (*ca*.50-200 ng/µl) and 0.5 µM of each primer. PCRs were performed on a T100 PCR thermal cycler (BioRad Laboratories Inc., Hemel Hempstead, UK) using the following cycling profile: initial denaturation at 94°C for 5 min, then 35 cycles of 94°C for 30 sec, annealing at 54°C for 30 sec, and extension at 72°C for 2 min, followed by a final 72°C extension for 7 min. Products derived from PCR were visualized on a 2% agarose/TBE gel with GreenSafe premium nucleic acid stain (NZYTech, Lisboa, Portugal). If samples contained a positive signal for *Sacculina*, amplicons were purified using HT ExoSAP-IT™ Fast high-throughput PCR product cleanup (Thermo Fisher Scientific, Altrincham, UK) in preparation for target sequencing. Amplicons were identified by DNA Sanger sequencing using both forward and reverse primers by Eurofins. A single sequence identified as *S. carcini* was archived in GenBank, MN961952.

2.5. Statistical analyses

All statistical analyses were undertaken on the crab population from the Dock (n = 221). Binomial logistic regression models with Logit link functions (following Bernoulli distributions) were used (MASS library) to determine whether specific predictor variables had a significant effect on the probability of finding crabs with *S. carcini* (in the population sampled). All logistic models were run in RStudio Version 1.2.1335 (©2009-2018 RStudio, Inc.) using R version 3.6.1. The information theoretic approach

was used for model selection and assessment of performance (Richards, 2005). Initial models are herein referred to as the full models. Once selected, each non-significant predictor variable from the full model was sequentially removed using the drop1 function to produce a final model with increased predictive power, herein referred to as the reduced model. The drop1 function compares the initial full model with the same model, minus the least significant predictor variable. If the reduced model is significantly different from the initial full model (in the case of binomial response variables, a Chi-squared test is used to compare the residual sum of squares of both models), then the removed predictor variable is kept out of the new, reduced model. This process continues hierarchically until a final reduced model is produced (Zuur et al., 2009). The full model included the input variables: season (winter [Dec ’17, Jan ’18, Feb ‘18], spring [Mar ’18, Apr ’18, May ’18], summer [Jun ’18, Jul ’18, Aug ’18], autumn [Sept ’18, Oct ’18, Nov ’17]), carapace width (numeric), sex (male or female), colour (green, yellow or orange), fouling (presence of epibionts, 0 or 1), limb loss (0 or 1) and haemocyte counts (numeric). Bacterial colony forming units were log transformed [Y=log(y+1)] and following testing for normality, a Mann-Whitney test (unpaired) was performed to compare ranks between *Sacculina* and *Sacculina*-free animals. All other statistics (tests of normality, transformations and t-test or non-parametric equivalent) as well as graphics, were produced using GraphPad Prism v. 8.2.1 (GraphPad Software, La Jolla California, USA).

2. Results

3.1. Determination of the prevalence of S. carcini *in shore crabs*

A total of 221 crabs were examined histologically across the year-long survey from the Prince of Wales Dock. Rootlets, externae and scars (absorbed externae) were

observed clearly in these crabs (Fig. 1A-C). The mean prevalence of *Sacculina,* as assessed by histological examination of the hepatopancreas and gonads, was 24%. In the case of externa and scars, 6.3% and 1.8% of all crabs from the Dock site were considered to be infected by *S. carcini,* respectively. *Sacculina*-affected crabs were found at all months between November 2017 and October 2018 with the maximum prevalence in April (32%) and the minimum prevalence in January (15%; Fig. 1A). No crabs were found bearing externae from January to April (Fig. 1B), and similarly, scars were absent from crabs collected from November to May and July (Fig. 1C).

The severity of infection, as judged by the number of rootlets observed in sections of hepatopancreas, is presented in Figure 2A. Initial observations suggested higher severity in tissues at the same time as the presence of externa, during the spring/summer months (April – August).

Binomial logistical Model 1 examined data from the Dock using the presence of *Sacculina* as the response variable, after reduction, revealed that size and crab colour were significant factors associated with the presence of the parasite (Table 1, Model 2). Smaller crabs were significantly more likely to have *Sacculina* compared to parasite-free crabs (P = 0.0356, mean = 49.2 *vs*. 53.4 mm, difference = 4.2 mm ± SEM 1.7 mm respectively, Fig. 2B and Model 2, Table 1). Crabs orange-red in colour were significantly more likely to have *Sacculina* than yellow or green crabs (P = 0.0336, Fig. 2C and Model 2, Table 1). Season, sex, fouling, limb loss and haemocyte count did not have a significant association with parasite presence (Models 1 and 2, Table 1).

3.2. Effect of Sacculina *parasitism on host (crab) gonadogenesis*

As one of the main effects of *S. carcini* infection on crabs is the feminisation of males and inhibition of gonadogenesis in both sexes (Zetlmeisl et al., 2011), the appearance

of gonadal tissue in histology was also evaluated. There was little systemic evidence of inhibition of gonadogenesis in male and female parasitized crabs. For instance, of the crabs with externae, 67% of males had mature spermatozoa in gonadal tissue despite invasion by rootlets (Figs. 3A, B). In the case of female crabs, 25% of individuals bearing externae had evidence of extensive egg development including mature forms with yolk (Fig. 3C). Only two of the female crabs affected by *Sacculina* had any morphological evidence of gonadal breakdown (Fig. 3D). With the exception of this putative gonadal breakdown, there were no obvious changes in the morphology of gonadal tissue in comparison to apparently disease-free crabs.

3.3. Host response of crabs to infection by Sacculina

Fifty two percent of *Sacculina* infected crabs examined showed evidence of host response to the presence of rootlets in the tissues. Of these, the majority (85%) showed only a limited response characterised by single cell thick sheaths sometimes with a small number of loosely associated haemocytes (Fig. 4A). In such crabs, not all rootlets found in the tissues showed evidence of this cellular reaction. A marked host response, characterised by the presence of multi-layered sheaths of haemocytes and evidence of degradation of the rootlets, was found in only 7.5% of the total number of infected crabs (Fig. 4B, C). In extreme cases, the encapsulated rootlets showed extensive structural degradation with accompanying melanisation (Fig. 4C).

Scars left after the degradation of externae also showed evidence of a host reaction to degraded tissues of the parasite. In some cases (*ca*. 50%), the rootlets were swollen and vacuolated and the surrounding tissues filled with infiltrating haemocytes from the host (Fig. 5A-C). In other cases, these rootlets became melanised and surrounded by thick sheaths composed of flattened haemocytes, i.e.,

encapsulation (Figs. 5D, E). No evidence of host response to tissues within externae was found although cells morphologically similar to host haemocytes were occasionally seen within the connective tissue (not shown) implying a connection with the host's haemocoel.

3.4. Bacterial and haemocyte numbers in the haemolymph of S. carcini*-infected crabs*

The number of bacterial CFUs in the haemolymph of parasitized crabs were significantly higher than in uninfected crabs (Mann-Whitney test, two-tailed, U = 2844, P = 0.0258, Fig. 6). No attempt was made to identify the bacteria found in haemolymph but their variable colony morphology suggested a high level of diversity. There was no significant difference in the total number of haemocytes in *Sacculina vs*. *Sacculina*-free crabs, 3 – 3.3 x10^7 mL^{-1} (Table 1, Model 1).

3.5. Preliminary molecular detection of S. carcini

Of the DNA extracted from four externae and corresponding haemolymph, no PCR with the selected primers gave a positive signal for the haemolymph. One positive signal from externa-derived DNA which shared >99% percentage identity with other *S. carcini* isolates in GenBank (e.g., KF649259 from Norway), was sequenced and submitted to GenBank under accession number MN961952 (see Supplementary Information)

4. Discussion

The main findings of the present study: (1) using the presence of externae and scars alone results in underestimation of *S. carcini* infections, and (2) although host

response was observed in *S. carcini* infected crabs, this does not appear to effectively impede the progression of the disease.

4.1. Determination of the prevalence of S. carcini *in shore crabs*

We have shown that recording the presence of externa and scars alone does not correctly reflect both the temporal dynamics of infection and the extent to which the population of crabs studied is infected. For example, rootlets were found in crab tissues throughout the year, yet externae and scars were mainly found in the late spring – early winter. Lützen (1984) in a study of externa production in shore crabs from Isefjord, Denmark, recorded the presence of these structures from June onwards with peak production of nauplii within the externa occurring from mid-July to October. Mouritsen et al. (2018) found high levels of externae in October to December, which is similar to our current findings on a smaller sample size and at one location.

Shore crabs go through a series of colour changes during the intermoult period. After the initial moult they are usually green in colour changing to yellowish and finally to orange-red (McGaw et al., 1992). We found that crabs infected by *Sacculina* are more likely to be of the orange-red morphotype. This observation is similar to that of Costa et al. (2013) in a study of *S. carcini* parasitization of shore crabs in the Mondego estuary in Portugal. Their explanation of this finding is that because parasitization causes inhibition of moulting this leaves crabs in the orange-red colour.

Our preliminary attempts to detect *S. carcini* in haemolymph using a PCR-based approach failed although the sample size used was small. Such approach, if it worked, could provide a rapid non-lethal diagnostic approach. Further studies are required to categorically demonstrate that this method is not appropriate.

4.2. Effect of S. carcini *on host gonadogenesis*

316 The inhibition of gonadogenesis within *S. carcini* infected crabs was variable in
317 severity with both mature eggs and sperm present in crabs displaying either externae
318 or with extensive infiltration of rootlets in the tissues. This is reflected in the existing
319 literature where variable effects have been reported in sacculinid-host interactions.
320 For example, studies on *Sacculina beauforti* infections of the edible mud crab, *Scylla*
321 *olivacea,* concluded that although mature spermatozoa are still produced the reduced
322 length of gonopods in infected males would decrease the chance of successful
323 copulation (Waiho et al., 2017) – hence reducing reproductive potential. Furthermore,
324 both reduced numbers of eggs and spermatozoa are produced in infected crabs
325 (Zetlmeisl et al., 2011; Mouritsen et al., 2018) although we saw no consistent evidence
326 of this in our study.

327 The mechanism of inhibition of gonadogenesis in infected crabs is not fully
328 understood. Although we found extensive infiltration of gonadal tissue by rootlets, this
329 appears to have no obvious localised effect on gonad production. Instead, sacculinid
330 parasites are considered to produce soluble factors that inhibit gonadogenesis via
331 interaction with the host's endocrine glands. An example of these potential factors are
332 the 25-30 kDa protein(s) produced by the rhizocephalan, *Loxothylacus panopei*
333 (Rubiliani, 1983). When these were injected into the natural crab host of this parasite,
334 *Rhithropanopeus harrisii*, they caused inhibition of spermatogenesis, androgenic
335 gland lysis and depletion of the sinus gland as observed in natural infections. The
336 identity of this/these factor(s) remains unknown. Low molecular weight organic
337 molecules may also be produced by some rhizocephalan parasites although their
338 identity and potential role in the inhibition of gonadogenesis is currently unproven
339 (Zacher et al., 2018).

340

4.3. Host response to S. carcini

Some of the rootlets observed in the hepatopancreas were surrounded by a thin sheath composed of a single layer of highly flattened cells. The identity of these cells is unknown; they may be haemocytic in origin or be the connective tissue cells described by Payen et al. (1981) on rootlets emerging around the ventral ganglionic mass. They clearly represent some form of host response but there is no evidence that it impedes disease progression. A small number of crabs (7.5% of the total infected crabs examined) showed evidence of rootlet degeneration following extensive encapsulation responses in the intertubular spaces in the hepatopancreas. Such rootlets were melanised and surrounded by thick haemocytic sheaths similar to those seen around a variety of pathogens and parasites in arthropods (Rowley, 2016). It is important to note that these parasitized individuals were not carrying mature externae or scars, so the response is not part of a systematic elimination of internal rootlets post-externa production. There was also no evidence that rootlets were hyper-parasitized by other disease-causing agents as has been occasionally observed (Russell et al., 2000) that could themselves elicit a host response. Therefore, the nature of the trigger of this event is unclear but it is apparent that most rootlets in their natural host inhibit and/or circumvent the attention of the crab's immune system. Of interest is the finding that experimental infection of non-target species for *S. carcini* although resulting in parasite penetration and production of rootlets, causes a strong melanisation (immune response) from these hosts (Goddard et al., 2005).

The small number of scars observed in our study following externa breakdown showed evidence of a strong host response with infiltration of haemocytes and encapsulation of rootlets. These rootlets appeared to show evidence of morphological changes prior to encapsulation implying that the trigger from this substantial host

response was the presence of damaged tissues rather than ‘healthy’ rootlets. None of the small number of externae examined histologically showed any evidence of host reactivity although free haemocyte-like cells were occasionally found in some of the connective tissue within these structures. The presence of rootlets in scars but not in externae, is currently unexplained.

Finally, our observation that the number of viable bacteria found in haemolymph in *Sacculina*-infected crabs was greater than that in the uninfected controls suggest that either parasitization compromises the immune system resulting in reduced ability to clear microbes from circulation, or parasite-derived factors induce dysbiosis.

5. Conclusions

Our study has reinforced the view that simply counting externae is not a suitable method to determine infection levels of crabs with sacculinid parasites and that there is a need to explore other approaches. In initial attempts to use molecular screening of whole haemolymph genomic DNA samples from *Sacculina*-infected crabs, we were unable to observe amplification of products using *S. carcini* specific primers, most likely because *Sacculina* does not circulate freely in the haemolymph and is cuticle-lined. Our histological approach, although with limitations in terms of processing and recording large numbers of samples, does have an advantage of giving information on interaction between the host and parasite. The observation that a small number of crabs show an immune response to the presence of *S. carcini* is interesting but the general lack of recognition and response by the host points to the possibility of molecular mimicry of host tissue determinants by the parasite and/or systematic inhibition of host defences. The higher numbers of culturable bacteria in the

haemolymph of *S. carcini* infected crabs may reflect modulation of host defences resulting in a reduction in bacterial clearance.

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Figures and Legends

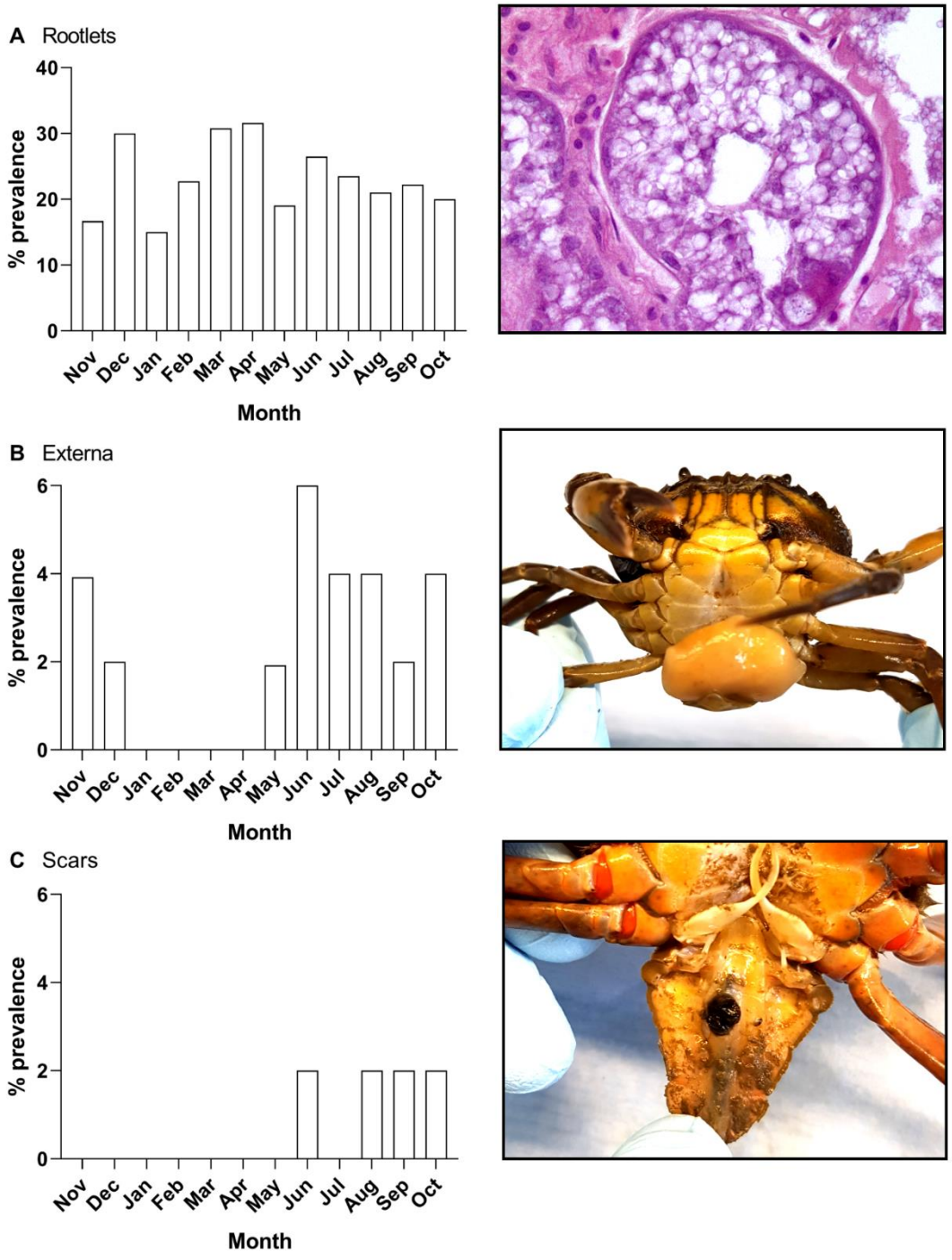
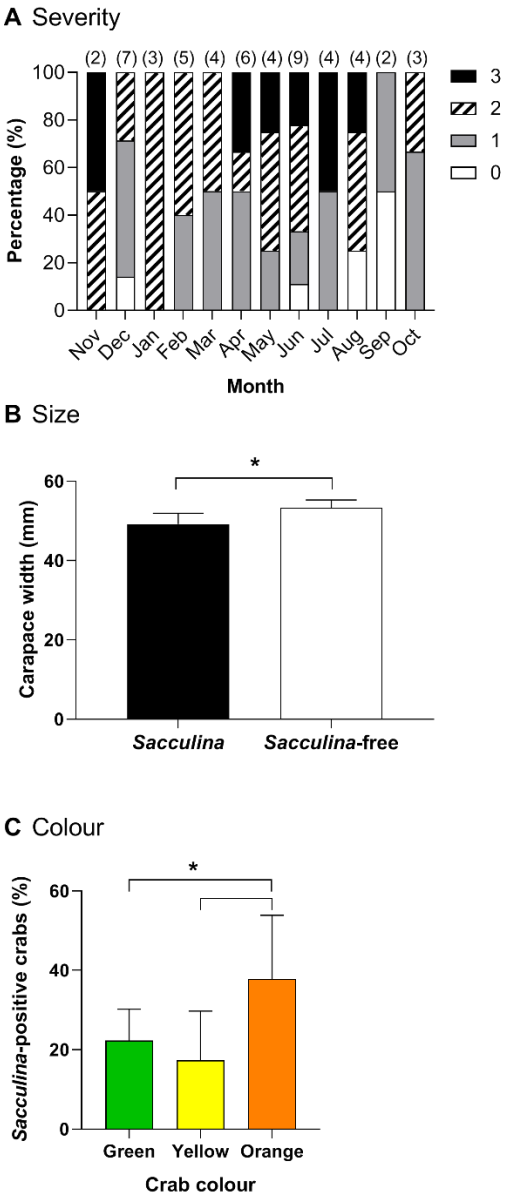


Fig. 1. Temporal dynamics of infections of shore crabs, *Carcinus maenas*, by *Sacculina carcini* at the Prince of Wales Dock, Swansea, U.K. using the presence of (A) rootlets, (B) externae and (C) scars as markers of infection.



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546 **Fig. 2A-C**. (A). Severity of *Sacculina carcini* infection of the hepatopancreas of
547 *Carcinus maenas* between November 2017 and October 2018 at the Prince of Wales
548 Dock, Swansea, U.K. The values above each column are the number of individuals
549 scored. For details of the severity rating, 0-3, see Supplementary Information. (B).
550 Carapace width (mm) of *C. maenas* presenting *S. carcini* and those *S. carcini* –free.
551 (C). Percentage of *S. carcini* in *C. maenas* according to crab colour. Values for (B)
552 and (C) represent mean + 95% CI, asterisk denotes significant difference ($P < 0.05$).

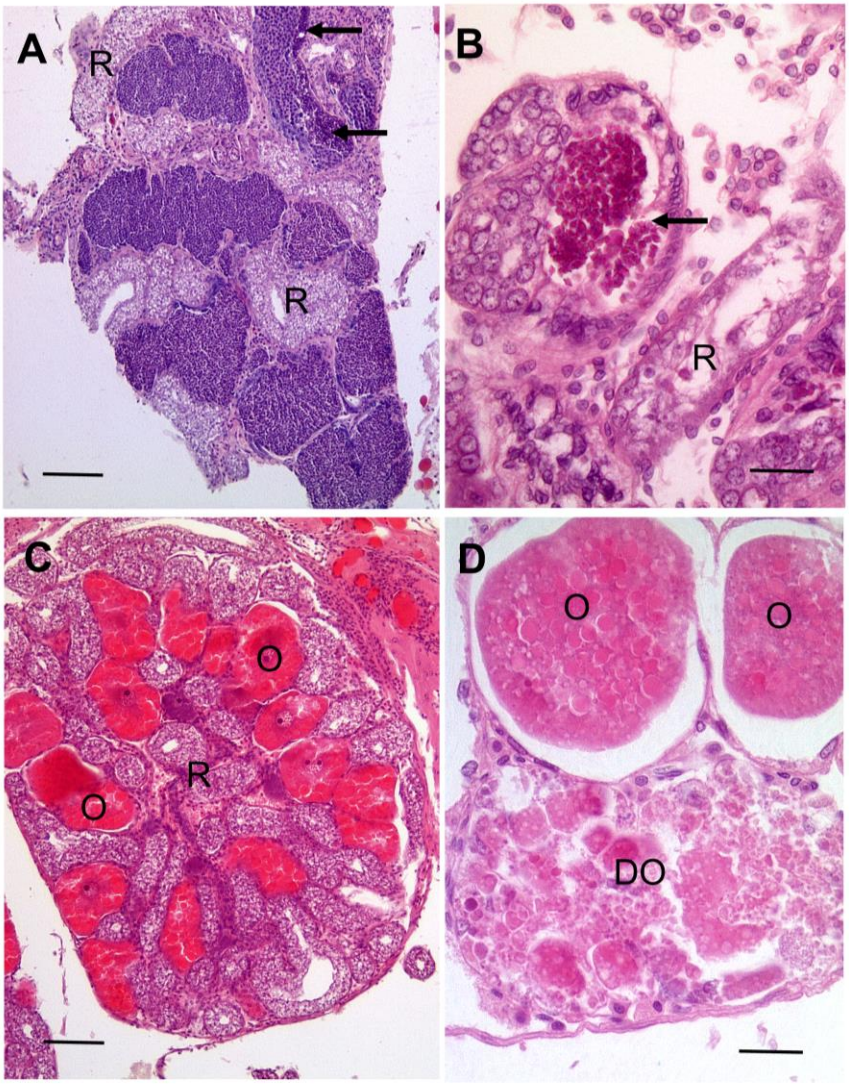

Fig. 3 A-D. Effect of *Sacculina carcini* on gonadogenesis in *Carcinus maenas* from the Prince of Wales Dock, Swansea, U.K. (A). Low power micrograph from a male crab with an externa showing infiltration of the gonadal tissue by rootlets (R). Note the presence of immature and mature (unlabelled arrows) spermatozoa. Scale bar = 100 µm. (B). High power view showing mature spermatozoa (unlabelled arrow) and adjacent rootlet of the parasite (R). Scale bar = 25 µm. (C). Low power micrograph of a female crab with externa showing extensive infiltration of gonadal tissue by rootlets (R) of *S. carcini*. Note presence of mature oocytes with yolk droplets (O). Scale bar = 100 µm. (D). Potential evidence of oocyte breakdown (DO) following parasitization. Mature oocytes (O). Scale bar = 25 µm.

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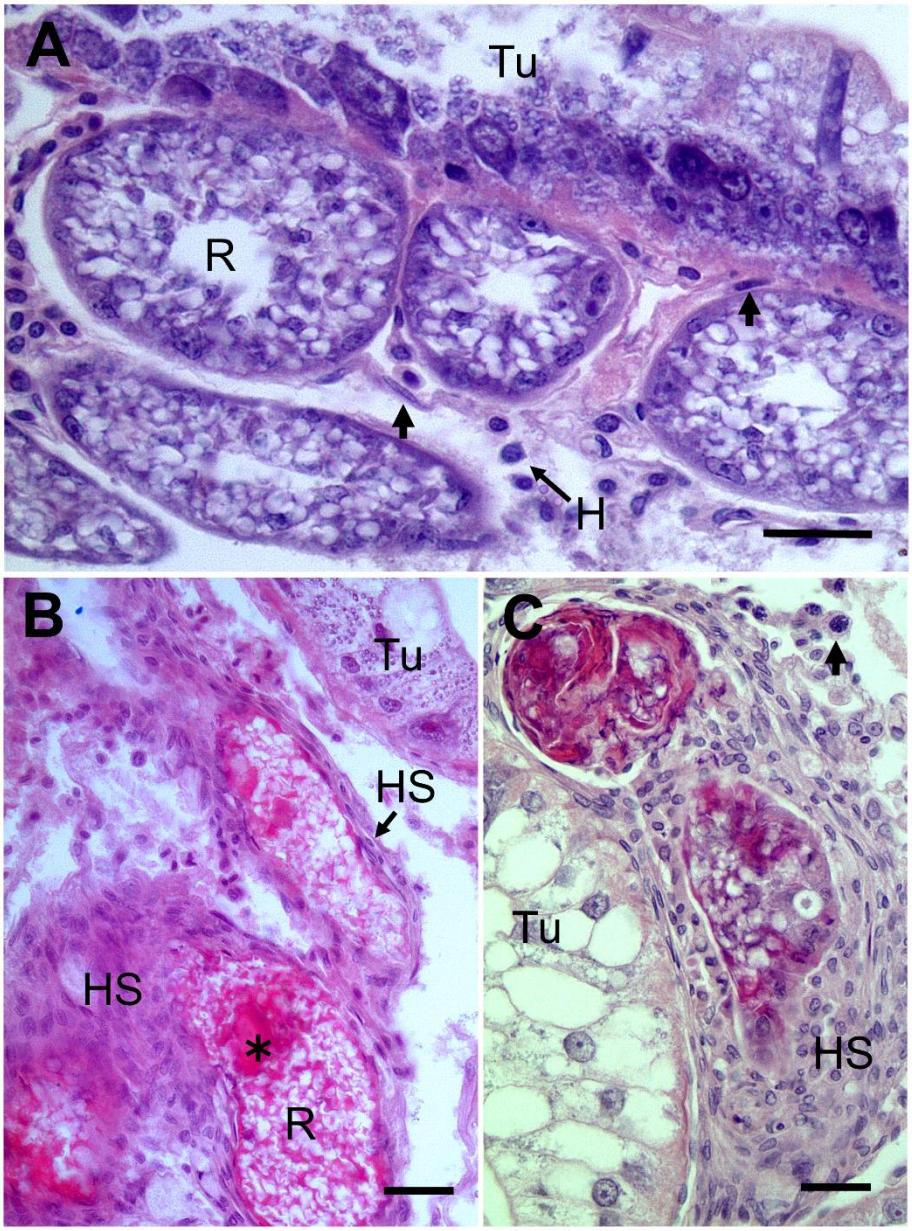

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565 **Fig. 4 A-C**. Host response in *Carcinus maenas* from the Prince of Wales Dock to
566 parasitization by *Sacculina carcini*. (A). Example of typical limited host response to the
567 presence of rootlets (R) in the intertubular spaces of the hepatopancreas. Note
568 flattened cells (unlabelled arrow) and small numbers of loosely associated
569 haemocytes (H). Hepatopancreatic tubule (Tu). (B, C). Strong encapsulation
570 responses to presence of rootlets in the hepatopancreas. Note sheath of
571 encapsulating haemocytes (HS) and degradation of internal structure of rootlets (R).
572 Some rootlets are melanised (*). Tubule (Tu) of hepatopancreas. Scale bars = 25 µm.

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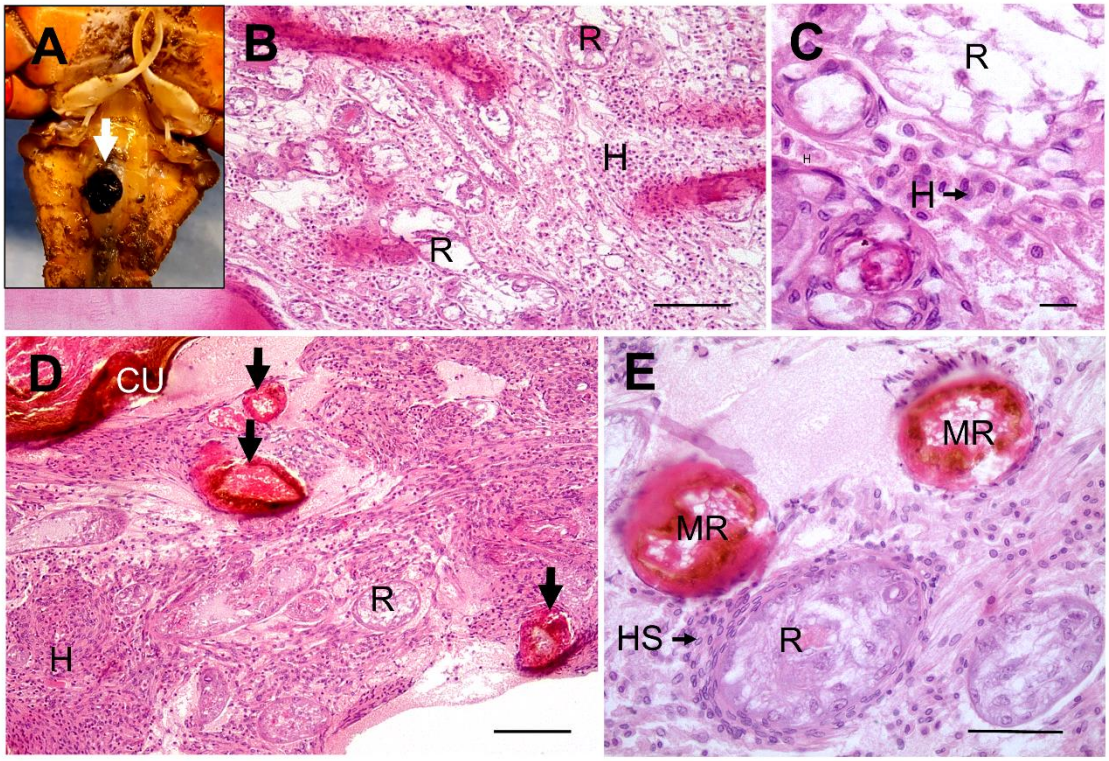

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575 **Fig. 5 A-E**. Host response in *Carcinus maenas* scars caused by *Sacculina carcini*. (A).
576 Macroscopic view of melanised scar examined histologically in Figs. 5B and C. (B).
577 Low power view of internal structure of scar showing swollen and necrotic rootlets (R)
578 surrounded by infiltrating haemocytes (H). Scale bar = 100 µm. (C). High power
579 micrograph showing swollen rootlet (R) and infiltrating haemocytes (H). Scale bar =
580 10 µm. (D). Low power micrograph of a section through a scar showing swollen
581 rootlets (R). Some of these are melanised (unlabelled arrows) and surrounded by
582 haemocytes (H). Melanised scar cuticle (CU). Scale bar = 100 µm. (D). High power
583 micrograph showing melanised (MR) and non-melanised (R) rootlets. Note large
584 numbers of infiltrating haemocytes and haemocytic sheath (HS). Scale bar = 50 µm.

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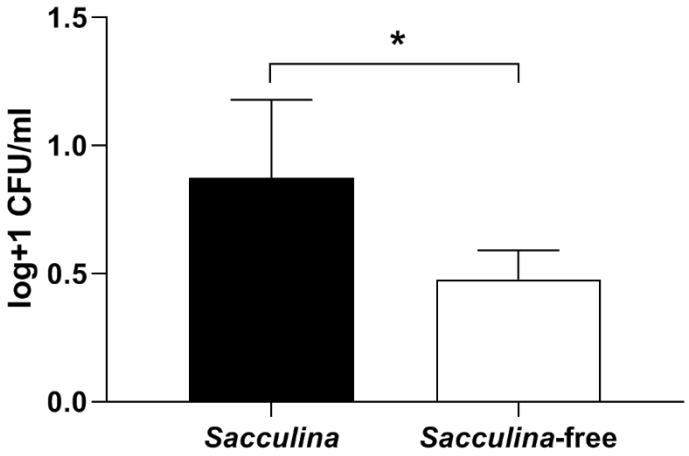

586

587 **Fig. 6**. Colony forming units (CFU) of culturable bacteria from the haemolymph of
588 *Sacculina*-positive (n = 52) and *Sacculina*-free (n = 158) crabs in the Prince of Wales
589 Dock site. *P = 0.0258, Mann-Whitney test.

590

591

592

593 **Table 1.** Binomial logistic regression models (Full Model 1, followed by reduced Model
594 2) testing the effects of biometric and environmental predictor variables on the overall
595 presence of *Sacculina*.

Model	Predictor variable	Estimate(slope)	± Standard Error	P value
Model 1 (Full)				
Sacculina ~ Season + CW	Season (Spring)	0.555480	0.590833	0.3471
+ Sex + Colour + Limb Loss	Season (Summer)	0.536650	0.544686	0.3245
+ Haemocyte count	Season (Winter)	0.281067	0.589537	0.6335
df = 208	Size (CW)	-0.041656	0.015901	0.0088**
AIC: 239.33	Sex (Male)	0.327296	0.405445	0.4195
	Colour (Orange)	1.100128	0.474302	0.0204*
	Colour (Yellow)	0.030777	0.465963	0.9473
	Fouling	0.170324	0.461480	0.7121
	Limb Loss	-0.619855	0.452209	0.1705
	Hemocyte Count	-0.006749	0.008919	0.4492
Model 2 (Reduced)				
Sacculina ~ Size (CW) +	Size (CW)	-0.03027	0.01440	0.0356*
Colour	Colour (Orange)	0.86877	0.40878	0.0336*
df = 218	Colour (Yellow)	-0.11380	0.43652	0.7943
AIC: 238.431				

596 Asterisks denotes significance ** P ≤ 0.01, * P ≤ 0.05
597