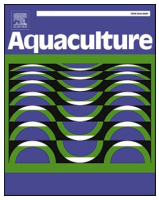
Aquaculture 610 (2026) 742882

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture



Mechanisms driving temperature-induced early sex change in barramundi (*Lates calcarifer*)

Alyssa M. Budd [a,b,c,d,e,*], Roger Huerlimann [b,c,f], Jarrod L. Guppy [b,c,i], Ricardo C.C. Pinto [g], Jose A. Domingos [b,h], Dean R. Jerry [b,h,i]

[a] Environomics Future Science Platform, Commonwealth Scientific and Industrial Research Organisation (CSIRO), Australia
[b] Centre for Sustainable Tropical Fisheries and Aquaculture, James Cook University Bebegu Yumba Campus, Townsville, Qld 4811, Australia
[c] Centre for Tropical Bioinformatics and Molecular Biology, James Cook University Bebegu Yumba Campus, Townsville, Qld 4811, Australia
[d] School of Molecular and Life Sciences, Curtin University, Perth, WA, Australia
[e] UWA Oceans Institute, The University of Western Australia, Perth, WA, Australia
[f] Marine Climate Change Unit, Okinawa Institute of Science and Technology Graduate University, Okinawa, Japan
[g] Federal University of Ceará, Marine Science Institute, Labomar, Avenida Abolição, 3207, Meireles, 60165-081 Fortaleza, Ceará State, Brazil
[h] Tropical Futures Institute, James Cook University Singapore Campus, Singapore 387380, Singapore
[i] ARC Research Hub for Supercharging Tropical Aquaculture through Genetic Solutions, James Cook University, Townsville, Qld 4811, Australia

ARTICLE INFO

Keywords:
Epigenetics
DNA methylation
Splicing
Plasticity
Differentiation
Hermaphroditism
Barramundi

ABSTRACT

Sex in fish is remarkably plastic. In some gonochoristic species, temperature exposure during early development can result in epigenetic changes and phenotypic reversal of an otherwise genetically encoded sex. In most of these cases, high temperatures result in an increased proportion of phenotypic males. In sequential hermaphrodites, such as the protandrous barramundi (*Lates calcarifer*), the effect of temperature on sex is largely unknown. Here, we reared 1-year-old barramundi under four different temperature profiles and examined the effects on mRNA expression, DNA methylation and phenotypic sex. Exposure to high temperature (34 °C) led to early sex change, skewing sex ratios toward transitional and female fish compared to the all-male control group. These phenotypic changes were accompanied by differences in methylation and/or expression of conserved sex-determining genes (e.g., *cyp19a1a* and *dmrt1*), and others with putative roles in cellular sensing and signal transduction. Global DNA methylation differences and alternative splicing were observed between sexes but not between temperatures, suggesting that these epigenetic mechanisms occur downstream of putative temperature-modulated signal transduction pathways. The results demonstrate that in contrast to gonochoristic fish, the gonads of sequential hermaphrodites maintain plasticity beyond the completion of sex differentiation, and that exposure to high temperatures leads to an increased proportion of females, rather than males. The findings may assist in the development of temperature as a consumer friendly strategy for sex control in aquaculture.

1. Introduction

Most fish species are gonochoristic, characterised by gonadal phenotypes that are typically genetically predetermined, stable and binary (male testes or female ovaries). However, in some gonochoristic species, genetically encoded sex can be modified in response to anomalous environmental conditions experienced preceding, or during primary gonadal development (Hattori et al., 2020). This process is termed 'sex reversal', and in most gonochorists leads to testes development in genetically female fish (e.g., XX or ZW). Additionally, about 5 % of fish species are sequentially hermaphroditic and may undergo protogynous (female to male), protandrous (male to female), or bidirectional sex change (either direction, multiple times) (Avise and Mank, 2009). In contrast to sex reversal in gonochoristic species, sex change in sequential hermaphrodites is part of typical reproductive development; it occurs after primary sexual development and maturation of the initial gonadal phenotype, under favourable environmental conditions and in the absence of sex chromosomes or major sex determining genes. This routine switch in reproductive mode requires that the gonads undergo a drastic and unparalleled change in organ morphology and function during adult life (Pla Quirante et al., 2018). Among vertebrates, sequential hermaphroditism is unique to teleost fish, providing powerful

* Corresponding author at: Environomics Future Science Platform, Commonwealth Scientific and Industrial Research Organisation (CSIRO), Australia.
E-mail address: alyssa.budd@my.jcu.edu.au (A.M. Budd).

https://doi.org/10.1016/j.aquaculture.2025.742882
Received 14 February 2025; Received in revised form 26 May 2025; Accepted 23 June 2025
Available online 29 June 2025

models with which to study molecular mechanisms underlying sexual development.

Temperature is a major driver of sexual phenotype in fish. For example, high temperature exposure during, or just prior, to sex differentiation leads to male development in genetically female European seabass (*Dicentrarchus labrax),* half-smooth tongue sole (*Cynoglossus semilaevis*), and Nile tilapia (*Oreochromis niloticus*) (Navarro-Martín et al., 2011; Shao et al., 2014; Wang et al., 2019b). In hermaphrodite fish, temperature may also influence primary sexual development, but the effects have instead been observed at low temperatures. For instance, in the simultaneous hermaphrodite mangrove killifish (*Kryptolebias marmoratus*) and protandrous barramundi (*Lates calcarifer*) it appears that low temperature exposure induces testes development (Budd et al., 2022a; Ellison et al., 2015; Harrington Jr, 1967). In all of these examples, changes in phenotypic sex were accompanied by changes in gene expression and DNA methylation (a well-studied epigenetic modification). In particular, an inverse relationship between DNA methylation and expression of male-associated *dmrt1* and female-associated *cyp19a1a* is consistently observed, suggesting that these genes may serve as useful epigenetic markers for sex across species (Piferrer et al., 2019).

The mechanisms by which temperature and other environmental cues initiate sex change in fish have been widely investigated, though they differ across reproductive systems. In socially controlled sex-changing teleosts, sex change is commonly regulated by the brain–pituitary–gonad (HPG) axis, where shifts in dominance hierarchy initiate neuroendocrine signalling cascades (Godwin et al., 2003; Nakamura et al., 1989). These are often accompanied by activation of the brain–pituitary–interrenal (HPI) axis, particularly in response to stress caused by social dominance or elevated temperature, resulting in increased cortisol, suppression of aromatase (*cyp19a1a*), and stimulation of androgen synthesis—mechanisms typically associated with masculinisation (Fernandino et al., 2013; Goikoetxea et al., 2017; Todd et al., 2019). Such pathways are well-characterised in gonochoristic and protogynous systems with social regulation. In contrast, protandrous species like barramundi (*Lates calcarifer*), white seabream (*Diplodus sargus*) and Black Porgy (*Acanthopagrus schlegii*) undergo mass spawning, where mating is random or near-random and transitions occur instead around size or age thresholds (Benvenuto et al., 2017; Shapiro, 1987; Warner, 1975; Wu et al., 2010). Furthermore, in barramundi, elevated temperature is associated with ovarian rather than testicular development, indicating that stress-induced androgen synthesis is not an underlying mechanism (Athauda et al., 2012). An alternative hypothesis proposes that environmental cues such as temperature are transduced via changes in intracellular calcium and redox (CaRe) status, which can activate signalling pathways and epigenetic processes relevant to sexual development (Castelli et al., 2020). While hormonal and CaRe pathways may interact in some systems, current evidence suggests that CaRe may provide a common cellular mechanism for environmental sex determination across both reptiles and teleosts, including species where high temperatures encourage female development, and sex is determined independent of social hierarchies.

The capacity for environmental cues to influence sexual development also varies across reproductive strategies. In gonochoristic fish, temperature exposure typically becomes ineffective once gonadal differentiation is complete (Ospina-Alvarez and Piferrer, 2008; Valenzuela et al., 2003). In contrast, phenotypic sex in sequential and bidirectional hermaphrodites often remains responsive to endogenous and/or exogenous environmental stimuli even much later in life (Godwin, 2009; Kobayashi et al., 2013; Warner, 1984). For example, sexually mature protandrous gilt-head seabream (*Sparus aurata*) and clownfish (*Amphiprion ocellaris*), as well as protogynous grouper (*Epinephelus* spp.) and rice field eel (*Monopterus albus*), exhibit changes in the timing of sex change in response to external factors like sex ratios, social hierarchies and stocking densities (Iwata et al., 2019; Mackie, 2000; Mackie, 2003; Quinitio et al., 1997; Sarter et al., 2006; Yuan et al., 2012; Zohar et al., 1984). In rice field eel and grouper, sex change is accompanied by substantial changes in gene DNA methylation and expression, but it remains unknown if these changes are induced by external factors (Guo et al., 2021; Hu et al., 2022; Zhang et al., 2013). In sexually differentiated barramundi, hormone (17β-estradiol) induces precocious male-to-female sex change as well as changes in DNA methylation and gene expression (Banh et al., 2021). Thus, unlike gonochoristic species, hermaphrodite fish appear to remain responsive to environmental stimuli even after sex differentiation, potentially allowing factors such as temperature to produce changes in DNA methylation and sexual phenotype later in life.

Sequential sex change creates a number of challenges for aquaculture. Firstly, it prevents same generation selection practices, slowing the rate of annual genetic progress (Bright et al., 2016; Robinson et al., 2010). Secondly, it exacerbates difficulties in controlling broodstock sex-ratios and parental contribution to spawning, leading to potential inbreeding (Kuo et al., 2014; Marc et al., 2024). Finally, it often necessitates costly long-term maintenance of large broodfish until sequential sex change takes place (Budd et al., 2015). As such, examining if the gonad of a given species is sensitive to endogenous and/or exogenous stimuli, what the precise stimuli are and when these stimuli could be most effectively applied are not only questions of biological interest, but are of economic importance as well. Temperature exposure treatments are particularly relevant, because of their potential to offer a consumer-friendly alternative to the use of steroidal hormones in aquaculture (Hoga et al., 2018) and the results have additional implications for fisheries management with predicted increases in ocean temperatures (Honeycutt et al., 2019).

In Australia, wild-caught barramundi were widely considered to mature as males from 2 to 4 years of age, and transition to female between 4 and 8 (Davis, 1982). However, more recent evidence suggests that transition to females typically occurs even later, at around 8 to 10 years, and may be influenced by environmental temperature (Budd et al., 2022b). Precocious sex change has been observed in cultured barramundi in Singapore as early as 2 years, but the causes are currently unknown (Terence et al., 2021). To date, barramundi is the only sequentially sex changing fish for which temperature has been reported to affect phenotypic sex (Athauda et al., 2012; Budd et al., 2022a; Budd et al., 2022b). There is also substantial evidence to suggest that male-to-female sex change in barramundi is under epigenetic control (Banh et al., 2021; Budd et al., 2022a; Budd et al., 2022b; Domingos et al., 2018). To better understand the molecular mechanisms underlying changes in phenotypic sex in sequential hermaphrodites after sex differentiation, we examine the effects of prolonged exposure to low, elevated, and fluctuating temperatures on gonadal gene expression, DNA methylation, and phenotypic sex in barramundi (*Lates calcarifer*). We assess the potential for temperature to induce precocious sex change and discuss the mechanisms through which these thermal cues may influence sexual development.

2. Methods

2.1. Fish rearing and temperature exposure

Barramundi larvae from a single spawning event were produced at an on-site hatchery (James Cook University, Townsville, QLD, Australia) and reared at 28 °C, 30–35 ppt and 12 h light:dark photoperiod (Dhert et al., 1992; Schipp et al., 2007). At 1 year of age, all individuals were phenotypically male (see Budd et al., 2022a) 1 year of age,all individuals were phenotypically male (see Budd et al., 2022a) and measured 34.5 cm ± 2.29 in length and 0.54 kg ± 0.11 in in weight (mean ± SD, n = 10). Fish were allocated into 2000 L tanks (n = 7 fish per tank; n = 2 tanks per treatment; total n = 14 fish per treatment). Fish were held at 28 °C for a period of 1 week, where they were acclimatized at a rate of 1 °C/ day to either one of three constant temperature exposures: 24 °C (low temperature; LT), 28 °C (control) and 34 °C (high temperature;

HT); or a fourth, fluctuating temperature (FT) of 24–34 °C. Fish in the FT group were continuously exposed to increasing temperatures of 1 °C/day with a 1 week hold at 34 °C, and then lowered at the same rate with a 1 week hold at 24 °C for a total of 11 cycles throughout the 12 month period. Temperatures were selected to allow for physiologically meaningful differences inadequate treatment intensity without compromising growth efficiency or survival (Athauda et al., 2012; Katersky and Carter, 2007; Williams et al., 2006). The fluctuating treatment of 24–34 °C was applied to simulate accelerated seasonal temperature fluctuations that may be associated with maturation in wild populations of L. *calcarifer* (Davis, 1985; Russell and Garrett, 1985).

Throughout the experiment, fish were kept in a recirculating aquaculture system with flow rates of 3–4 L per minute giving a total of 8–11 h for total exchange. Fish were stocked below commercial densities (<10 kg/m^3) and were fed ad-libitum twice daily with a commercial barramundi diet (Ridley Corporation). Any unconsumed pellets were removed after approximately 30 min. A total of four mortalities occurred during the experiment, all of which were in the HT treatment. Another three fish, one per treatment (excluding controls), were sampled at 6 months into the treatment period and macroscopic sex was assessed to determine the date at which the experiment would be terminated. At this timepoint, all fish ($n = 3$) appeared phenotypically male. Temperature was measured twice daily to ensure that the tanks were as close as practical to their target temperatures. Research was conducted under James Cook University Animal Ethics Committee Approval Number A2014.

2.2. Tissue sampling and histology

After 1 year of temperature exposure (i.e., at 2 years old) the experiment was terminated and fish were humanely euthanized using AQUI-S aquatic anaesthetic (AQUI-S New Zealand Ltd), followed by immersion in an ice slurry and severance of the head at the junction of the skull and the first vertebra. Total fish length and weight were recorded. Tissue from the middle-most section along the length of the right gonad was removed and immersed in RNAlater (Thermo Fisher Scientific). The neighbouring section of each gonad was fixed in 10 % neutral buffered formalin containing 4 % formaldehyde for at least 24 h and subject to standard histology procedures with preparations sectioned serially at a thickness of 5 μm and stained with hematoxylin-eosin. After staining, slides were examined using an Olympus cellSens Microscope Digital Camera System (Olympus). Gonad development stages were assessed according to Guiguen et al. (1994), where M1-M4 represent male stages, T1-T4 represent transitional stages and F1-F4 represent female stages. Histological examination was performed on the subset of individuals selected for sequencing (see Section 2.4).

2.3. Nucleic acid extraction

Gonad tissue was removed from RNAlater (Thermo Fisher Scientific), washed once in PBS and dried with a KimWipe (Kimberly-Clark) before immediate immersion into either TRIzol (Thermo Fisher Scientific) for RNA extraction, or CTAB for DNA extraction (Doyle and Doyle, 1987). Total RNA was extracted from homogenised gonad tissue using TRIzol (Thermo Fisher Scientific) following the manufacturer's protocol with the exception of the precipitation step, in which 0.5 volumes of isopropyl alcohol and 0.5 volumes of RNA precipitation solution [1.2 M sodium chloride, 0.8 M disodium citrate (Sambrook and Russell, 2001)] were added to increase RNA yield. To remove any contaminating gDNA, isolated RNA was then treated using TURBO DNase (Ambion), followed by an ammonium acetate precipitation. Quantification and purity of RNA was measured on an ND-1000 spectrophotometer (Nanodrop technology) based on absorbance at 260 nm and 260/280 nm ratio. RNA integrity was analysed using a TapeStation 2200 (Aligent). Genomic DNA (gDNA) was extracted following the CTAB protocol, including an overnight digestion with proteinase K (Doyle and Doyle, 1987). Quantification of gDNA was carried out as per RNA, and integrity was assessed by visualisation on a 0.8 % agarose gel with lambda standards at 50, 20, 10 and 5 ng/μl.

2.4. Library preparation and sequencing

Following extraction and quality checks, total RNA was subject to TruSeq Stranded mRNA library preparation low sample (LS) protocol (Illumina) and 100 bp paired end reads were sequenced across two lanes on two flow cells using a HiSeq2500 (approx. 17–25 million reads/sample; Illumina). Whole genome bisulfite sequencing (WGBS) libraries were prepared using a TruSeq DNA Methylation Kit (Illumina) and 125 bp pair end reads were sequenced across five lanes on a single flow cell using a HiSeq2500 (approx. 24 x coverage; Illumina). Library preparation and sequencing was performed at the Australian Genome Research Facility (AGRF) for high quality samples from three individuals per temperature group (total $n = 12$). To increase statistical power library preparation the TruSeq Stranded mRNA library preparation low sample (LS) protocol was performed in-house for another two individuals per treatment (total $n = 8$) and sent to AGRF for RNA-sequencing as described above. The extra samples were not sent for WGBS sequencing, due to the higher cost. This resulted in 5 samples per treatment for RNA-seq (total $n = 20$) and 3 samples per treatment for WGBS (total n = 12). Only those samples with sufficient tissue quality, total RNA and DNA yield, purity and integrity were able to be sequenced.

2.5. RNA-Seq analysis

Read quality was assessed using FastQC version 0.11.7 (Andrews, 2010). Raw reads were subject to sequencing error correction using the *k*-mer based method, Rcorrector using jellyfish version 2.2.10 (Marçais and Kingsford, 2011; Song and Florea, 2015). Corrected reads were then quality filtered using Trimmomatic (Bolger et al., 2014) within Trinity version 2.6.6 (Haas et al., 2013), trimming nucleotides below a Phred quality score of 2 (MacManes, 2014) and discarding reads less than 25 bp long. Overlapping read pair data was merged with non-overlapping or unpaired read data for use in subsequent transcriptome assembly and differential expression analysis.

Alignment and assembly processes were performed following recommendations in Pertea et al. (2016). Specifically, trimmed, error corrected reads were aligned and assembled using HISAT2 version 2.1.0 (Kim et al., 2015) using the *L. calcarifer* chromosome-level genome assembly version 3 as reference (Vij et al., 2016). A custom index was built to aid the mapping of RNA-Seq reads using *extract_splice_sites.py, extract_exons.py,* and the *hisat2-build* function. Filtered reads were then aligned to the *L. calcarifer* genome specifying to report alignments tailored for transcript assemblers and reverse stranded reads as inferred by the *infer-experiment.py* script from the RSeQC package (Wang et al., 2012). Aligned transcripts were assembled and merged using StringTie version 2.0.4 and used for re-estimation of transcript abundances for each sample (Pertea et al., 2015).

Expression analysis was performed in R version 4.1.3. Tximport (Soneson et al., 2015) was used to compute counts from the coverage information given by StringTie and then import the data into DESeq2 (Love et al., 2014). To assess the influence of technical variation, surrogate variable analysis (SVA) was performed using known covariates including tank, sequencing run, and library preparation batch. No major structure attributable to these factors was detected (Fig. S1), and batch terms were therefore not included in the final differential expression model. Reads with zero counts, or only a single count across all samples, were removed and a variance stabilising transformation was applied to the DESeq2 matrix to reduce over influence of high-count transcripts using the DESeq2 function *vst* (Anders and Huber, 2010; Huber et al., 2003; Tibshirani, 1988). To test differences between the four groups (HT, CT, FT and control), the DESeq2 function *contrasts* was used to extract test results for log2 fold changes for all six possible treatment-to-

treatment comparisons. For each comparison, *p*-values were corrected using the Benjamini-Hochberg FDR method (Benjamini and Hochberg, 1995) and results were subset to only include those with an adjusted *p*-value of less than 0.05 and an absolute fold change greater than one.

2.6. WGBS analysis

Read quality was assessed using FastQC version 0.11.5 (Andrews, 2010). Raw reads were subject to trimming using Trim Galore Version 0.6.5 (Krueger, 2015) removing standard Illumina adapters, specifying the default minimum Phred quality score of 20 and a length cut off of 20 bp. Alignment and assembly were performed using Bismark version 0.19.0 (Krueger and Andrews, 2011). First, the *L. calcarifer* chromosome-level genome assembly version 3 (Vij et al., 2016) was in silico bisulphite converted (C-to-T conversion in the forward read, G-to-A conversion in reverse) and indexed using bowtie2 to allow for alignment of bisulphite sequencing reads. To maximise sensitivity, the trimmed reads were aligned to the reference genome specifying 1 mismatch per seed alignment and a 20 bp seed substring length. A minimum alignment score of L's,0, −0.2 (roughly two mismatches) was applied. All other specifications left to default settings.

Bismark coverage files were imported into R using the methylKit *methRead* function (Akalin et al., 2012), including a treatment vector of all four temperature groups and the *L. calcarifer* reference genome. Samples were filtered such that any bases with less than 10 x or more than the 99th percentile of coverage were removed. Coverage was normalised using median scaling and data were merged to extract bases covered by reads in all the samples. Non-variable CpGs were removed using the standard deviation method, keeping only CpGs with a standard deviation of >2 %. Differential methylation was calculated using the methylKit's *calculateDiffMeth* function applying basic overdispersion correction and correcting p-values for multiple testing using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995). Pairwise comparisons between temperature groups were performed by first creating all possible combinations and then applying methylKit's *reorganise* function to subset the data for each. Differential methylation was re-calculated for each pair-wise test. Significantly differentiability methylated CpG sites (DMCs) among all groups (using the full dataset) were identified as those with *q*-values smaller than 0.05 and with percent methylation difference larger than 25 %. These steps were carried out to minimise false positives and increase robustness in the context of limited replication, as is common in WGBS studies constrained by sequencing costs.

2.7. Functional annotation and analysis

Functional annotation was carried out for the DEG and DMCs in the HT vs control comparison, as well as the top 20 overall. BED files for the DMG and DMCs were exported from R, and BEDtools was used to obtain fasta files of the DEGs, and the sequences overlapping or closest to the DMCs (referred to as DMGs, herein). For the top 20 DEGs, CD-HIT was used to cluster the sequences to reduce redundancy. A ray-finned fish-specific database was created by filtering the full National Centre for Biotechnology Information (NCBI) non-redundant protein (nr) database for taxonomic identifier 7898 (Actinopterygii). The *blastx* command was then used to query the database for the DEGs and DMGs. Functional analysis was performed for the DEG and DMGs in the HT vs control comparison. The results were imported into OmicsBox version 3.1.11 (BioBam) for mapping and annotation following the suggested gene ontology (GO) annotation workflow including merging of InterProScan and BLAST GO terms to produce the final annotation and combined pathway analysis to search Kyoto Encyclopaedia of Genes and Genomes (KEGG) and Reactome databases.

2.8. Paired methylation and expression analysis

A fasta file was created containing three ovary-associated (*cyp19a1a, esr1, foxl2)* and three testis-associated (*dmrt1, nr5a2 and amh*) genes for which full length annotated sequences were available. To identify their genomic coordinates, the six genes were mapped to the *L. calcarifer* chromosome-level genome assembly version 3 (Vij et al., 2016) using minimap2 (Li, 2018). For expression data, BEDTools (Quinlan, 2014) *intersect* was then used to identify overlapping genomic coordinates and retrieve MSTRG StringTie IDs that corresponded to each of the mapped sex genes. Using the resultant StringTie sex gene IDs, the DESeq2 *plotCounts* function was used to obtain expression values for the sex genes of interest. Count data was transformed using a log base 10 transformation and modelled using a negative binomial regression using the *glm.nb* function in from the MASS package in R (Ripley et al., 2013). For the methylation data, sex genes were mapped to the *L. calcarifer* genome annotation file (.gff3) and the regions of interest were extracted from the methylKit object using the *selectByOverlap* function. Percentages were converted to proportions and logit transformed to allow subsequent modelling using ANOVA. For both methylation and expression, pair-wise comparisons between temperatures were performed using Tukey's Honest Significant Difference, adjusted for multiple testing using the Benjamini-Hochberg procedure and represented using compact letter display output, all from the *multcomp* package v1.4–8 (Hothorn et al., 2016).

To undertake fine-scale analysis of methylation over the length of *cyp19a1a* and *dmrt1*, the methods and scripts provided by Todd et al. (2019) were followed. Briefly, mapped reads, Bismark coverage files, a GTF file containing the genomic coordinates of *cyp19a1a* and *dmrt1* and the barramundi reference genome and annotation were imported into SeqMonk (www.bioinformatics.babraham.ac.uk/projects/seqmonk/). Typically, the proximal promoter region of a gene is assumed to be located up to 2000 bp upstream unless experimentally proven otherwise (Chen et al., 2018; Hu et al., 2019; Zheng et al., 2017). As such, probes were created to target predicted promoter regions and coding sequences (from −2000 bp to stop codon) of the target sex genes using the feature probe generator, specifying to remove exact duplicates. Running windows of 2 bp with a 2 bp step size were created over the gene and predicted proximal promoter and the SeqMonk's methylation pipeline 'bisulphite methylation over features' analysis was used to generate percent methylation values. Paired mRNA expression values were created similarly, quantifying the 2 bp running window probes by total read count and normalising per million reads. Plots were created in R using the Gvis package (Hahne and Ivanek, 2016).

2.9. Analysis of body size and sex change

To assess the effect of temperature and body size on the probability of feminisation, a Firth logistic regression model was fitted using the logistf package in R, which applies penalized maximum likelihood estimation to reduce small-sample bias in logistic models. To enable comparison of length at sex change between our experimental fish and that of wild-caught barramundi, we also analysed the relationship between length and sex for 8923 individuals collected by Fisheries Queensland Department of Agriculture and Fisheries (DAF) barramundi biological monitoring program (Queensland, 2010) as part of a previous study (Budd et al., 2022b). First, logistic regression models were fitted for length and age frequency distributions for all individuals using the *glm* function in R and specifying the family as binomial. The coefficients were then extracted from the model to calculate the point of inflection of the logistic curve, which represents the point at which the curve changes most rapidly, i.e., the age and length at which most barramundi transition from male to female.

3. Results

3.1. Phenotypic effects of temperature

Following 1 year of temperature exposure, HT barramundi exhibited a skewed distribution toward transitional and female gonad stages, while in all other groups fish were primarily male (Fig. 1A). Control fish largely featured early testes stages (M1 and M2) with predominantly spermatogonia and spermatocytes (Fig. 1C). Similarly, the LT fish mostly possessed M2 stage testes, with one instance of an M3 stage individual as indicated by a predominance of spermatozoa (Fig. 1C). In the FT group, male stages M1 to M3 were primarily observed, as well as one transitioning individual with T2 stage gonads, characterised by degenerating testes tissue concurrent with the emergence of ovarian tissue (Fig. 1B). HT fish displayed the most significant variation in gonad development, with one individual remaining in the male phase (M2), while the majority of individuals (80 %) were in transitional phases (T1 or T2). A single fish in the HT group was also observed to possess vitellogenic oocytes (F2 female stage; Fig. 1C). Histological images for all individuals are provided in Supplemental Fig. S2.

Higher temperature positively influenced fish length and weight, with the HT group achieving the largest size by the conclusion of the 1 year exposure period (Fig. 1B). HT fish were markedly heavier and longer than those from the LT group but did not differ significantly in size from the control group (Fig. 1B). Fish from the LT group were significantly smaller in length compared to the controls, whereas FT fish did not significantly deviate from the controls in either growth metric (Fig. 1B). The HT female was both notably younger and smaller (60 cm; 2 yo) relative to the average size (88 cm) and age (8 yo) of transition for wild-caught barramundi (Fig. 1D, Supplemental Fig. S3).

A firth logistic regression was conducted to examine the effect of treatment on sex, where transitional and female stages were combined into a single “non-male” category and all male stages were combined into a single “male” category ($n = 20$). The model was statistically significant, likelihood ratio $\chi^2(3) = 8.59$, $p = .035$. Compared to the control group, HT individuals had significantly higher odds of being non-male,

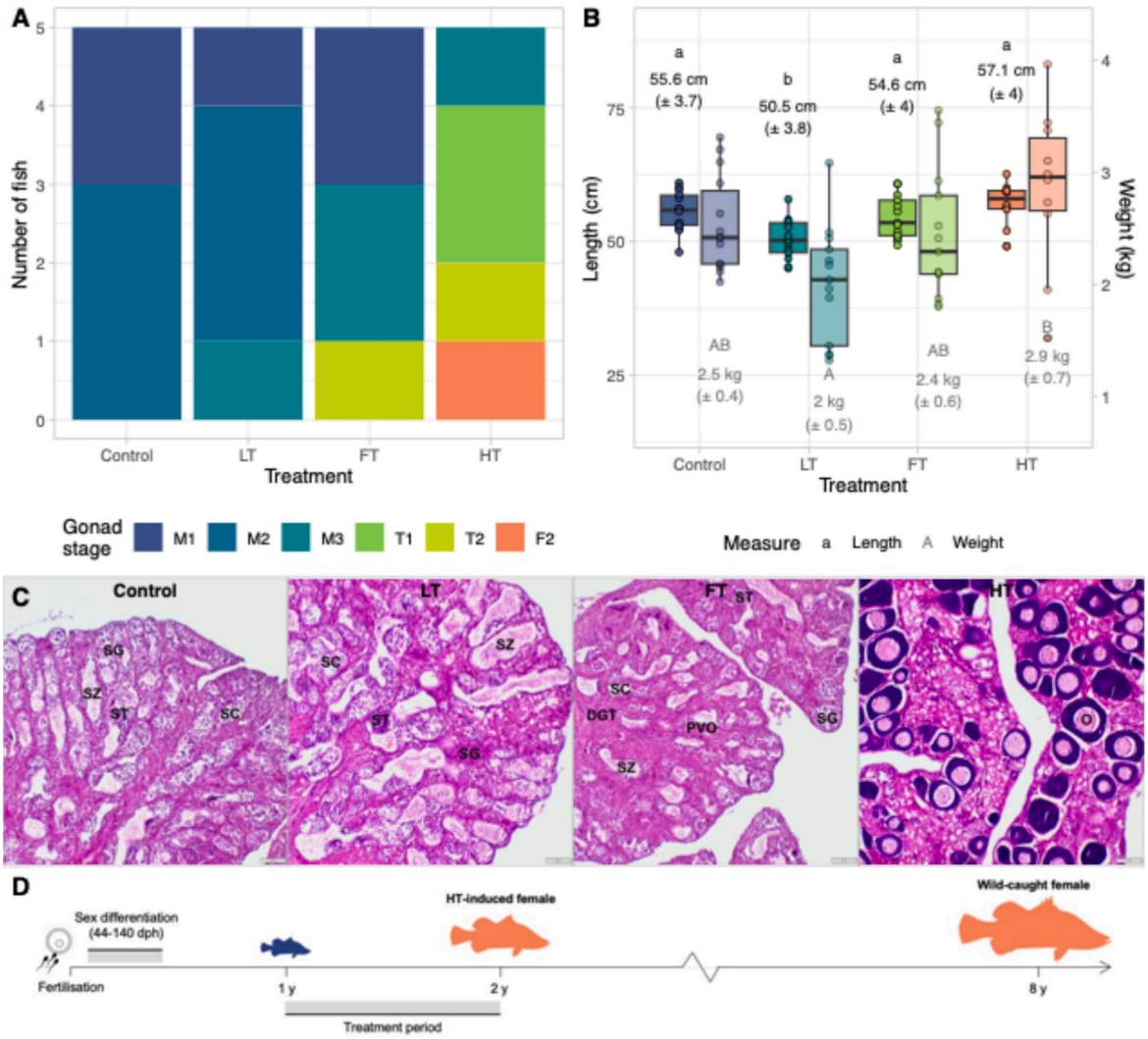


Fig. 1. Phenotypic effect of temperature on barramundi (*Lates calcarifer*) following exposure from 1 to 2 years of age. Control: 28 °C, LT: low temperature; 24 °C, FT: fluctuating temperature 24–34 °C, HT: high temperature; 34 °C. **A.** Gonadal development stage based on Guiguen et al. (1994) where M1-M4 represent male stages, T1-T4 represent transitional stages and F1-F4 represent female stages. **B.** Comparison of length and weight, where letters denote pair-wise comparisons using Tukey’s Honest Significant Difference following ANOVA ($P = .05$) and values show mean with standard deviation. **C.** Hematoxylin and eosin staining on the gonads. Spermatogonia (SG), spermatocytes (SC), spermatids (ST), spermatozoa (SZ), oocyte (O), pre-vitellogenic oocyte (PVO) and degenerating tissue (DGT). Scale bars = 20 μm. **D.** Diagram illustrating the age (2 yo) and size (60 cm) of the HT-induced female and the age (8 yo) and size (88 cm) at which wild-caught barramundi typically transition from male to female. Values for the wild-caught female represent the point of inflection of fitted binomial GLMs for 8923 sexed samples (see Supplemental Fig. S3 for details).

OR = 32.99, 95 % CI [1.90, 5502.93], p = .013. No significant differences were observed for the LT or FT groups. An additional Firth logistic regression tested the effect of treatment and body weight. The overall model was statistically significant, likelihood ratio $\chi^2(4) = 12.16$, p = .016. The effect of treatment was not statistically significant after controlling for weight (all $p > .33$), however the trend remained directionally consistent. Weight was a significant predictor, with higher weight associated with increased odds of being non-male, OR = 1.0025, 95 % CI [1.0000, 1.0146], p = .048.

3.2. Global gene expression and methylation patterns

Genome-wide DNA methylation patterns in barramundi revealed global hypomethylation in the female individual compared to males (Fig. 2). Linkage groups ASB_LG5, ASB_LG17, ASB_LG3, ASB_LG24, and ASB_LG9 exhibited relatively lower methylation levels across all samples, whereas ASB_LG7_2 and ASB_LG14 showed consistently higher average methylation (Fig. 2). However, global DNA methylation levels did not show clear separation among treatments. This indicates that at the whole methylome level, DNA methylation was more strongly influenced by phenotypic sex than by temperature treatment directly.

Analysis of differential gene expression and methylation revealed patterns that were strongly associated with their respective temperature treatments and stages of gonadal development (Fig. 3). Clustering of samples based on all DMCs and the top 20 DEGs resulted in pronounced and early divergence of female and transitional fish, which subsequently formed a distinct group (Fig. 3). Conversely, male staged individuals were grouped more ambiguously (Fig. 3). Within the top 20 DEGs, HT individuals segregated distinctly from those in other groups, with the exception of one transitional fish in the FT group, which aligned with the other transitional individuals from the HT group (Fig. 3). FT individuals clustered less distinctly, highlighting the variability in gonadal stages (ranging from M1 to T2) previously mentioned (Fig. 1, Fig. 3). In contrast, DMC analysis resulted in defined clustering for the HT and FT groups, whereas individuals from the LT and control groups were more diffuse. Overall, patterns of clustering for both DEGs and DMCs consistently show a marked difference between HT-treated fish and all other groups.

Annotation of the top 20 DEGs and DMGs indicated that these genes were primarily involved in cellular sensing and/or signalling, as well as epigenetic and other regulatory pathways (Fig. 3; Supplemental Tables S1 and S2). Two of the DMGs identified were involved in G protein coupled-receptor activity, including *adgra2* (a positive regulator of Wnt signalling, which has been implicated in sexual development) and *celsr1* (predicted to enable calcium ion binding activity) (Fig. 3; Supplemental Table S1). An additional two DMGs were involved in redox and calcium sensing, namely *ndor* (predicted to enable oxide reductase activity by catalysing the transfer of electrons from NADPH to potential redox partners) and *unc79* (a subunit of the NALCN channel that contributes its calcium ion sensitivity of the cell). Other DMGs were involved in epigenetic or other regulatory processes, including *smyd2* (implicated in RNA polymerase II binding and histone methyltransferase activity), *tada1* (subunit of the human STAGA chromatin-modifying multi protein complex) as well as *hox10a* and *lbw* (regulators of the Wnt signalling pathway). Finally, two DMGs were involved in degeneration and regeneration, namely *znf503* and *spire1* (involved in G1 to G0 transition of the cell and positive regulation of double-strand break repair, respectively) (Fig. 3).

Within the top 20 DEGs, genes with regulatory roles were common (Fig. 3; Supplemental Table S2). These included: *znf271* (regulator of transcription), *srrm1* and *ddx17* (with functions in mRNA binding and processing, including splicing) as well as *naca* (involved in binding nascent proteins). Three zona pellucida proteins *zpd, zp3f.2, zpxc* were also differentially expressed, which are predicted to enable sperm binding (Fig. 3, Supplemental Table S2). Two genes with specific epigenetic regulatory functions were among the top 20 DEGs, namely

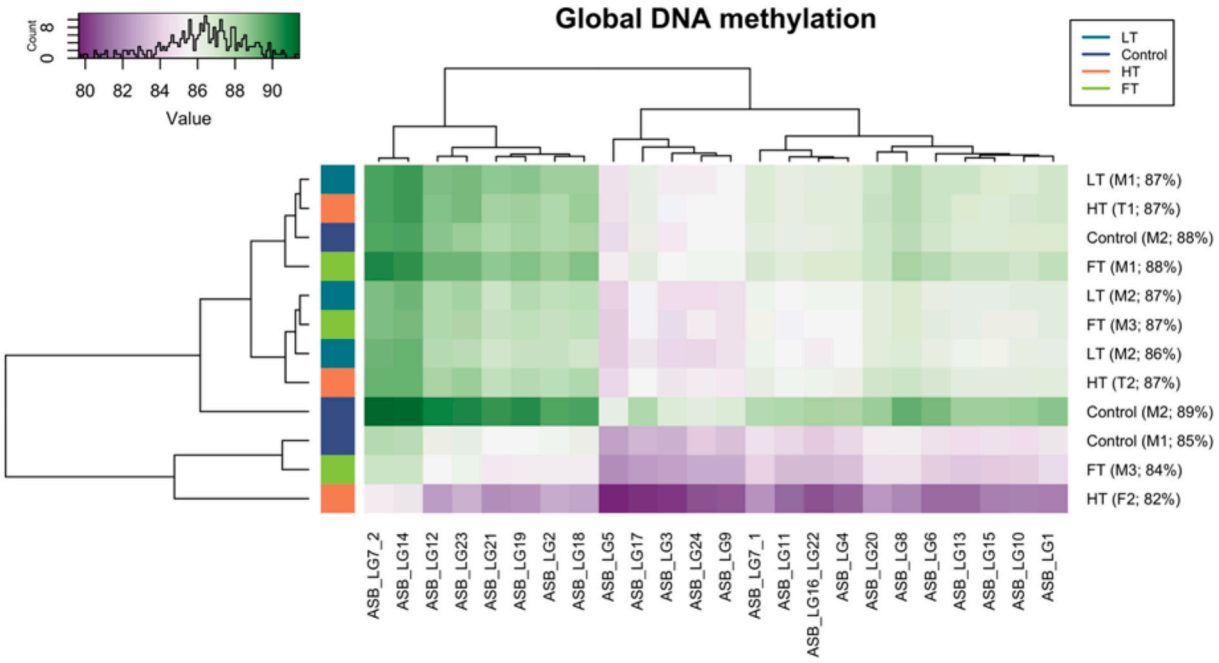


Fig. 2. Heatmap of DNA methylation across chromosomes among treatments and gonadal development stages in barramundi (*Lates calcarifer*). Chromosomes (columns) are labelled as ASB_LG#, where 'ASB' denotes Asian seabass, and 'LG#' refers to linkage groups derived from genetic linkage mapping (Vij et al., 2016). Samples (rows) are labelled by treatment, and in brackets by gonadal development stage, followed by the total percent methylation across all chromosomes, i.e., whole-genome methylation percentage. M1–M4 represent male stages, T1–T4 represent transitional stages, and F1–F4 represent female stages, as described by Guiguen et al. (1994). Tissue samples were collected from barramundi gonads following temperature exposure between 1 and 2 years of age. Row colours indicate different treatments (Control: 28 °C, LT: low temperature; 24 °C, FT: fluctuating temperature 24–34 °C, HT: high temperature; 34 °C). Hierarchical clustering was performed using Euclidean distance to calculate pairwise similarities and complete linkage for agglomeration, grouping both rows (samples) and columns (chromosomes) based on the most dissimilar elements within each cluster.

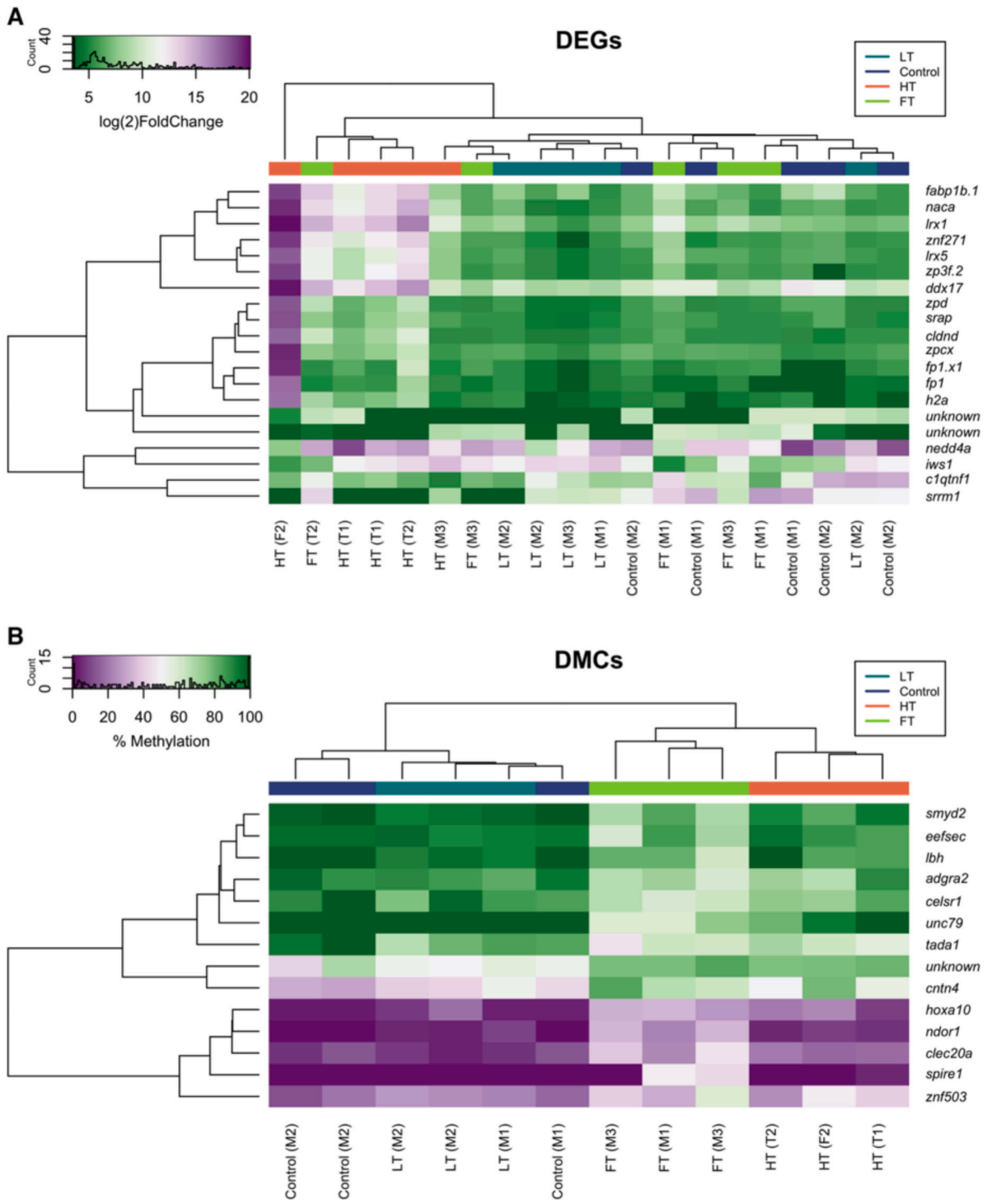


Fig. 3. Heatmap of the top 20 differentially expressed genes (DEGs; **A**) and all differentially methylated CpG sites (DMCs; **B**) among treatments. Tissue was obtained from barramundi (*Lates calcarifer*) gonads following temperature exposure from 1 to 2 years of age. DEGs were annotated by performing blastx sequence searches against NCBI's non-redundant (nr) protein database and retaining the top hit for each gene and are labelled accordingly. For DMCs, gene sequences either overlapping or closest to the CpG site were used. NB: the exact identity and function of these sequences is not yet known. Control: 28 °C, LT: low temperature; 24 °C, FT: fluctuating temperature 24–34 °C, HT: high temperature; 34 °C. Heatmaps use Euclidean to obtain distance matrices and complete agglomeration for clustering of both rows and columns. Bracketed information indicates gonadal development stage based on Guiguen et al. (1994) where M1-M4 represent male stages, T1-T4 represent transitional stages and F1-F4 represent female stages.

h2a (proteins involved in the structure of chromatin in eukaryotes) and *iws1* (involved in regulation of histone modification). Several DEGs encoding membrane proteins with potential roles in cellular sensing were also identified, namely *cldnd* and *srap*, as well as membrane receptor *nedd4a* (enables sodium channel inhibitor activity). Finally, *c1qtnf1* (involved in positive regulation of signal transduction), was also identified (Fig. 3, Supplemental Table S2).

3.3. Functional results

A total of 8257 DEGs and 50 DMCs were identified between HT and control groups (Supplemental Fig. S4). Overlapping Gene Ontology (GO) terms were predominantly categorized under Molecular Functions, with an emphasis on various binding activities including ATP, protein, DNA, calcium, metal, and zinc ion binding (Fig. 4A). Notably, two of these GO terms were specifically attributed to RNA polymerase II DNA-binding activities, either associated with transcription factor activity, or cis-regulatory region specificity (Fig. 4A). For Cellular Components, both DEGs and DMGs were predominantly assigned to structures including the cell membrane, nucleus, plasma membrane, and cytoplasm (Fig. 4B). Biological Processes implicated included the regulation of transcription by RNA polymerase II, signal transduction, and anatomical structure development (Fig. 4A). Reactome pathway analysis highlighted that DEGs were primarily involved in metabolic pathways, whereas DMGs showed a heightened association with signal transduction pathways (Fig. 4B). Additional Reactome pathways

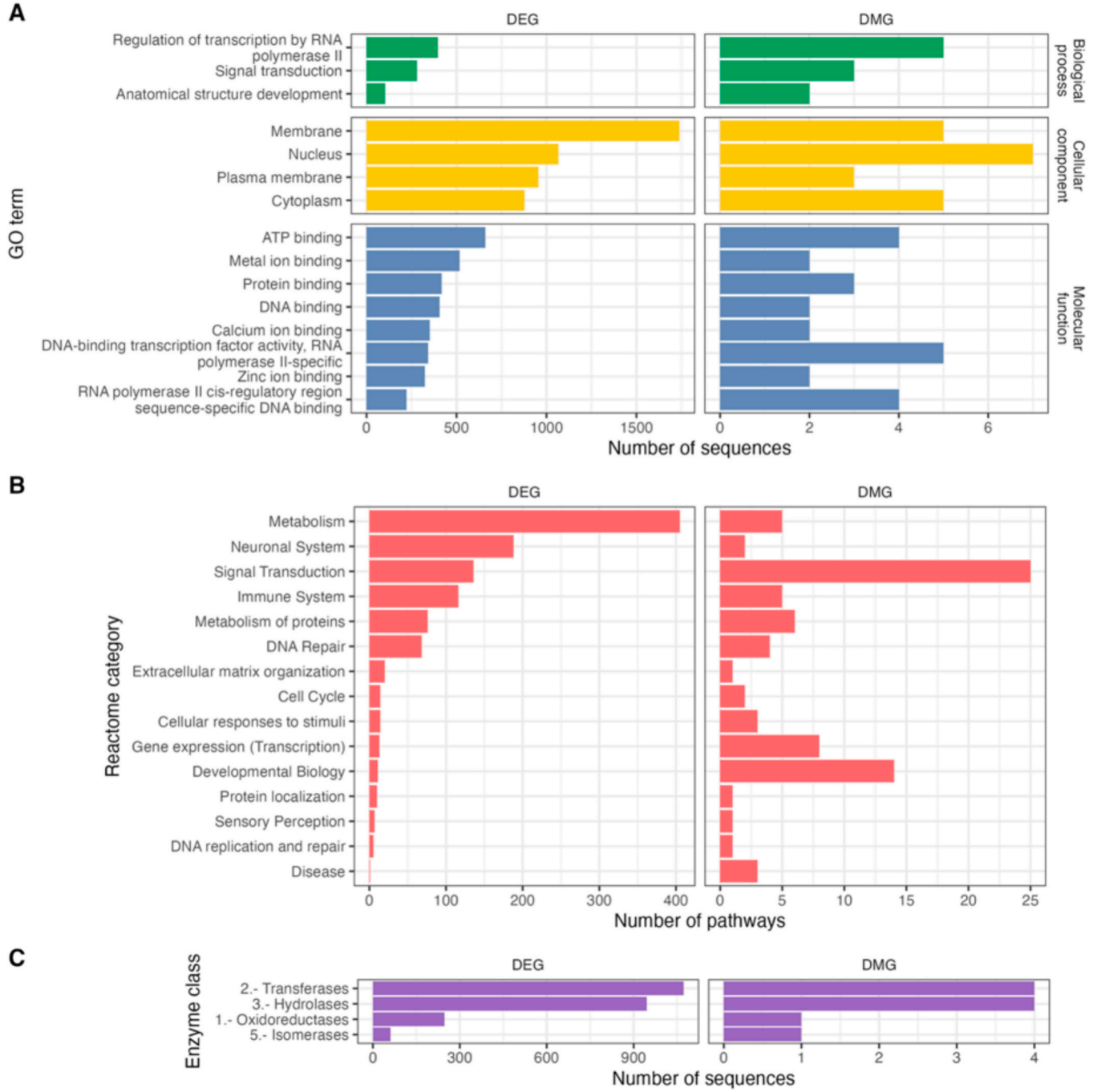


Fig. 4. Comparative functional analysis of differentially expressed genes (DEGs) and differentially methylated genes (DMGs) in *Lates calcarifer* gonads subject to high temperature exposure (34 °C) from 1 to 2 years of age. **A.** Shared Gene Ontology (GO) terms are categorized into Biological Process, Molecular Function, and Cellular Component. Bars represent the number of sequences associated with each GO term. **B.** Shared pathway categories based on Reactome analysis, where bars depict the number of pathways associated with each category **C.** Enzyme class distribution, were bars show the number of sequences corresponding to each enzyme class. All results are shown for DEGs (left) and DMGs (right). The figure illustrates only those functions that were common to both DEGs and DMGs, highlighting the potential molecular intersections of gene expression regulation and DNA methylation in the gonads in response to temperature exposure.

implicated included metabolism of proteins, cellular response to stimuli, gene expression, and developmental biology (Fig. 4B). Enzyme classes common to both DEG and DMGs included transferases, hydrolases, oxidoreductases, and isomerases (Fig. 4C).

3.4. DNA methylation and expression in sex genes

Targeted examination of six annotated sex-related genes highlighted differential expression patterns across temperatures. Specifically, HT fish exhibited an upregulation of all three genes associated with ovarian

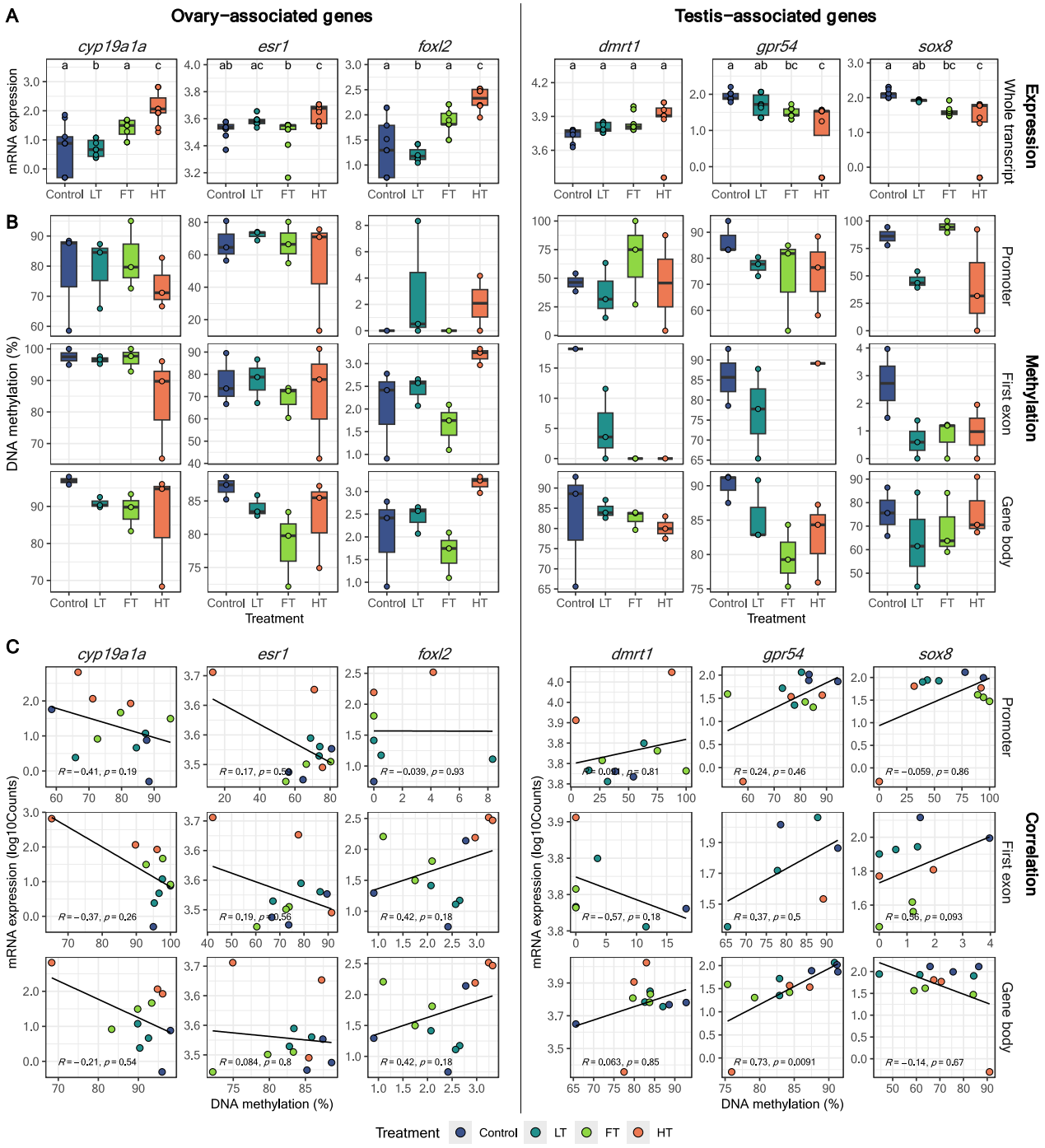


Fig. 5. Gene expression and DNA methylation in *Lates calcarifer* gonads following temperature exposure from 1 to 2 years of age. **A.** Expression plots show upregulation of ovary-associated genes and downregulation of two testis-associated genes in the high temperature (HT) group. **B.** No significant difference in average DNA methylation between treatment groups or regions (promoter, first exon, or gene body) was observed **C.** A consistent inverse correlation between methylation and expression was not apparent. Data is shown for three ovary-associated (top three rows) and three testis-associated (bottom three rows) sex genes. Gene expression data ($n = 5$ fish per group) are presented as normalised, log-transformed counts. Methylation data ($n = 3$ fish per group) were obtained from whole genome bisulphite sequencing and represent average percent methylation. Letters on expression plots denote pair-wise comparisons using Tukey's Honest Significant Difference corrected for false discovery rate following negative binomial generalised linear modelling. Methylation differences were calculated by ANOVA on logit transformed data, where the absence of labelling indicates no significant differences were observed. Relationships between expression and methylation are examined using Spearman correlations.

function—*cyp19a1a*, *esr1*, and *foxl2*. Accordingly, HT fish also showed a reduction in expression for two of the three genes typically associated with male development (*gpr54* and *sox8*) (Fig. 5A). In contrast, LT fish displayed the opposite pattern, with a decrease in the expression of *cyp19a1a* and *foxl2* and an increase in *gpr54* and *sox8* relative to the HT group (Fig. 5A).

WBGS analysis did not reveal significant differences in average DNA methylation profiles across temperatures for the promoter regions, first exons, or gene bodies of any of the six annotated sex genes (Fig. 5B). Nevertheless, a non-significant trend toward decreased gene body methylation was evident for *cyp19a1a* and *dmrt1* in the HT group (Fig. 5B). Correlation analyses revealed a positive correlation for gene body methylation in *gpr54* (Fig. 5C). No significant inverse relationships between methylation and expression were observed, although the general trend of decreasing expression with increasing methylation was apparent for most regions in *cyp19a1a* and *esr1* (Fig. 5C). Correlations for other genes and regions displayed no significant patterns, with an equal distribution of negative and positive correlation coefficients (Fig. 5C). It should be noted that although an inverse correlation between DNA methylation and expression of the sex genes was not seen (Fig. 5), in general, DMCs exhibited decreased methylation and DEGs exhibited increased expression between HT and control groups (Fig. S5).

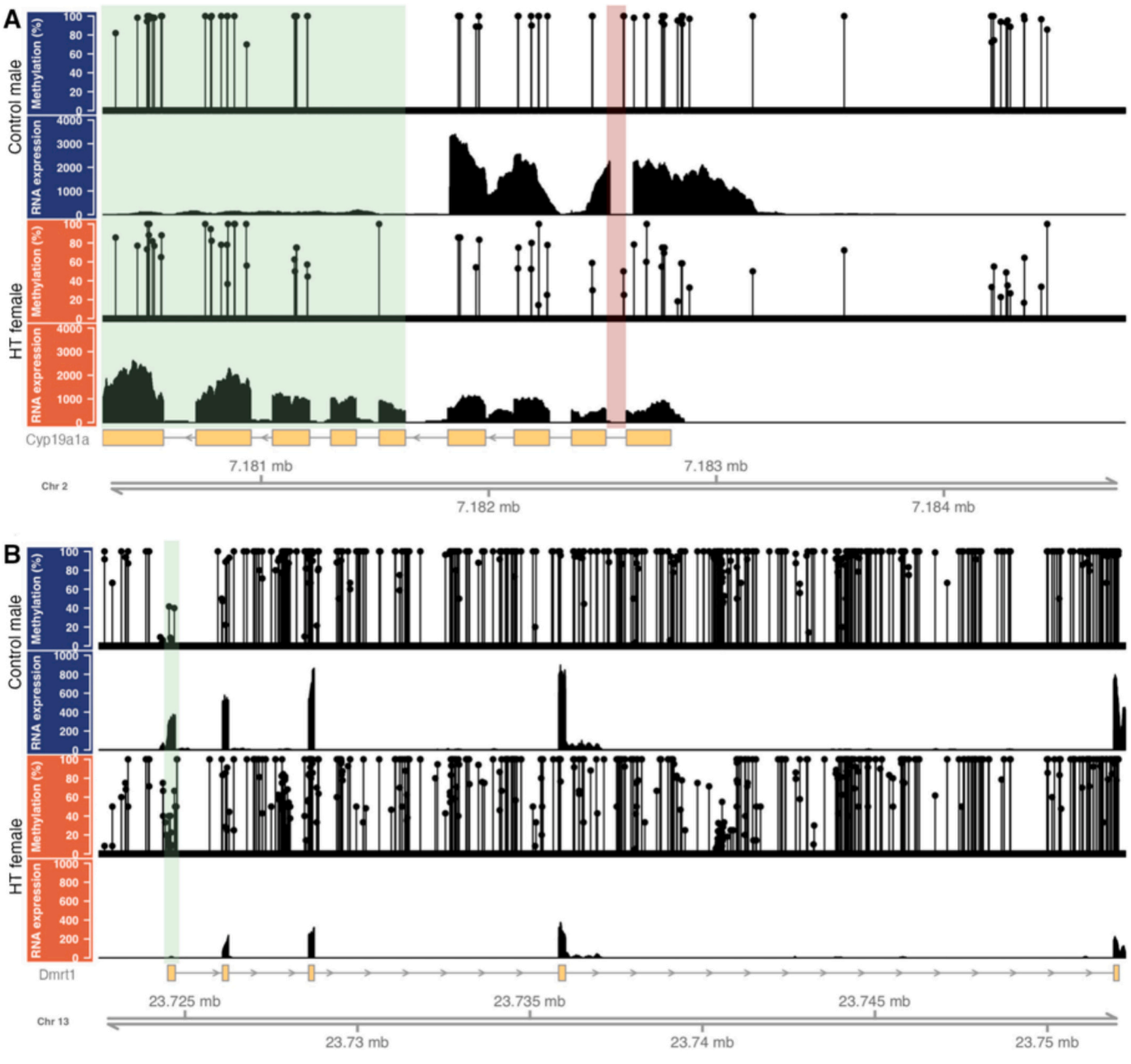


Fig. 6. Base-pair resolution DNA methylation and RNA expression highlighting alternative splicing in *Lates calcarifer* genes *cyp19a1a* (A) and *dmrt1* (B) in male and female fish following temperature exposure from 1 to 2 years of age. Presented DNA methylation and RNA expression levels for one control-treated male (28 °C; blue highlight) and one high temperature (HT) treated female (34 °C; orange highlight). Vertical bars show the methylation percentage at CpG sites (upper histograms) and gene expression normalised counts (lower histograms) within 2 bp windows. The green shaded regions highlight exons exhibiting alternative splicing in both genes and the red shaded region highlights differential expression of exon 1 and 2 in *cyp19a1a*. Gene structure is depicted with yellow boxes for exons, and grey lines for introns. Grey arrows indicate the transcriptional direction. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. Single base-pair resolution analysis of dmrt1 and cyp19a1a

Base-pair resolution analyses across the length of sex-determining genes *cyp19a1a*, associated with ovarian function, and *dmrt1*, linked to testicular differentiation, revealed variation in methylation and expression patterns and evidence of alternative splicing between male and female fish (Fig. 6, Supplemental Figs. S6 and S7). For example, mRNA expression was relatively uniform across all exons of *cyp19a1a* in the female, whereas males showed a marked overrepresentation of mRNA transcripts within the initial four exons and an absence of expression in exons 5–9 (Fig. 6). Additionally in males, expression was identified within the intronic region separating exons 3 and 4 and was reduced in regions of exon 1 and 2 producing a known short-form mRNA *cyp19a1a* sequence. Expression of *dmrt1* was present in the female, contrary to anticipated trends for a gene typically expressed in testes. However, there was a complete lack of expression in exon 1 for the female, indicative of alternative splicing (Fig. 6). Exon 1 of *dmrt1* contains the active and highly conserved DNA-binding motif (DM domain). Methylation levels were comparatively elevated in *dmrt1* exon 1 of the female, as well as at CpGs situated at −122 and −130 base pairs, close to potential transcription factor binding sites for E2F and SP1 (Supplemental Fig. S8). Transitional stage fish generally paralleled male methylation and expression patterns (Fig. S6, Supplemental Fig. S7). However, all HT treated fish (female and transitional) demonstrated a trend of reduced methylation in the exons of *cyp19a1a*, as well as within a cluster of CpG sites located in the upstream proximal promoter region (Fig. 6; Supplemental Figs. S6 and S7). Overall, although there were some differences in methylation and expression observed between temperatures, alternative splicing was only observed between the sexes.

4. Discussion

Here, 1-year-old barramundi were exposed to four different temperature regimes for a period of 1 year and the influence of temperature on sequential sex change, gonadal DNA methylation and gene expression was examined. High temperature exposure led to an increased proportion of transitional and female individuals, accompanied by downregulation of testes-associated and upregulation of ovary-associated sex genes. Although the number of individuals assessed using histological examination and WGBS was contrained, aconsistent pattern of feminisation in the HT group—supported by gene expression and methylation data—strengthens confidence in these observations. Both global and gene-specific differential DNA methylation were observed between male and female individuals, accompanied by alternative splicing in ovary-associated *cyp19a1a* and testes-associated *dmrt1*. Among treatments, an inverse relationship between methylation and expression was observed for *cyp19a1a* and *esr1*, although the correlations were not significant. Functional analyses and annotation of DEGs and DMGs among treatments revealed a predominance of genes and biological functions associated with cellular sensing and signalling pathways, instead of those directly involved in DNA methylation, or other epigenetic and/or regulatory mechanisms as was expected. The results suggest that temperature most strongly affects the expression and DNA methylation of genes involved in cellular signalling pathways, and that the downstream completion of transition from male to female involves differential methylation and alternative splicing of sex-associated genes, including *cyp19a1a* and *dmrt1*.

4.1. Effect of temperature on phenotypic sex

High temperature exposure resulted in largely transitional and female fish, whereas low and control temperatures resulted in exclusively male fish after 1 year of temperature exposure (at 2 years of age). This morphological result was supported by DNA methylation and gene expression profiles, whereby transitional and female fish (largely from the high temperature group) were found to cluster together based on DMC and DEG profiles. There was also significant upregulation of ovary-associated genes and downregulation of testis-associated genes in the high temperature group. Our results are consistent with previous research which showed that high temperature (31 °C) induced both transitional and female 18 month old barramundi following 3.5 months of temperature exposure (Athauda et al., 2012). Here, we showed that wild-caught barramundi typically transition at 8 years and 88 cm, whereas all experimental individuals were under 65 cm and 2 years old, demonstrating a substantial reduction in the time taken to reach male-to-female transition. These results indicate that, in contrast to gonochoristic species, the gonads of barramundi remain sensitive to temperature exposure well beyond the period of primary sexual differentiation (as males), which occurs from approximately 40 to 140 days post hatch (Banh et al., 2017).

The finding of high temperature leading to early female development in barramundi is also in contrast to the pattern known to most gonochoristic fish (Geffroy and Wedekind, 2020; Ospina-Alvarez and Piferrer, 2008), where high-temperature exposure during primary sex differentiation typically leads to decreased *cyp19a1a* expression, and subsequent testis development (e.g., Hattori et al., 2009; Hayashi et al., 2010). However, this pattern is disrupted in species where sex differentiation is strongly linked to size and/or growth (Ospina-Alvarez and Piferrer, 2008). In barramundi, increased juvenile growth rate has been shown to influence the timing of male-to-female sex change (Roberts et al., 2021), suggesting that high temperature could promote feminisation indirectly through accelerated growth. Consistent with this, treatment effects were not statistically significant when controlling for weight; however, the effect of high temperature remained directionally consistent. Moreover, Although HT fish were larger than LT fish, their size did not differ significantly from controls, despite exhibiting marked differences in gene expression and DNA methylation. Given that the optimal thermal range for metabolic efficiency in barramundi is 28–32 °C, the reduced size observed at 24 °C is more likely a result of suppressed metabolic activity and reduced feed intake, rather than enhanced growth at 34 °C, where signs of metabolic stress begin to emerge (Katersky and Carter, 2007). These findings suggests that while growth may contribute to temperature-induced feminisation, it cannot fully explain the observed effects. Instead, our results point to the involvement of additional temperature-sensitive regulatory mechanisms in barramundi. Such mechanisms may resemble those present in TSD reptiles for which high temperature similarly induces feminisation (see section 4.5).

4.2. Sex-specific global DNA methylation

Global hypomethylation in female barramundi is consistent with previous observations in tongue sole (*Cynoglossus semilaevis*), where females exhibited lower DNA methylation levels in gonadal tissues compared to males. For tongue sole, this comparison included both genetic (ZZ) and temperature-derived (ZW) ‘pseudo’ males and demonstrates that sex correlates with epigenetic but not genetic differences (Shao et al., 2014). This consistency in methylation status of males and females across species and irrespective of temperature treatment points to the existence of a conserved global sex-specific epigenetic mechanism. The absence of global methylation differences between male and transitional stages in our study may be attributed to the dynamic nature of gonadal tissue during these phases. Transitional stages encompass a mix of cell types at various stages of differentiation, and male gonads at different stages of spermatogenesis, potentially masking distinct methylation patterns. Similar challenges have been noted in previous research, where the heterogeneity of gonadal tissue complicates the detection of clear epigenetic markers in hermaphrodite fish (Ortega-Recalde et al., 2020). Future studies employing the use of single cell sequencing will help to elucidate cell-specific patterns of gonadal DNA methylation during transitional stages.

4.3. Relationship between DNA methylation and mRNA expression

The present analyses revealed a general trend of increased gene expression and decreased DNA methylation between HT and control groups but did not identify a consistent inverse correlation among the six annotated sex-associated genes examined. The major biological consequence of DNA methylation is thought to be gene silencing (Colot and Rossignol, 1999) and there are reports of inverse relationships between promoter, first exon and first intron methylation (Anastasiadi et al., 2018; Brenet et al., 2011; Kuroda et al., 2009) and positive relationships with gene body methylation (Ball et al., 2009). Nevertheless, there are many exceptions to these general trends (Suzuki and Bird, 2008) and the absence of an inverse correlation between methylation and expression in sex-related genes in fish is commonly reported. For example, sex-related genes in temperature sex-reversed tongue sole (*C. semilaevis*) showed a variety of relationships between expression and gene body methylation, including positive, negative or no change (Shao et al., 2014). Similarly, previous research in barramundi showed upregulated promoter methylation and gene expression in *amh*, as well as no relationship between promoter methylation and gene expression in *cyp19a1a* (Domingos et al., 2018). Here a single inverse correlation was observed between methylation in the first exon of *cyp19a1a*, but not for any other sex gene or region (i.e., promoter or gene body). However, we observed an overall decrease in methylation and increase in expression between HT and control groups. These results highlight that although there are general trends, the relationship between methylation and expression is unique to many genes and may be obscured by the presence of multiple transcripts.

4.4. Alternative splicing in cyp19a1a and dmrt1

Base-pair resolution analysis of DNA methylation and expression along the length of *cyp19a1a* revealed that HT-treated fish exhibited lower *cyp19a1a* methylation in exonic regions, but also in a cluster of CpG sites in the upstream promoter region. Similar analysis in bluehead wrasse (*Thalassoma bifasciatum*) revealed hypermethylation in males compared to females, most notably in a CpG island close to the transcription start site (Todd et al., 2019). In the present study, transcripts from *cyp19a1a* in male and transitional fish were heavily overrepresented in the first four exons and introns but absent for the remaining coding sequence (exons 5 to 9). Only in the female fish was the complete gene expressed. For *dmrt1*, the female individual lacked expression of the first exon almost entirely, which was also heavily methylated. Notably, this exon contains the active DM domain, likely rendering the gene non-functional in females (Zarkower and Murphy, 2021). This finding is supported by our previous work which showed that wild-caught female barramundi lack the first exon of *dmrt1* (Domingos et al., 2018). In addition to altered patterns of gene expression, DNA methylation has a known role in the regulation of splicing (Maor et al., 2015) and may lead to the sex specific splice dynamics observed in barramundi and other teleosts (e.g., Guo et al., 2005; He et al., 2003; Shao et al., 2014; Yu et al., 2003; Zhang et al., 2008). Because methylation and expression patterns were similar in the control males and HT transitional fish (Supplemental Fig. S6, Supplemental Fig. S7), it is likely that temperature does not directly affect alternative splicing, and that this is a downstream effect of sex change. However, in the absence of functional studies, it is challenging to determine causative factors.

4.5. Putative upstream mechanisms of high temperature-induced feminisation

While DNA methylation has been identified as the mechanism linking changes in environmental temperature to differences in phenotypic sex in fish (Ellison et al., 2015; Navarro-Martín et al., 2011; Shao et al., 2014; Wang et al., 2019b), as well as many reptiles (Matsumoto et al., 2013; Parrott et al., 2014), specifically how temperature affects DNA methylation is not known. Temperature has been proposed to elicit an effect on methylation through the activity of DNA methyltransferases (DNMTs); temperature-sensitive enzymes responsible for the physical transfer of methyl groups onto nucleic acids (Navarro-Martín et al., 2011). In this study, GO terms associated with genes differentially methylated and expressed between HT and control treated barramundi did not reveal a marked overrepresentation in DNA methylation or methyltransferase-related gene functions. Additionally, none of the top 20 DEGs or DMGs among temperature groups were associated with DNA methyltransferases. These findings indicate that the observed DNA methylation changes in barramundi are unlikely to result directly from the effect of temperature on methyltransferase expression.

Elevated temperature is also known to induce heat shock proteins, which may act as upstream temperature sensors in sex determination pathways. For example, in tongue sole, high temperature treatment has been shown to upregulate several HSP family members, including hsp 70 and hsp90, in sex-reversed males (Wang et al., 2019a). Because hsp90β is upregulated in olive flounder testis, and hsp70 is associated with testis development in tilapia, these have been identified as candidate temperature sensors for further investigation (Fan et al., 2014; Tao et al., 2018). However, the present analysis did not identify any HSPs among differentially expressed or methylated genes, nor were any HSP-related GO terms (e.g., *response to heat*, *response to stress*, *protein folding*) enriched, providing no evidence for this mechanism in barramundi.

High temperature exposure has also been shown to induce masculinisation in several teleost species, including medaka and pejerrey, through upregulation of cortisol biosynthesis, increased expression of androgen-producing enzymes, and suppression of aromatase activity (Hattori et al., 2009; Hayashi et al., 2010). Cortisol-associated masculinisation also occurs during protogynous sex change in bluehead wrasse (Todd et al., 2019). While we did not measure cortisol directly, our findings contrast with these patterns: elevated temperature in barramundi was associated with upregulation of gonadal *cyp19a1a* (aromatase), *esr1*, and *foxl2*, along with downregulation of *sox8* and *gpr54*, and a corresponding increase in the proportion of female and transitional fish. The fluctuating treatment may have introduced a different form of thermal stress; however, this treatment did not show a consistently strong femininizing or masculinising effect. Taken together, our observations indicate that high-temperature-induced feminisation in barramundi operates through mechanisms distinct from the cortisol-mediated masculinisation observed in other fish species.

Alternatively, it has been proposed that cells can sense environmental temperature through changes in calcium and redox balance, which initiates downstream epigenetic and transcriptional responses that influence sex differentiation in temperature sex-determined (TSD) reptiles (Ahn and Thiele, 2003; Castelli et al., 2020; Singh et al., 2020). Notably, evidence for changes in calcium and redox balance have been observed in the absence of increased cortisol (Mukai et al., 2022) and in species for which high temperature induces feminisation (Whiteley et al., 2021). In the present research, many differentially methylated and expressed genes in barramundi exposed to high temperature were associated with GO terms related to signal transduction, transcriptional regulation and ion binding, including calcium. Reactome analysis similarly highlighted enrichment in signal transduction, responses to stimuli and metabolic pathways known to modulate cellular redox status (Gong et al., 2020). Furthermore, enzyme classifications of differentially expressed and methylated genes were dominated by hydrolases and transferases, key mediators of intracellular signal transduction, as well as oxidoreductases, which are directly involved in redox signalling (Rack et al., 2020). These data support the hypothesis that redox-mediated signals may be driving temperature-driven sex differentiation across vertebrates.

Additional support for the CaRe hypothesis in barramundi emerged from the annotation of the top 20 differentially methylated and expressed genes. A minority of these were involved in epigenetic (e.g.,

histone modification) or regulatory pathways, but the majority had putative roles in cellular sensing. For example in humans, *unc-79* encodes a protein that contributes to NALCN calcium channel sensitivity, and *ndor1*, a redox enzyme closely related to cytochrome P450 reductase (*por*), which is an obligate electron donor for steroidogenic enzymes, including *cyp19a1* (Banci et al., 2013). Other genes identified included *eefsec*, predicted to be involved in selenocystine incorporation critical for redox maintenance (Labunskyy et al., 2014) and *clnd4*, encoding a transmembrane protein potentially involved in ion regulation (Berselli et al., 2022). Leucine-rich repeat extension proteins, known in plants to function in perceiving extracellular signals and relaying this information to the cytoplasm (Herger et al., 2019), were also found to be differentially expressed. Together these findings support a role for cellular calcium and redox signalling pathways as transducers of environmental temperature into sex-specific gene regulation in barramundi (Castelli et al., 2020).

4.6. Future directions

Although this study is largely observational, the results suggest that understanding how temperature exerts an effect on sex in barramundi and other fish species requires investigating beyond the repressive relationship between DNA methylation and gene expression. Future research should explore additional roles of DNA methylation, other regulatory mechanisms, and cellular signalling dynamics. Theories like the calcium and redox hypothesis offer improved understanding of the genes and cellular functions that may be involved in temperature-induced sex change and reversal, and can help direct future functional studies (Castelli et al., 2020). For example, calcium channels are known to respond to pharmacological compounds, which may inhibit the effect of temperature on the cell (Herger et al., 2019). In corals, it has been shown that suppressing the supply of extracellular calcium by chelation with EGTA can deplete active calcium signalling and inhibit the loss of zooxanthellae in response to high temperatures (i.e., bleaching) (Huang et al., 1998). Although similar studies have not been conducted in fish, there is evidence to show that oxidative stress and differential calcium regulation are involved in sex change in bluehead wrasse (Todd et al., 2017), and that genes involved in ion transport processes are upregulated in sex-reversed male Nile tilapia (Wang et al., 2019b). A deeper understanding of how exogenous stimuli influence sex determination could lead to more effective sex control in aquaculture.

The present results corroborate previous work suggesting that temperature may provide a viable method of sex control for barramundi aquaculture (Athauda and Anderson, 2014; Budd et al., 2022a). Here, culture at high temperatures resulted in one female and three transitional fish, corresponding to a frequency of 20 % female and 60 % transitional individuals. Increasing numbers to a commercial scale could validate temperature as an effective method for sex control in aquaculture. This is particularly relevant if the effects of temperature on sex are stable in offspring, meaning that temperature exposure would not need to be repeated for newly spawned broodfish. Transgenerational effects of temperature on phenotypic sex have previously been observed in tongue sole, where high temperatures resulted in sex reversal of both the parent and offspring (to which no temperature exposure was applied) (Shao et al., 2014). Future work should look to understand if the effects of temperature on barramundi are similarly retained in offspring, leading to early development of females in the absence of temperature exposure. The work could further be extended to examine possible cumulative effects of temperature when applied to consecutive generations, as transgenerational effects have been observed in other fish species (Pierron et al., 2021; Shao et al., 2014; Valdivieso et al., 2020). Alternatively, breeders could select for temperature sensitive families which may allow for reduced temperatures or exposure periods (Baroiller and d'Cotta, 2016; Baroiller et al., 2009; Saillant et al., 2002; Wessels et al., 2017). Such initiatives could ultimately establish temperature as an economically and environmentally sustainable method for sex control in aquaculture.

5. Conclusion

This study demonstrates that elevated temperature induces feminisation in barramundi through coordinated physiological, epigenetic and transcriptional changes, providing insight into the molecular basis of sex change in a protandrous fish. Understanding these environmentally responsive mechanisms is key to improving sex control in aquaculture, informing fisheries management and advancing our knowledge of sexual development across vertebrates. Our findings support the idea that sequential hermaphrodites retain prolonged sensitivity to environmental cues and provide evidence to support the existence of evolutionary conserved cellular mechanisms that detect and transduce environmental temperature. Reproductive plasticity may enable climate-driven shifts in sex ratios and population structure - effects that are already evident across environmental gradients for this species (Budd et al., 2022b). In aquaculture, these insights highlight the potential for temperature-based sex control in barramundi and other commercially important species.

Animal and human rights statement

The authors declare that all applicable international, national, and/or institutional guidelines for sampling, care, and experimental use of organisms for the study have been followed and all necessary approvals have been obtained.

CRediT authorship contribution statement

Alyssa M. Budd: Validation, Methodology, Formal analysis, Conceptualization, Writing – original draft, Investigation, Data curation, Writing – review & editing, Visualization, Project administration. **Roger Huerlimann:** Software, Formal analysis, Writing – review & editing, Methodology. **Jarrod L. Guppy:** Writing – review & editing, Data curation, Investigation. **Ricardo C.C. Pinto:** Writing – review & editing, Investigation. **Jose A. Domingos:** Supervision, Funding acquisition, Writing – review & editing, Investigation, Conceptualization. **Dean R. Jerry:** Writing – review & editing, Project administration, Funding acquisition, Supervision, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Dean Jerry reports financial support was provided by Australian Research Council. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All code used for the analyses and figure generation can be found at github.com/dr-budd/adult_meth. Raw sequence data are available in the NCBI Sequence Read Archive under BioProject accession PRJNA1263116, with BioSample accessions SAMN48511396-SAMN48511415. SRA accessions for RNA-seq data are SRR33638345 - SRR33638356, and for WGBS data are SRR33696300 - SRR33696311.

Acknowledgements

This project was funded through Australian Research Council Linkage scheme Project LP130100007. The experimental component could not have been completed without the contribution of James Cook University staff, students and volunteers including Jonathan Bester, Callum Bower, Tahi Jackson Fu, Julie Goldsbury, Melissa Joyce, Shannon

Kjeldsen, Nicholas Langley, Teva Lechat, Adrien Marc, Aiden Mellor, Olivia Mei Lin Lee, Joana Ferreira Mendes, Maria Nayfa, Anna Purcell, Kessia Virah-Sawmy, Daniel Small, Hillary Smith, David Vaughn and Julian Wilson who either helped to rear and sample the fish. Special thanks to Valentin Thepot for leading the successful culture of the barramundi used in this experiment and to the staff of the Marine and Aquaculture Research Facility Unit for their assistance throughout the experiment. Additional thanks to Lit Chien Cheah for his histology expertise as well as molecular laboratory manager Carolyn Smith-Keune for sharing her knowledge and her dedication to a safe and productive work environment. Our gratitude extends to Queensland Facility for Advanced Bioinformatics (QFAB), Gareth Price, Mike Thang and Anne Bernard, and to Oscar Ortega Recalde for sharing his code and expertise. We extend our sincere thanks to our sequencing partner the Australian Genome Research Facility (AGRF) and industry partner, Mainstream Aquaculture, for their support, which significantly enhanced the scope and impact of our project. Thank you to Julie Robins, Olivia Whybird, and Wayne Hagedoorn from the Queensland Department of Agriculture and Fisheries who provided the field data that facilitated comparison to our cultured animals. We additionally thank Paul O'Brien and David Jones for reviewing earlier drafts of the manuscript, and Nick Wade for providing mentorship throughout the project. Finally, our sincere appreciation goes to the anonymous reviewers who contribute their time and expertise to improve the work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2025.742882.

References

Ahn, S.-G., Thiele, D.J., 2003. Redox regulation of mammalian heat shock factor 1 is essential for *Hsp* gene activation and protection from stress. Genes Dev. 17 (4), 516–528.

Akalin, A., Kormaksson, M., Li, S., Garrett-Bakelman, F.E., Figueroa, M.E., Melnick, A., Mason, C.E., 2012. methylKit: a comprehensive R package for the analysis of genome-wide DNA methylation profiles. Genome Biol. 13 (10), R87. https://doi.org/10.1186/gb-2012-13-10-r87.

Anastasiadi, D., Esteve-Codina, A., Piferrer, F., 2018. Consistent inverse correlation between DNA methylation of the first intron and gene expression across tissues and species. Epigenet. Chromatin 11 (1), 37.

Anders, S., Huber, W., 2010. Differential expression analysis for sequence count data. Genome Biol. 11 (10), R106.

Andrews, S., 2010. FastQC: A Quality Control Tool for High Throughput Sequence Data. Babraham Bioinformatics, Babraham Institute, Cambridge, United Kingdom.

Athauda, S., Anderson, T., 2014. Effect of temperature and salinity on sex inversion in Asian seabass (*Lates calcarifer*): relationship with plasma sex steroids concentration and aromatase activity of gonad and brain. Aquacult. Res. 45 (5), 787–797.

Athauda, S., Anderson, T., de Nys, R., 2012. Effect of rearing water temperature on protandrous sex inversion in cultured Asian seabass (*Lates calcarifer*). Gen. Comp. Endocrinol. 175 (3), 416–423. https://doi.org/10.1016/j.ygcen.2011.11.040.

Avise, J., Mank, J., 2009. Evolutionary perspectives on hermaphroditism in fishes. Sex. Dev. 3 (2–3), 152–163.

Ball, M.P., Li, J.B., Gao, Y., Lee, J.-H., LeProust, E.M., Park, I.-H., Xie, B., Daley, G.Q., Church, G.M., 2009. Targeted and genome-scale strategies reveal gene-body methylation signatures in human cells. Nat. Biotechnol. 27 (4), 361–368. https://doi.org/10.1038/nbt.1533.

Banci, L., Bertini, I., Calderone, V., Ciofi-Baffoni, S., Giachetti, A., Jaiswal, D., Mikolajczyk, M., Piccioli, M., Winkelmann, J., 2013. Molecular view of an electron transfer process essential for iron-sulfur protein biogenesis. Proc. Natl. Acad. Sci. U. S. A. 110 (18), 7136–7141. https://doi.org/10.1073/pnas.1302378110.

Banh, Q.Q., Domingos, J.A., Zenger, K.R., Jerry, D.R., 2017. Morphological changes and regulation of the genes *dmrt1* and *cyp11b* during the sex differentiation of barramundi (*Lates calcarifer* Bloch). Aquaculture 479, 75–84.

Banh, Q.Q.T., Guppy, J.L., Domingos, J.A., Budd, A.M., Pinto, R.C.C., Marc, A.F., Jerry, D.R., 2021. Induction of precocious females in the protandrous barramundi (Lates calcarifer) through implants containing 17β-estradiol-effects on gonadal morphology, gene expression and DNA methylation of key sex genes. Aquaculture 539, 736601.

Baroiller, J.-F., d'Cotta, H., 2016. The reversible sex of gonochoristic fish: insights and consequences. Sex. Dev. 10 (5–6), 242–266.

Baroiller, J.F., D'Cotta, H., Bezault, E., Wessels, S., Hoerstgen-Schwark, G., 2009. Tilapia sex determination: where temperature and genetics meet. Comparat. Biochem. Physiol. Part A: Mol. Integr. Physiol. 153 (1), 30–38. https://doi.org/10.1016/j.cbpa.2008.11.018.

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. B. Methodol. 57 (1), 289–300.

Benvenuto, C., Capellini, I., Mariani, S., Piferrer, F., Pla Quirante, S., 2017. Ecological and evolutionary consequences of alternative sex-change pathways in fish. Sci. Rep. 7.

Berselli, A., Alberini, G., Benfenati, F., Maragliano, L., 2022. Computational study of ion permeation through claudin-4 paracellular channels. Ann. N. Y. Acad. Sci. 1516 (1), 162–174. https://doi.org/10.1111/nyas.14856.

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30 (15), 2114–2120.

Brenet, F., Moh, M., Funk, P., Feierstein, E., Viale, A.J., Socci, N.D., Scandura, J.M., 2011. DNA methylation of the first exon is tightly linked to transcriptional silencing. PloS One 6 (1), e14524. https://doi.org/10.1371/journal.pone.0014524.

Bright, D., Reynolds, A., Nguyen, N.H., Knuckey, R., Knibb, W., Elizur, A., 2016. A study into parental assignment of the communal spawning protogynous hermaphrodite, giant grouper (Epinephelus lanceolatus). Aquaculture 459, 19–25. https://doi.org/10.1016/j.aquaculture.2016.03.013.

Budd, A.M., Banh, Q.Q., Domingos, J.A., Jerry, D.R., 2015. Sex control in fish: approaches, challenges and opportunities for aquaculture. J. Marine Sci. Eng. 3 (2), 329–355.

Budd, A.M., Guppy, J.L., Thépot, V., Domingos, J.A., Jerry, D.R., 2022a. Delayed effect of low rearing temperature on gonadal DNA methylation in juvenile barramundi (Lates calcarifer). Aquaculture 560, 738541.

Budd, A.M., Robins, J.B., Whybird, O., Jerry, D.R., 2022b. Epigenetics underpins phenotypic plasticity of protandrous sex change in fish. Ecol. Evol. 12 (3), e8730. https://doi.org/10.1002/ece3.8730.

Castelli, M.A., Whiteley, S.L., Georges, A., Holleley, C.E., 2020. Cellular calcium and redox regulation: the mediator of vertebrate environmental sex determination? Biol. Rev. 95 (3), 680–695.

Chen, Y., Wang, J., Wang, X., Liu, Y., Gu, B., Zhao, G., Li, Y., 2018. Methylation of TP53BP2 and Apaf-1 genes in embryonic lung cells and their impact on gene expression. Ann. Transl. Med. 6 (23).

Colot, V., Rossignol, J.L., 1999. Eukaryotic DNA methylation as an evolutionary device. Bioessays 21 (5), 402–411.

Davis, T.L.O., 1982. Maturity and sexuality in barramundi, *Lates calcarifer* (Bloch), in the Northern Territory and South-Eastern Gulf of Carpentaria. Mar. Freshw. Res. 33 (3), 529–545.

Davis, T.L.O., 1985. Seasonal changes in gonad maturity, and abundance of larvae and early juveniles of barramundi, *Lates calcarifer* (Bloch), in Van Diemen gulf and the Gulf of Carpentaria. Mar. Freshw. Res. 36 (2), 177–190.

Dhert, P., Lavens, P., Sorgeloos, P., 1992. State of the art of Asian seabass *Lates calcarifer* larviculture. J. World Aquacult. Soc. 23 (4), 317–329.

Domingos, J.A., Budd, A.M., Banh, Q.Q., Goldsbury, J.A., Zenger, K.R., Jerry, D.R., 2018. Sex-specific *dmrt1* and *cyp19a1* methylation and alternative splicing in gonads of the protandrous hermaphrodite barramundi. PloS One 13 (9), e0204182.

Doyle, J.J., Doyle, J.L., 1987. Isolation of plant DNA from fresh tissue. Phytochem. Bullet. 19 (11), 11–15.

Ellison, A., Rodriguez Lopez, C.M., Moran, P., Breen, J., Swain, M., Megias, M., Hegarty, M., Wilkinson, M., Pawluk, R., Consuegra, S., 2015. Epigenetic regulation of sex ratios may explain natural variation in self-fertilization rates. Proc. R. Soc. B Biol. Sci. 282 (1819), 20151900.

Fan, Z., You, F., Wang, L., Weng, S., Wu, Z., Hu, J., Zou, Y., Tan, X., Zhang, P., 2014. Gonadal transcriptome analysis of male and female olive flounder (*Paralichthys olivaceus*). Biomed. Res. Int. 2014, 10. https://doi.org/10.1155/2014/291067.

Fernandino, J.I., Hattori, R.S., Moreno Acosta, O.D., Strussmann, C.A., Somoza, G.M., 2013. Environmental stress-induced testis differentiation: androgen as a by-product of cortisol inactivation. Gen. Comp. Endocrinol. 192, 36–44. https://doi.org/10.1016/j.ygcen.2013.05.024.

Geffroy, B., Wedekind, C., 2020. Effects of global warming on sex ratios in fishes. J. Fish Biol. 97 (3), 596–606. https://doi.org/10.1111/jfb.14429.

Godwin, J., 2009. Social Determination of Sex in Reef Fishes, Seminars in Cell & Developmental Biology. Elsevier, pp. 264–270.

Godwin, J., Luckenbach, J.A., Borski, R.J., 2003. Ecology meets endocrinology: environmental sex determination in fishes. Evol. Dev. 5 (1), 40–49.

Goikoetxea, A., Todd, E.V., Gemmell, N.J., 2017. Stress and sex: does cortisol mediate sex change in fish? Reproduction 154 (6), R149–R160.

Gong, Q.-Y., Yang, M.-J., Yang, L.-F., Chen, Z.-G., Jiang, M., Peng, B., 2020. Metabolic modulation of redox state confounds fish survival against vibrio alginolyticus infection. J. Microbial. Biotechnol. 13 (3), 796–812. https://doi.org/10.1111/1751-7915.13553.

Guiguen, Y., Cauty, C., Fostier, A., Fuchs, J., Jalabert, B., 1994. Reproductive cycle and sex inversion of the seabass, *Lates calcarifer*, reared in sea cages in French Polynesia: histological and morphometric description. Environ. Biol. Fishes 39 (3), 231–247.

Guo, C.-Y., Tseng, P.-W., Hwang, J.-S., Wu, G.-C., Chang, C.-F., 2021. Potential role of DNA methylation of cyp19a1a promoter during sex change in protogynous orange-spotted grouper, *Epinephelus coioides*. General Compar. Endocrinol. 311, 113840. https://doi.org/10.1016/j.ygcen.2021.113840.

Guo, Y., Cheng, H., Huang, X., Gao, S., Yu, H., Zhou, R., 2005. Gene structure, multiple alternative splicing, and expression in gonads of zebrafish *dmrt1*. Biochem. Biophys. Res. Commun. 330 (3), 950–957. https://doi.org/10.1016/j.bbrc.2005.03.066.

Haas, B.J., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P.D., Bowden, J., Couger, M.B., Eccles, D., Li, B., Lieber, M., 2013. De novo transcript sequence reconstruction from RNA-seq using the trinity platform for reference generation and analysis. Nat. Protoc. 8 (8), 1494.

Hahne, F., Ivanek, R., 2016. Visualizing genomic data using Gviz and bioconductor. In: Statistical Genomics. Springer, pp. 335–351.

Harrington Jr., R.W., 1967. Environmentally controlled induction of primary male gonochorists from eggs of the self-fertilizing hermaphroditic fish, *Rivulus marmoratus* Poey. Biol. Bullet. 132 (2), 174–199.
Hattori, R.S., Fernandino, J.I., Kishii, A., Kimura, H., Kinno, T., Oura, M., Somoza, G.M., Yokota, M., Strüssmann, C.A., Watanabe, S., 2009. Cortisol-induced masculinization: does thermal stress affect gonadal fate in pejerrey, a teleost fish with temperature-dependent sex determination? PloS One 4 (8), e6548.
Hattori, R.S., Castañeda-Cortés, D.C., Arias Padilla, L.F., Strobl-Mazzulla, P.H., Fernandino, J.I., 2020. Activation of stress response axis as a key process in environment-induced sex plasticity in fish. Cell. Mol. Life Sci. 77 (21), 4223–4236. https://doi.org/10.1007/s00018-020-03532-9.
Hayashi, Y., Kobira, H., Yamaguchi, T., Shiraishi, E., Yazawa, T., Hirai, T., Kamei, Y., Kitano, T., 2010. High temperature causes masculinization of genetically female medaka by elevation of cortisol. Mol. Reprod. Dev. 77 (8), 679–686.
He, C.L., Du, J.L., Wu, G.C., Lee, Y.H., Sun, L.T., Chang, C.F., 2003. Differential *dmrt1* transcripts in gonads of the protandrous black porgy, *Acanthopagrus schlegeli*. Cytogenet. Genome Res. 101 (3–4), 309–313 https://doi.org/74354.
Herger, A., Dünser, K., Kleine-Vehn, J., Ringli, C., 2019. Leucine-rich repeat extensin proteins and their role in cell wall sensing. Curr. Biol. 29 (17), R851–R858.
Hoga, C.A., Almeida, F.L., Reyes, F.G., 2018. A review on the use of hormones in fish farming: analytical methods to determine their residues. CyTA-J. Food 16 (1), 679–691.
Honeycutt, J.L., Deck, C.A., Miller, S.C., Severance, M.E., Atkins, E.B., Luckenbach, J.A., Buckel, J.A., Daniels, H.V., Rice, J.A., Borski, R.J., Godwin, J., 2019. Warmer waters masculinize wild populations of a fish with temperature-dependent sex determination. Sci. Rep. 9 (1), 6527. https://doi.org/10.1038/s41598-019-42944-x.
Hothorn, T., Bretz, F., Westfall, P., Heiberger, R.M., Schuetzenmeister, A., Scheibe, S., Hothorn, M.T., 2016. Package 'Multcomp'. Simultaneous inference in general parametric models. Project for Statistical Computing, Vienna, Austria.
Hu, G., Feng, Z., Bai, Y., Yang, C., Guo, Y., Lin, C., Zhang, Y., He, Q., Li, X., 2019. Study of promoter methylation patterns of HOXA2, HOXA5 and HOXA6 and its clinicopathological characteristics in colorectal cancer. Front. Oncol. 9, 394.
Hu, Q., Lian, Z., Xia, X., Tian, H., Li, Z., 2022. Integrated chromatin accessibility and DNA methylation analysis to reveal the critical epigenetic modification and regulatory mechanism in gonadal differentiation of the sequentially hermaphroditic fish, *Monopterus albus*. Biol. Sex Differ. 13 (1), 73. https://doi.org/10.1186/s13293-022-00484-6.
Huang, S.-P., Lin, K.-L., Fang, L.-S., 1998. The involvement of calcium in heat-induced coral bleaching. Zool. Stud.-Taipei 37, 89–94.
Huber, W., von Heydebreck, A., Sültmann, H., Poustka, A., Vingron, M., 2003. Parameter estimation for the calibration and variance stabilization of microarray data. Stat. Appl. Genet. Mol. Biol. 2 (1).
Iwata, E., Suzuki, N., Ohno, S., 2019. Influence of social stability on the sex determination process in false clown anemonefish (Amphiprion ocellaris). Mar. Freshw. Behav. Physiol. 52 (3), 107–119. https://doi.org/10.1080/10236244.2019.1655408.
Katersky, R.S., Carter, C.G., 2007. High growth efficiency occurs over a wide temperature range for juvenile barramundi *Lates calcarifer* fed a balanced diet. Aquaculture 272 (1–4), 444–450.
Kim, D., Langmead, B., Salzberg, S.L., 2015. HISAT: a fast spliced aligner with low memory requirements. Nat. Methods 12 (4), 357.
Kobayashi, Y., Nagahama, Y., Nakamura, M., 2013. Diversity and plasticity of sex determination and differentiation in fishes. Sex. Dev. 7 (1–3), 115–125. https://doi.org/10.1159/000342009.
Krueger, F., 2015. Trim galore. A wrapper tool around Cutadapt and FastQC to consistently apply quality and adapter trimming to FastQ files 516, 517.
Krueger, F., Andrews, S.R., 2011. Bismark: a flexible aligner and methylation caller for bisulfite-Seq applications. Bioinformatics 27 (11), 1571–1572.
Kuo, H.C., Hsu, H.H., Chua, C.S., Wang, T.Y., Chen, Y.M., Chen, T.Y., 2014. Development of pedigree classification using microsatellite and mitochondrial markers for Giant grouper broodstock (Epinephelus lanceolatus) management in Taiwan. Mar. Drugs 12 (5), 2397–2407. https://doi.org/10.3390/md12052397.
Kuroda, A., Rauch, T.A., Todorov, I., Ku, H.T., Al-Abdullah, I.H., Kandeel, F., Mullen, Y., Pfeifer, G.P., Ferreri, K., 2009. Insulin gene expression is regulated by DNA methylation. PloS One 4 (9), e6953. https://doi.org/10.1371/journal.pone.0006953.
Labunskyy, V.M., Hatfield, D.L., Gladyshev, V.N., 2014. Selenoproteins: molecular pathways and physiological roles. Physiol. Rev. 94 (3), 739–777. https://doi.org/10.1152/physrev.00039.2013.
Li, H., 2018. Minimap2: pairwise alignment for nucleotide sequences. Bioinformatics 34 (18), 3094–3100.
Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15 (12), 550.
Mackie, M., 2000. Reproductive biology of the halfmoon grouper, Epinephelus rivulatus, at Ningaloo reef, Western Australia. Environ. Biol. Fish. 57 (4), 363–376.
Mackie, M.C., 2003. Socially controlled sex-change in the half-moon grouper, *Epinephelus rivulatus*, at Ningaloo reef, Western Australia. Coral Reefs 22 (2), 133–142. https://doi.org/10.1007/s00338-003-0296-3.
MacManes, M.D., 2014. On the optimal trimming of high-throughput mRNA sequence data. Front. Genet. 5, 13.
Maor, G.L., Yearim, A., Ast, G., 2015. The alternative role of DNA methylation in splicing regulation. Trends Genet. 31 (5), 274–280.
Marc, A.F., Guppy, J.L., Goldsbury, J., Rudd, D., Cate, H.S., Paris, D.B.B.P., Jerry, D.R., 2024. Characterization of sperm quality in captive-bred barramundi (Lates calcarifer): effect on spawning performance and paternal contribution in mass-spawning events. Aquaculture 585, 740717. https://doi.org/10.1016/j.aquaculture.2024.740717.
Marçais, G., Kingsford, C., 2011. A fast, lock-free approach for efficient parallel counting of occurrences of k-mers. Bioinformatics 27 (6), 764–770.
Matsumoto, Y., Buemio, A., Chu, R., Vafaee, M., Crews, D., 2013. Epigenetic control of gonadal aromatase (*cyp19a1*) in temperature-dependent sex determination of red-eared slider turtles. PloS One 8 (6). https://doi.org/10.1371/journal.pone.0063599.
Mukai, K., Hara, S., Sakima, K., Nozu, R., Yazawa, T., Kitano, T., 2022. Oxidative stress causes masculinization of genetically female medaka without elevating cortisol. Front. Endocrinol. 13, 878286.
Nakamura, M., Hourigan, T., Yamauchi, K., Nagahama, Y., Grau, E.G., 1989. Histological and ultrastructural evidence for the role of gonadal steroid hormones in sex change in the protogynous wrasse *Thalassoma duperrey*. Environ. Biol. Fishes 24 (2), 117–136. https://doi.org/10.1007/BF00001282.
Navarro-Martín, L., Viñas, J., Ribas, L., Díaz, N., Gutiérrez, A., Di Croce, L., Piferrer, F., 2011. DNA methylation of the gonadal aromatase (*cyp19a*) promoter is involved in temperature-dependent sex ratio shifts in the European sea bass. PLoS Genet. 7 (12), e1002447. https://doi.org/10.1371/journal.pgen.1002447.
Ortega-Recalde, O., Goikoetxea, A., Hore, T.A., Todd, E.V., Gemmell, N.J., 2020. The genetics and epigenetics of sex change in fish. Ann. Rev. An. Biosci. 8, 47–69. https://doi.org/10.1146/annurev-animal-021419-083634.
Ospina-Alvarez, N., Piferrer, F., 2008. Temperature-dependent sex determination in fish revisited: prevalence, a single sex ratio response pattern, and possible effects of climate change. PloS One 3 (7), e2837.
Parrott, B.B., Kohno, S., Cloy-McCoy, J.A., Guillette Jr., L.J., 2014. Differential incubation temperatures result in dimorphic DNA methylation patterning of the *sox9* and aromatase promoters in gonads of alligator (*Alligator mississippiensis*) embryos. Biol. Reprod. 90 (1), 1–11.
Pertea, M., Pertea, G.M., Antonescu, C.M., Chang, T.-C., Mendell, J.T., Salzberg, S.L., 2015. StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. Nat. Biotechnol. 33 (3), 290.
Pertea, M., Kim, D., Pertea, G.M., Leek, J.T., Salzberg, S.L., 2016. Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown. Nat. Protocols 11 (9), 1650.
Pierron, F., Lorioux, S., Héroin, D., Daffe, G., Etcheverria, B., Cachot, J., Morin, B., Dufour, S., Gonzalez, P., 2021. Transgenerational epigenetic sex determination: environment experienced by female fish affects offspring sex ratio. Environ. Pollut. 277, 116864. https://doi.org/10.1016/j.envpol.2021.116864.
Piferrer, F., Anastasiadi, D., Valdivieso, A., Sánchez, N., Moraleda, J., Ribas, L., 2019. The model of the conserved epigenetic regulation of sex. Front. Genet. 10, 857.
Pla Quirante, S., Capellini, I., Benvenuto, C., Mariani, S., Piferrer, F., 2018. Switches, Stability and Reversals: The Evolutionary History of Sexual Patterns in Teleosts.
Queensland, F., 2010. Fisheries Long Term Monitoring Program Sampling Protocol–Barramundi (2008 onwards) Section 1. In: Queensland, F. (Ed.), Department of Employment, Economic Development and Innovation, Brisbane.
Quinitio, G.F., Caberoy, N.B., Reyes Jr., D.M., 1997. Induction of sex change in female *Epinephelus coioides* by social control. Israeli J. Aquac. Bamidgeh 49 (2), 77–83.
Quinlan, A.R., 2014. BEDTools: the Swiss-army tool for genome feature analysis. Curr. Protoc. Bioinformatics 47 (1), 11.12. 11–11.12. 34.
Rack, J.G.M., Palazzo, L., Ahel, I., 2020. (ADP-ribosyl) hydrolases: structure, function, and biology. Genes Dev. 34 (5–6), 263–284.
Ripley, B., Venables, B., Bates, D.M., Hornik, K., Gebhardt, A., Firth, D., Ripley, M.B., 2013. Package 'mass'. Cran R 538.
Roberts, B.H., Morrongiello, J.R., Morgan, D.L., King, A.J., Saunders, T.M., Crook, D.A., 2021. Faster juvenile growth promotes earlier sex change in a protandrous hermaphrodite (barramundi Lates calcarifer). Sci. Rep. 11 (1), 1–10.
Robinson, N.A., Schipp, G., Bosmans, J., Jerry, D.R., 2010. Modelling selective breeding in protandrous, batch-reared Asian sea bass (*Lates calcarifer*, Bloch) using walkback selection. Aquacult. Res. 41 (10).
Russell, D., Garrett, R., 1985. Early life history of barramundi, <I>*Lates calcarifer*</I> (Bloch), in north-eastern Queensland. Mar. Freshw. Res. 36 (2), 191–201. https://doi.org/10.1071/MF9850191.
Saillant, E., Fostier, A., Haffray, P., Menu, B., Thimonier, J., Chatain, B., 2002. Temperature effects and genotype-temperature interactions on sex determination in the European sea bass (*Dicentrarchus labrax* L.). J. Exp. Zool. 292 (5), 494–505.
Sambrook, J., Russell, D.W., 2001. Molecular Cloning. Cold Spring Harbor Laboratory Press, New York.
Sarter, K., Papadaki, M., Zanuy, S., Mylonas, C.C., 2006. Permanent sex inversion in 1-year-old juveniles of the protogynous dusky grouper (*Epinephelus marginatus*) using controlled-release 17α-methyltestosterone implants. Aquaculture 256 (1–4), 443–456.
Schipp, G., Bosmans, J., Humphrey, J., 2007. Barramundi farming handbook. Department of Primary Industry, Fisheries and Mines, Northern Territory Government.
Shao, C., Li, Q., Chen, S., Zhang, P., Lian, J., Hu, Q., Sun, B., Jin, L., Liu, S., Wang, Z., Zhao, H., Jin, Z., Liang, Z., Li, Y., Zheng, Q., Zhang, Y., Wang, J., Zhang, G., 2014. Epigenetic modification and inheritance in sexual reversal of fish. Genome Res. 24 (4), 604–615. https://doi.org/10.1101/gr.162172.113.
Shapiro, D.Y., 1987. Differentiation and evolution of sex change in fishes. Bioscience 37 (7), 490–497.
Singh, S.K., Das, D., Rhen, T., 2020. Embryonic temperature programs phenotype in reptiles. Front. Physiol. 11, 35.
Soneson, C., Love, M.I., Robinson, M.D., 2015. Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences. F1000Research 4.
Song, L., Florea, L., 2015. Rcorrector: efficient and accurate error correction for Illumina RNA-seq reads. GigaScience 4 (1), 48.

Suzuki, M.M., Bird, A., 2008. DNA methylation landscapes: provocative insights from epigenomics. Nat. Rev. Genet. 9 (6), 465–476.

Tao, W., Chen, J., Tan, D., Yang, J., Sun, L., Wei, J., Conte, M.A., Kocher, T.D., Wang, D., 2018. Transcriptome display during tilapia sex determination and differentiation as revealed by RNA-Seq analysis. BMC Genomics 19 (1), 363. https://doi.org/10.1186/s12864-018-4756-0.

Terence, C., Domingos, J.A., Uichanco, J.A., Monteil, Y., Monteil, L., De Braux, E., Banh, Q.Q.T., Gibson-Kueh, S., Jerry, D.R., 2021. Early sex change of barramundi Lates calcarifer farmed in Singapore. Aquac. Rep. 20, 100712. https://doi.org/10.1016/j.aqrep.2021.100712.

Tibshirani, R., 1988. Estimating transformations for regression via additivity and variance stabilization. J. Am. Stat. Assoc. 83 (402), 394–405.

Todd, E.V., Liu, H., Lamm, M.S., Thomas, J.T., Rutherford, K., Thompson, K.C., Godwin, J.R., Gemmell, N.J., 2017. Female mimicry by sneaker males has a transcriptomic signature in both the brain and the gonad in a sex-changing fish. Mol. Biol. Evol. 35 (1), 225–241. https://doi.org/10.1093/molbev/msx293.

Todd, E.V., Ortega-Recalde, O., Liu, H., Lamm, M.S., Rutherford, K.M., Cross, H., Black, M.A., Kardailsky, O., Graves, J.A., Hore, T.A., 2019. Stress, novel sex genes, and epigenetic reprogramming orchestrate socially controlled sex change. Sci. Adv. 5 (7), eaaw7006.

Valdivieso, A., Ribas, L., Monleón-Getino, A., Orbán, L., Piferrer, F., 2020. Exposure of zebrafish to elevated temperature induces sex ratio shifts and alterations in the testicular epigenome of unexposed offspring. Environ. Res. 186, 109601. https://doi.org/10.1016/j.envres.2020.109601.

Valenzuela, N., Adams, D.C., Janzen, F.J., 2003. Pattern does not equal process: exactly when is sex environmentally determined? Am. Nat. 161 (4), 676–683.

Vij, S., Kuhl, H., Kuznetsova, I.S., Komissarov, A., Yurchenko, A.A., Van Heusden, P., Singh, S., Thevasagayam, N.M., Prakki, S.R.S., Purushothaman, K., 2016. Chromosomal-level assembly of the Asian seabass genome using long sequence reads and multi-layered scaffolding. PLoS Genet. 12 (4), e1005954.

Wang, J., Liu, Y., Jiang, S., Li, W., Gui, L., Zhou, T., Zhai, W., Lin, Z., Lu, J., Chen, L., 2019b. Transcriptomic and epigenomic alterations of Nile tilapia gonads sexually reversed by high temperature. Aquaculture 508, 167–177.

Wang, L., Wang, S., Li, W., 2012. RSeQC: quality control of RNA-seq experiments. Bioinformatics 28 (16), 2184–2185.

Wang, Q., Liu, K., Feng, B., Zhang, Z., Wang, R., Tang, L., Li, W., Li, Q., Piferrer, F., Shao, C., 2019a. Transcriptome of gonads from high temperature induced sex reversal during sex determination and differentiation in Chinese tongue sole, *Cynoglossus semilaevis*. Front. Genet. 10, 1128. https://doi.org/10.3389/fgene.2019.01128.

Warner, R.R., 1975. The adaptive significance of sequential hermaphroditism in animals. Am. Nat. 109 (965), 61–82.

Warner, R.R., 1984. Mating behavior and hermaphroditism in coral reef fishes. Am. Sci. 72 (2), 128–136.

Wessels, S., Krause, I., Floren, C., Schütz, E., Beck, J., Knorr, C., 2017. ddRADseq reveals determinants for temperature-dependent sex reversal in Nile tilapia on LG23. BMC Genomics 18 (1), 531.

Whiteley, S.L., Holleley, C.E., Wagner, S., Blackburn, J., Deveson, I.W., Marshall Graves, J.A., Georges, A., 2021. Two transcriptionally distinct pathways drive female development in a reptile with both genetic and temperature dependent sex determination. PLoS Genet. 17 (4), e1009465.

Williams, K.C., Barlow, C.G., Rodgers, L., Agcopra, C., 2006. Dietary composition manipulation to enhance the performance of juvenile barramundi (*Lates calcarifer Bloch*) reared in cool water. Aquacult. Res. 37 (9), 914–927.

Wu, G.-C., Tomy, S., Lee, M.-F., Lee, Y.-H., Yueh, W.-S., Lin, C.-J., Lau, E.-L., Chang, C.-F., 2010. Sex differentiation and sex change in the protandrous black porgy, *Acanthopagrus schlegeli*. General Compar. Endocrinol. 167 (3), 417–421.

Yu, H., Cheng, H., Guo, Y., Xia, L., Zhou, R., 2003. Alternative splicing and differential expression of P450c17 in gonads during sex transformation in the rice field eel. Biochem. Biophys. Res. Commun. 307 (1), 165–171.

Yuan, H.W., Chen, F., Xu, Q.Q., Gong, S.Y., Chu, Z.J., Yang, D.Q., 2012. Effects of different stocking densities on growth, serum steroid hormone concentrations, gonadosomatic index and sex reversal in the rice field eel, Monopterus albus (Zuiew), applied mechanics and materials. Trans. Tech Publ. 233–237.

Zarkower, D., Murphy, M.W., 2021. DMRT1: an ancient sexual regulator required for human Gonadogenesis. Sex. Dev. 16 (2–3), 112–125. https://doi.org/10.1159/000518272.

Zhang, Y., Zhang, W., Yang, H., Zhou, W., Hu, C., Zhang, L., 2008. Two cytochrome P450 aromatase genes in the hermaphrodite ricefield eel Monopterus albus: mRNA expression during ovarian development and sex change. J. Endocrinol. 199 (2), 317–331.

Zhang, Y., Zhang, S., Liu, Z., Zhang, L., Zhang, W., 2013. Epigenetic modifications during sex change repress gonadotropin stimulation of *Cyp19a1a* in a teleost ricefield eel (*Monopterus albus*). Endocrinology 154 (8), 2881–2890.

Zheng, Y., Joyce, B.T., Liu, L., Zhang, Z., Kibbe, W.A., Zhang, W., Hou, L., 2017. Prediction of genome-wide DNA methylation in repetitive elements. Nucleic Acids Res. 45 (15), 8697–8711.

Zohar, Y., Billard, R., Weil, C., 1984. La reproduction de la daurade (*Sparus aurata*) et du bar (*Dicentrarchus labrax*): connaissance du cycle sexuel et controle de la gametogenese et de la ponte. L'Aquaculture du Bar et des Sparides 3–24.