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Epigenetic regulation of sex-ratios may explain natural variation in self-fertilisation rates

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Abstract: Self-fertilisation (selfing) favours reproductive success when mate availability is low but renders populations more vulnerable to environmental change by reducing genetic variability. A mixed-breeding strategy (alternating selfing and outcrossing) may allow species to balance these needs but requires a system for regulating sexual identity. We explored the role of DNA methylation as a regulatory system for sex-ratio modulation in the mixed-mating fish *Kryptolebias marmoratus.* We found a significant interaction between sexual identity (male or hermaphrodite), temperature and methylation patterns when two selfing lines were exposed to different temperatures during development. We also identified several genes differentially methylated in males and hermaphrodites that represent candidates for the temperature-mediated sex regulation in *K. marmoratus*. We conclude that an epigenetic mechanism regulated by temperature modulates sexual identity in this selfing species, providing a potentially widespread mechanism by which environmental change may influence selfing rates. We also suggest that *K. marmoratus*, with naturally inbred populations, represents a good vertebrate model for epigenetic studies.

Introduction

Self-fertilisation (selfing) has been an evolutionary enigma ever since Darwin[1]. Selfing is a particularly cost-effective breeding mode compared to outcrossing (bi-parental reproduction)[2]. The ability of hermaphrodites to reproduce without a mate avoids the cost and uncertainty of gonochoric reproduction, and should be particularly advantageous when mate availability is low[3]. Conversely, outcrossing preserves populations allelic diversity and heterozygosity that help to confer resilience against future epidemiological or environmental challenges. Many hermaphrodite animals and plants that are able to self-fertilise seem to balance the contrasting needs for reproductive assurance (favouring selfing) and allelic diversity (favoured by outcrossing) by operating a mixed-mating strategy where selfing and outcrossing occur at variable rates[4]. Thus, in mixed-mating species the main genetic consequences of selfing (i.e. accumulation of deleterious mutations, reduced rate of adaptation, inbreeding depression and loss of heterozygosity) can be counteracted by the relative advantages of outcrossing, such as greater offspring plasticity and genetic variability[5]. Species with a mixed mating model could thereby circumvent the issue of inbreeding depression as a critical evolutionary constraint of self-fertilisation[6]. However, selfing rates are very variable in natural populations and non-genetic and ecological factors seem to influence them[7]. Among the non-genetic factors influencing selfing, mate availability and the need for reproductive assurance have received particular attention[4, 8], although the regulatory mechanism remains unclear[9]. Mate availability plays an important role in regulating selfing; in cestodes and nematodes, low population density induces delayed selfing[10, 11] whereas stress alters both sex ratios and outcrossing rates[12]. Yet, the mechanisms that modulate the sex ratios of mixed mating species in the face of changes to population structure and the environment remain elusive.

Epigenetic modifications (such as DNA methylation or histone modification) are influenced by fluctuations in the environment and mediate changes in gene expression that contribute to phenotypic diversity in populations without altering the underlying genetic code[13]. Changes in temperature can alter DNA methylation in plants at specific genomic loci [14] and in animals, dietary regimes have been shown to affect DNA methylation and therefore expression of specific genes [15]. Epigenetic modifications thereby can create phenotypic variability in asexual and selfing organisms that, without involving DNA mutations, allows them to adapt to environmental change[16]. In addition, there is a relationship between inbreeding depression and DNA methylation that suggests that epigenetic modulation may influence the magnitude of environment-dependent inbreeding depression [17, 18] and therefore could contribute to polymorphism in selfing rates[19]. We therefore hypothesized that in species with mixed mating epigenetic control systems could play an important role in determining sex ratios, hence the balance between selfing and outcrossing rates. *Kryptolebias marmoratus* (the mangrove killifish) is an androdioecious fish with a mixed mating reproductive strategy that is moderated by temperature[20]. Here, we compare sex ratios and methylation patterns in isogenic lines under different environmental conditions (incubation temperatures) to test the hypothesis that DNA methylation plays a role in determining temperature-dependent sex ratios, and ultimately influences selfing rates. In this and other androdioecious species, hermaphrodites coexist with males, with the latter being usually present at low frequencies. Hermaphrodites are able to self-fertilise but cannot outcross, and so the frequency of males is critical in determining outcrossing rates. The ability of *K. marmoratus* to self-fertilise makes it easy to control for genetic variation when studying epigenetic effects. Its natural populations live under highly variable environmental conditions and, although predominantly consist of highly homozygous selfing hermaphrodites, the presence of males has been observed in some populations at proportions

varying from 1% to 20%[21]. Factors determining the frequency of males in natural populations of *K. marmoratus* are not well known but the proportion of males can be manipulated by modifying egg incubation temperature (lower incubation temperature increases male percentage) [22]. Although selfing is the predominant mode of reproduction, outcrossing is common in the populations with the highest proportion of males, resulting in high individual heterozygosity and low population linkage disequilibria, that contrast with the patterns of high homozygosity characteristic of the populations with the lowest percentage of males[21]. We have previously demonstrated that outcrossing with males increases genetic diversity and parasite resistance in the offspring compared with selfed progeny [23, 24], suggesting a potential adaptive value of maintaining males for outcrossing. In addition, males show a behavioural preference for hermaphrodites that are genetically dissimilar to them, a potential mechanism to increase genetic diversity in this species through outcrossing [25].

Material and Methods

(a) Sex-ratios of K. marmoratus at different incubation temperatures

The study fish had two different selfing backgrounds; the R strain derived originally from Belize in the early 1990's and the DAN strain derived also from Belize in early 2000's [26]. These strains were chosen due to known differences in their levels of genotypic diversity; i.e. DAN strain has higher levels of individual and within-strain heterozygosity than R [24]. A total of 240 eggs (30 from each strain and temperature) were collected on the day of oviposition and incubated at four different temperatures between 18°C-25°C, within the natural temperature range experienced by this species. Eggs were allowed to develop for 750 degree-days before being hatched by dechorionation. Newly hatched fry were then reared under standard growing conditions of 25°C and 12:12 hour light/dark. Fish were euthanized

according to Home Office Schedule One methods 60 days post-hatching, and fish heads were removed and preserved in ethanol for brain tissue dissection. Sexual identity (male or hermaphrodite) was determined by histological examination of the gonads; hermaphrodites possess oocytes and spermatogenic tissue whereas males are characterized by pure spermatogenic tissue. Logistic regression was used to analyse variation in sex ratios (proportion of males) in relation to selfing strain and temperature. Logistic regression was used to analyse variation in sex ratios (proportion of males) in relation to strain and temperature.

(b) Genome-wide methylation analysis (MSAP)

Genome-wide DNA methylation was assessed in 79 fish from both genetic strains incubated at the 2 temperature extremes (18°C and 25°C). Total genomic DNA was extracted from whole brains (which can show sex differences before gonadal hormones start acting), and analysed for differential patterns of DNA methylation using a modification of the methylation-sensitive amplified fragment length polymorphism (MS-AFLP) method [27]. A single organ was chosen in order to minimize tissue specific differences in methylation. The method involved the digestion of genomic DNA with restriction enzymes, subsequent ligation of linkers and selective PCR amplification with primers complementary to the linkers but with unique 3' overhangs. Genomic DNA was cleaved using restriction enzymes *HpaII* + *EcoRI* or restriction enzymes *MspI* + *EcoRI* in two separate reactions. *Hpa*II and *Msp*I vary in their sensitivity to methylation. Both enzymes recognise CCGG sequences but cleaving by *Msp*I is blocked when the inner C is methylated whereas cleaving by *Hpa*II is blocked when either or both cytosines are fully or hemimethylated. Comparison of the two restriction profiles for each individual allowed assessment of the methylation state of the restriction sites in *K. marmoratus*. The four possible types of variation were pooled in methylated and not

methylated restriction sites [28]. For the selective amplification step the combination of primers used was *HpaII* + CA (5'-GATGAGTCTAGAACGGCA-3') / *EcoRI*+ AAA (5′-GACTGCGTACCAATTCAAA). Fragments were run on an ABI PRISM 3100® (Applied Biosystems) and resultant profiles were analysed using GeneMapper® v 4.0 (Applied Biosystems). Only fragments larger than 100 bp in size were considered, to reduce the potential impact of size homoplasy[29]. Singleton observations were excluded from the dataset, i.e. markers with only one non-consensus sample.

We used hierarchical Analyses of Molecular Variance (AMOVA) in GenAlEx v.6 (Peakall & Smouse 2006) on both enzyme combinations to compare genetic and epigenetic variances among selfing lineages (Φ_{RT}), among sex and incubation temperature groups within selfing lineages (Φ_{PR}) and among individuals (Φ_{PT}). We also performed independent hierarchical AMOVAs to compare variances with the individuals grouped by sex or incubation temperatures separately. Groups with less than five individuals were not considered. For AMOVA analyses, we used 9999 permutations to estimate statistical significance, adjusting for multiple comparisons by the sequential Bonferroni method. Differences in the presence/absence MS-AFLP profiles were also explored using principal coordinate analysis (PCA) in GenAlEx v.6 (Peakall & Smouse 2006). PCA was first carried out to compare methylation-sensitive (*Hpa*II/*Eco*RI) and methylation-insensitive (*Msp*I/*Eco*RI) fragment presence/absence profiles. The variance of the scores for the first two coordinates of profiles from *Hpa*II/*Eco*RI and *Msp*I/*Eco*RI was calculated and 95% confidence intervals were determined using jack-knifing[30]. PCA was then used to analyse epigenetic variation between groups (separated by sex, temperature and lineage) from combined presence/absence profiles from *Hpa*II enzyme combinations. In addition, to assess the statistical significance in the differences of principal coordinate scores between genetic strains, sexes and temperatures, general linear modelling (GLM) was performed on the scores

of the first 2 coordinates, with individual as random effect. The initial models (where 1st or 2nd coordinate score was the dependant variable) used all factors (sex, temperature and genotype, where genotype represented the *MspI* scores of the first principal component) as independent variables plus all two-term interactions. Model selection was then carried out based on the AICc (AIC corrected for small sample size) criterion[31]. All statistical modelling was carried out using R (version 2.11.0) with the MuMIn package [32].

(c) Detection of Differentially Methylated Regions (DMRs) using Methyl-Capture Sequencing (MethylCap-seq)

Genomic DNA was isolated from a male (18°C incubation) and a hermaphrodite (25°C incubation) from the R selfing line, sheared to 200-400 bp by sonication and enriched for methylated DNA using MethylMiner™ Methylated DNA Enrichment Kit (Invitrogen Inc., Carlsbad, CA, USA). This method also reduces the amount of template and sequencing needed to measure the methylated regions compared to bisulfite-based methylome sequencing, representing an advantage given the small size of the mangrove killifish brain. Following methyl capture, the recovered DNA was diluted to 0.2ng/μl and used to create a Nextera XT library (Illumina, Inc.) for each sample. Libraries were uniquely indexed for multiplexing on the Illumina MiSeq NGS platform. Insert size was 200-500 bp. Libraries were pooled at 10nM and diluted to 8pM for sequencing. The sequencing run produced paired-end 2x150bp reads that were automatically trimmed to remove adaptors. DNA was also isolated from a single R hermaphrodite and sequenced in a similar way to provide a reference draft sequence for the alignment of the methyl-captured DNA. Since the genome sequence for *K. marmoratus* is not yet available, resulting sequences were aligned to the published genome from tilapia (*Oreochromis niloticus*). Read information was extracted from

BAM files and methylation levels across the genome were extrapolated from the sequencing coverage for each region (i.e., more coverage indicates higher level of methylation).

Two approaches with different stringencies were used to determine the significance of the observed methylation differences. First, DMRs were calculated for each window by simple subtraction of each (normalized) sample count. Differential methylation was deemed significant when the calculated difference in methylation between both samples was 2 ($p<0.01$) or 3 standard deviations away from the mean ($p<0.001$). Loci counts were calculated based on 1,857,793 500bp non-overlapping sliding windows covering the entire aligned genome. Read counts were normalized to window size (reads per base) to account for windows smaller than 500bp. Windows with no counts for either sample (819,840) were discarded. Normalized counts for both samples were plotted to identify the correlation between the loci count from each sample. Second, an additional analysis with a higher level of stringency was used to identify significant differentially methylated regions. Analysis was carried out using a model-based peak-calling algorithm previously been used to analyse methyl capture data [32]. Model-based Analysis of ChIP-Seq version 2 (MACS2 [33]) was run on both samples, using default parameters, pvalue and qvalue cut-offs and an effective genome size of $9.27e^{8}$. DMRs resulting from both approaches were inspected for the presence of *K. marmoratus* sex-determining related sequences [33] aligned to tilapia with a minimum BLAST bit score of 192, in particular: *cyp19a* (DQ339107.1), *ER alpha* (AB251458.1), *ER beta* (AB251457), *aromatase B* (AB251459), *Sox9a* (DQ683739.1), *Sox9b* (DQ683740.1), *Sox9c* (DQ683741.1), *figalpha* (DQ683743.1), *dmrt1* (DQ683742.1), *foxl2* (DQ683738.1) and *GnRHR* (DQ996268.2).

(d) Validation of putative DMRs using High Resolution Melting Analysis

We then used methylation sensitive High Resolution Melting (MS-HRM) to validate the observed differences in methylation between males and hermaphrodites in three of the

genes: *cyp19a*, *Sox9a* and *dmrt1*. For this analysis, DNA from 17 individuals from the R strain (males (5) and hermaphrodites (5) incubated at low temperature (18°C) and males (2) and hermaphrodites (5) incubated at high temperature (25°C)) was extracted, and sodium bisulphite treated using the EZ DNA Methylation-Gold kit (Zymo Research) according to the manufacturer's instructions. Converted DNA concentration was then assessed using a NanoDrop® 1000 spectrophotometer with the RNA setting. PCRs for *cyp19a* and *dmrt1* were carried out using primers designed for Japanese flounder as described by[34]. PCRs for *Sox9*a were carried out using primers designed for alligator by Parrott et al (2013). PCR conditions for the *Sox9a* and *dmrt1* promoters were as follows: 5 min at 95°C; 5 cycles of 20 s at 95°C, 15 s at 55°C, 40 s at 68; 30 cycles of 15 s at 95°C, 30 s at 53°C, 40 s at 68°C. The *cyp19a* promoter was analysed by nested PCR with external cycle as follows: 95° C for 5 min, followed by 7 cycles of 10 s at 95°C, 60 s at 55°C, 40 s at 72; 35 cycles at 95° C for 30 s, 52° C for 30 s, 72° C for 30 s and final extension at 72° C for an additional 10 min period. Nested PCR conditions for *cyp19a* were: 5 min at 95°C; 40 cycles of 30s at 95°C, 60 s at 50°C, 60 s at 72°C. After PCR resultant fragments were directly subjected to the same HRM conditions. In brief, melting curve analysis was conducted on the Rotor-Gene 6000® (software version 1.7, Qiagen, U.K.) using the Cycling A-Green channel. During HRM, temperature was increased from 50 to 90 °C in 0.1 °C incremental steps, with each step held for 2s. Using Rotor-Gene 6000 software, melting curves were normalized by calculation of the 'line of best fit' between two normalization regions selected before and after the major decrease in fluorescence (representing the 'fragment melting'). Comparisons were made between sex/temperature DNA samples in terms of Tm or by a combination of Tm and altered curve shape.

(e) Analysis of expression of differentially methylated sex determination genes

A total of 17 fish were selected for RNA extraction: males (5) and hermaphrodites (5) incubated at low temperature and males (2) and hermaphrodites (5) incubated at high temperature. RNA was extracted with the ISOLATE II RNA Mini Kit® (Bioline UK). Fish were euthanized following Schedule 1 using benzocaine, the head of each fish was removed and transferred into 350µl lysis buffer RLY and kept on ice. Samples were homogenized using a micro pestle, 3.5 µl of β-mercaptoethanol were added and the rest of the extraction followed the manufacturer's instructions. RNA was eluted in 60µl of RNase-free water and 2 µl were used for quantification using the NanoDrop 2000. In order to validate the methylation results, the expression profiles of 2 genes (*sox 9a* and *cyp 19a1a*), were analysed by Reverse Transcription Quantitative-PCR (RT-qPCR) using the primers and TM conditions detailed in [33]. The reactions were performed in duplicate per sample using the SensiFAST™ SYBR No-ROX One Step kit (Bioline Ltd, UK) in a Bio Rad CFX 96™ Real-Time System®. Reaction mixes of 20µl were prepared following the manufacturer's instructions with 2µl of extracted RNA. 18S rRNA was used as standard. PCR efficiency was estimated by linear regression analysis of the logarithm of SYBR Green fluorescence versus cycle number. We then used Pfaffl's method [35] to estimate the ratio of gene expression of each sample relative to that of the most common phenotype, used as calibrator (hermaphrodites incubated at high temperature). Normalisation was carried out against the 18S rRNA housekeeping gene. In addition, for each experimental group we estimated the ΔCt of each gene normalised relative to the standard and then compared them using a Kruskal-Wallis test [36] in Systat v.11.

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Results

(a) Sex-ratios

In the DAN strain, the proportion of males ranged from 0% at 25°C to 83.33% at 18°C. In contrast, the R strain yielded 6.66% males at 25°C but only 50.00% at 18°C. Logistic regression showed both genetic strain (P = 0.003) and temperature (P <0.001) to be significant predictors of the probability of being male (Table S1). We also found a significant interaction (P = 0.006) between temperature and strain (Figure 1).

(b) Genome-wide analysis of temperature induced methylation changes using MSAPs

The MS-AFLP analysis of the 77 (from 2 strains at 4 incubation temperatures: DAN: 13 hermaphrodites at 25°C and 5 at 18°C, 14 males at 18°C; R: 13 hermaphrodites at 25°C and 15 at 18°C; 4 males at 25°C and 13 at 25°C) samples yielded 105 scorable polymorphic fragments, 91 generated by *Msp*I (restriction enzyme insensitive to methylation) and 76 by *Hpa*II (sensitive to methylation) (63 common to both enzymes; Table S2; Figure S1). AMOVA analysis of the *Msp*I profiles indicated that 89.29% of the variation was explained by differences between genetic strains (ΦRT = 0.893, P = 0.001); 0.12% by differences between groups defined by incubation temperature and sex (ΦPR = 0.011, P = 0.357) and 10.58% by individual differences (ΦPT = 0.894, P = 0.001) (Table S3). In contrast, for *Hpa*II profiles, 44.16% of the variation was explained by differences between genetic strains (ΦRT = 0.442, P = 0.001); 2.88% to differences between temperature/sex groups (ΦPR = 0.052, P = 0.018) and 52.97% was due to individual differences (ΦPT = 0.470, P = 0.001), implying a methylation basis for the differences between sex and temperature groups as well as more epigenetic than genetic individual differences. *Hpa*II AMOVAs grouping the fish by either sex or temperature within genetic strains indicated that 2.07% and 3.63% of the variance was explained by differences between sexes (ΦPR = 0.037, P = 0.034) and temperature (ΦPR = 0.064, P = 0.015) respectively (Table S3). To evaluate whether genetic strain, egg incubation temperature and/or sex produced different epigenetic profiles, PCA was used to describe and visualize the variation contained in the polymorphic MS-AFLP loci. The first two coordinates

of the PCA explained 47.47% and 15.58% of variation in MS-AFLP profiles, respectively (Figure 2). General linear modelling selection showed that for the coordinate 1 PCA scores, genotype ($P < 0.001$) and sex ($P = 0.014$) had a significant influence with an additional significant interaction between and sex and genotype ($P = 0.007$) (Table 1; Figure S2). For coordinate 2 scores, only temperature was included in the final GLM ($P = 0.045$)

(c) Characterization of Putative DMRs associated to sex determination using Methyl-Cap-seq

After sequencing and subsequent alignment, the coverage of the read mappings across the tilapia genome was calculated from a samtools "pileup" file: 96.5 % of the genome was covered by less than 1 read, and about 1 % of the genome had an average coverage of more than 4 reads. In total, 19.65 million *K. marmoratus* reads of genomic DNA reads mapped to the tilapia genome of a total of 29.04 million, i.e. 67.65 %. For the *K. marmoratus* methyl captured DNA, 4.39 million reads (male) mapped of a total of 7.24 million, (60.75%) and 4.04 million reads (hermaphrodite) mapped from a total of 6.67 million (60.66%). Read numbers showed a simple linear correlation between both captured genomes (Figure S5).

A total of 18,225 windows across the compared genomes presented significantly different normalised number of reads ($p<0.001$) and were considered DMRs. We then analysed genome locations where 11 *K. marmoratus* sequences related to sex-determination or sex differentiation[33] aligned to tilapia with a minimum BLAST bit score of 192 (Figure S3). Six of these sequences were present among the methylated-enriched sequences and overlapped with DMRs. These included homologous to sex determination genes, namely *cyp19a* (GL831420.1 on the tilapia genome), *Sox9a* (two different loci on the tilapia genome GL831217.1 and GL831136.1), *dmrt1* (locus GL831221 on the tilapia genome), *foxl2* (locus GL831570 on the tilapia genome) and gonadotropin-releasing hormone receptor (*GnRHR*) (locus GL831133 on the tilapia genome). A further 35,894 windows were significant DMRs at $p<0.01$. Using the MACS2 peak calling algorithm [33], 827 peaks with statistically

significant different number of reads were called between the two samples. Of these, 482 were located in gene regions, while 30 overlapped with promoter regions (defined as 1.5kb before the transcription start site) (Table S4; Figure S3). Observed methylation level differences in these DMRs ranged from 2 to almost 300 fold (Figure S3A).

(d) Validation of putative DMRs using High Resolution Melting Analysis

We then used methylation sensitive High Resolution Melting (MS-HRM) to validate the methylated status of three of the genes (*cyp19a, Sox9a and dmrt1*) in 17 individuals from the R strain from all experimental groups. These genes had been previously identified as being involved in sex-reversal regulated by temperature and controlled by DNA methylation [34, 37]. We found significantly higher levels of methylation in males incubated at low temperature compared to the other three groups (hermaphrodites at low temperature and males and hermaphrodites at high temperature for *Sox9a* (Figure 3a)). Conversely differences in methylation related to incubation temperature during embryo development but not sex in *cyp19a* (Figure 3b). Finally, HRM analysis for *dmrt1* did not display methylation differences between sexes or temperatures (Figure 3c).

(e) Analysis of temperature induced differential expression of differentially methylated sex determination genes

Relative expression of differentially methylated genes *sox 9*a and *cyp 19a1a* determined by quantitative RT-qPCR indicated differences in relative expression for *sox9a* with males at both temperatures showing lower expression in relation to all hermaphrodites (Figure 3d). Based on the ΔCt values the differences among the four groups were significant (KW=11.7, P=0.008) and paired test indicated that there were significant differences between males and hermaphrodites incubated at low temperatures (Mann-Withney U=6.8 P=0.009), hermaphrodites incubated at high temperature and males at low (U=6.0 P=0.014) and high

temperatures (U=3.4 P=0.000), but not between hermaphrodites incubated at different temperatures (U=1.5 P=0.221) or males incubated at different temperatures (U=0.6 P=0.439). In contrast, the differences in expression of *cyp19a* were less clear, males incubated at both temperatures displayed lower relative expression with males at high temperature presenting the lowest levels of expression (Figure 3e) but the ΔCt values indicated no significant differences among the four groups (KW=3.1 P=0.376).

Discussion

Taken together, our results show that temperature-related variation in the sex-ratio of the mixed-mating fish *Kryptolebias marmoratus* is related to changes in methylation patterns. between males and hermaphrodites in this species results in offspring with increased genetic diversity and lower parasite loads compared to selfed offspring [23, 24]. Therefore, changes in the proportion of males are likely to influence outcrossing rates, offspring genetic diversity and ultimately population fitness. Differences in the proportion of males found in natural populations of *K. marmoratus* are attributed to environment-dependent sex determination[38], with population sex ratios controlled by local environmental conditions[39], but a regulatory mechanism has thus far not been described. However, the proportion of males varies significantly between selfing lines even under constant rearing conditions, suggesting a genetic component of sex determination in this species[40]. The combination of environmental and genetic factors in sex regulation, a mechanism more common to fish than previously recognised[41], seems the most plausible explanation for our results. Epigenetic regulation of gene expression during gonad differentiation is involved in the adaptive sex reversal observed in some fish and other organisms with genetic sex determination[42]. For example, methylation has been related to sexual reversal in relation to

incubation temperature in the half-smooth tongue sole[34]. This is because, although also linked to genomic imprinting [43], transposon immobilization [44] and suppression of transcriptional noise [45], the major biological consequence of DNA methylation throughout plants, fungi and animals is gene silencing [46]. Thus, transcription of sex-related genes can be directly inhibited by methylated cytosine bases that preclude the association of DNA transcription factors [47] or indirectly, mediated by methyl-CpG binding proteins [48]. DNA methylation states are affected by the environment, providing a potential link between phenotypic variation and genotype-environment interaction [49]. Epigenetic mechanisms, especially DNA methylation, may be critical factors in sex determination and reproductive development of certain plants and animals [50] and *K. marmoratus*, because of its life history and genetic architecture, represents a particularly good model for epigenetic studies. We have identified a number of genes involved in temperature sex-regulation potentially modulated by DNA methylation in the mangrove killifish. We tested three of them, *cyp19a*, *sox9a* and *dmrt1* using three methods (MethylCap-seq, well suited to assess relative differences between samples [51] particularly when used in combination with next-generation sequencing [52], MS-HRM and RT-qPCR of total RNA), and found evidence suggesting that two of them (*cyp19a, sox9a*) could be involved in modulating K. marmoratus sex-ratios in response to environmental change during embryo development. However, although methylation changed significantly at a local level, these changes were not always in the same direction (i.e, all DMRs showing higher methylation levels in males when compared with hermaphrodites, or vice versa). Therefore, no significant global changes in methylation levels were observed across the studied genomes. Differential DNA methylation of promoters can suppresses the transcription of either male or female specific loci, thus determining an individual's sex [53]. The inactivation of one X chromosome in female mammals [43] is a well-known example of epigenetic control and there is indication that there is a similar mechanism in some dioecious

plants [54]; experimental modification of DNA methylation in plants has also been shown to induce sex reversal [55]. For some time DNA methylation was hypothesized to be involved in sex determination of fish [56] and more recently methylation of the gonadal aromatase promoter (*cyp 19a*) has been related to the regulation of temperature-mediated sex ratios in two fish with environmental and genetic sex-determination, the European sea bass (*Dicentrarchus labrax*)[37] and the half-smooth tongue sole (*Cynoglossus semilaevis*)[34], suggesting that DNA methylation is indeed a crucial mechanism linking environmental temperature and sex determination in some fish species.

The duplicated *sox9a* and *sox9b* are critical for testis differentiation in fish mammals, but also seem to be highly expressed in the brain[57], particularly *sox9a*. We found that *sox9a* was hypermethylated in *K. marmoratus* males incubated at low temperature and the expression of the gene was lower in males than in hermaphrodites, providing for the first time an explanation for the increase of males at low incubation temperatures. We also found that the expression of *cyp19a1a* was down-regulated in the brain of males incubated at 25°C, but lower levels of methylation at higher temperatures in both sexes. This contradicts previous studies in this species where *cyp19a1a* was downregulated in fish incubated at 20°C [33] but coincides with those in seabass where high incubation temperatures result in higher methylation of the promoter and decreased the expression of this gene in males [37]. Expression levels of *cyp19a1a* vary temporally and between tissues during fish embryogenesis [37] and possibly in relation to the genotype in *K. marmoratus* [33]. In contrast to mammals, adult teleost fish display a very intense expression of aromatase genes (*cyp19a1a* and *cypa19a1b*) in the brain, but less is known regarding differences in brain aromatase activities between sexes[58]. A more detailed temporal study including different tissues may be needed to fully understand the role of methylation in the regulation of this gene in *K. marmoratus* under different environmental conditions. Finally, we found that

dmrt1 was differentially methylated between male and hermaphrodite in the Methyl-cap-seq analysis, but the MS-HRM results did not show any differences between any of the sex/temperature groups, possibly reflecting individual differences that could not be taken into account with the initial sequencing.

Sex ratios also vary with environmental conditions in other mixed mating species, for example the proportion of males increases in *Caenorhabditis elegans* under stressful conditions[59], and populations that strictly outcross with males show a clear fitness advantage with respect to obligate selfed populations, displaying better ability to adapt to environmental change[59, 60]. Thus, males may play an important adaptive role in mixed-mating species where hermaphrodites can self-fertilise but not outcross with other hermaphrodites. The environmental instability of the *K. marmoratus* habitat[61], widely different availability of males in different areas (<1% to >20%) and the uniform distribution of isogenic lineages, without evidence of high local frequencies[62], suggest that reproductive assurance[63] may be the main adaptive advantage of selfing for *K. marmoratus*, as for other selfing species[62].

Our results serve to illustrate the role of an epigenetic mechanism (methylation) in modulating sex ratios in a selfing species, and provide a potential mechanistic explanation for the influence of the environment in maintaining variable selfing rates by modulating mate availability. Methylation has also been associated to environment-dependent inbreeding depression[18], that in turn can result in variable selfing rates. We suggest that this form of epigenetic modification could provide a mechanism of control of sexual identity in species exhibiting mixed mating systems and thereby a means by which environmental change may directly influence the balance between selfing and outcrossing, optimising population resilience in the light of inbreeding depression, mate availability and environmental instability.

Ethics statement

All fish experimentation was carried out following the Association for the Study of Animal Behaviour / Animal Behavior Society Guidelines for the Use of Animals in Research (Animal Behaviour, 2006, 71, 245-253), in consultation with the NACWO officer of Aberystwyth University.

Data accessibility

Sequences were archived under SRA project accession number - SRP033207.

Competing interests

We have no competing interests.

Authors' contributions

Conceived the work SC; carried out lab experiments AE, CMRL, PM, MM, RP; analysed the data AE, CMRL, JB, MH, MS; wrote the manuscript SC, AE with help from CMRL, JB, MS and MW.

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Figure legends

Figure 1. Logistic regression model predicting the probability of producing primary males across a range of egg incubation temperatures. Black lines represent DAN strain and grey lines represent R strain. Dotted lines indicate the 95% confidence interval bands.

Figure 2. Principal coordinates analysis (PCA) of variation in methylation epigenotypes between hermaphrodite and male fish from R and DAN selfing lines, incubated at high and low temperatures, based on presence⁄absence scores of polymorphic methylation-sensitive amplified (MS-AFLP) markers. High: 25°C egg incubation temperature, low: 18°C egg incubation temperature.

Figure 3. (A-C) Determination of gene methylation level using High Resolution Melting analysis. Normalised melting curves for *Sox9a* (A), of *cyp19a* (B), and *dmrt1* (C) promoters in hermaphrodite (solid line) and male (dotted line) fish incubated at 25°C (red) or 18°C (blue) from a single selfing line (R). Small squares represent the average melting curves for each sex/temperature combination. (D-E) Determination of expression using RT-qPCR. Relative expression of *Sox9a* (D), of *cyp19a* (D) genes in males (dotted bar) and hermaphrodites (full bar) incubated at different temperatures (25°C (red) or 18°C (blue)) using 18S rRNA as endogenous control and normalised against the expression value of the most common phenotype in nature (hermaphrodites incubated at 25°C).

Table 1. Results of generalized linear models (GLMs) evaluating the effect of genotype, sex and temperature on principal coordinates (PCA) scores. PCA scores derived from variation in methylation epigenotypes between samples (R and DAN males and hermaphrodites incubated at high and low temperatures), based on presence⁄absence scores of 63 polymorphic methylation-sensitive amplified fragment length polymorphism (MS-AFLP) markers. Significant values shown in **bold**. Genotype represents the scores of the first principal component of the *MspI* PCA analysis.

Parameter	**df**	***F***	***P***
Coordinate 1			
Genotype	1	38.922	**<0.001**
Sex	1	2.512	**0.014**
Temperature	1	0.802	0.425
Genotype x Sex	1	2.796	**0.007**
Temperature x Sex	1	-1.620	0.108
Error	71		
Coordinate 2			
Temperature	1	-2.045	**0.045**
Error	75		