Epigenetic effects of temperature on sex change in barramundi, *Lates calcarifer*

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STATEMENT OF THE CONTRIBUTION OF OTHERS

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ABSTRACT

How and why do fish change sex? Whilst sexual development is nearly universal among eukaryotes, phenotypic sex can be established through a variety of complex and rapidly evolving regulatory pathways. In particular, teleost fish display remarkable variety in their systems of sexual development, as well as the molecular mechanisms by which these systems are governed. For example, fish can be gonochoristic, developing as either male or female, or simultaneous hermaphrodites, possessing both functional male and female reproductive organs. Alternatively, fish can be sequential hermaphrodites, undergoing primary development as one sex, but later sex changing to the other, or serial hermaphrodites where sex change occurs not once, but multiple times. The molecular mechanisms governing sex in fish are equally diverse, whereby sex can be determined by endogenous factors (e.g. genetic components), exogenous factors (e.g. environmental or experimental stimuli), or as a result of an interaction between the two. How these differences in phenotypic sex arise from the same genetic template, and what the environmental drivers of this may be, form the basis of the more specific questions that this thesis addresses.

Changes in phenotype in the absence of changes in genotype, particularly in response to environmental cues, are hallmarks of epigenetic control. Epigenetic changes are those that occur in the structural or chemical composition of DNA, rather than the sequence itself, and cause changes in gene expression. One particular epigenetic modification, DNA methylation, has been shown to underlie both temperature effects on sexual development in gonochoristic and simultaneous hermaphrodite fish species (i.e. those with stable sexual phenotypes), and sex change in sequential hermaphrodite species. Such evidence is rapidly accumulating to suggest that DNA methylation is a common mechanism underlying the remarkable plasticity of sex in fish. It is unknown, however, if temperature can induce changes in DNA methylation, and thus influence development of the primary sex and/or mediate transition to the secondary sex, in sequential hermaphrodites.

Barramundi, *Lates calcarifer*, are protandrous (male-first) hermaphrodites and, at present, are the only sequentially sex changing fish for which temperature has been reported to affect phenotypic sex. Very recently, it has been shown that male and female barramundi also exhibit differential DNA methylation in key sex-related genes. With a known effect of temperature on

sexual development and differential DNA methylation between the sexes, barramundi presents an experimental model with which to test the hypothesis that temperature-induced DNA methylation is an experimental and/or environmental driver of sex change in a sequential hermaphrodite. In this species, sex change is strongly associated with body length, but substantial differences in length-at-sex change are observed between regions and culture environments, the causes of which are unknown. The resulting inability to predict and control sex change in barramundi presents the single biggest impediment to the instigation of commercial breeding programs and invalidates key assumptions regarding sex ratio, maturity and fecundity used in fisheries modelling for this species. As such, understanding the environmental causations and underlying molecular mechanisms that drive sex change in barramundi is likely to lead to significant improvements in aquaculture production and enable better fisheries management.

The overarching aim of this thesis was to investigate the effect of temperature on DNA methylation and sexual phenotype in barramundi. First, the research explored if there is an association between DNA methylation patterns, fish total length, and sex in wild-caught barramundi, and in doing so, investigated the hypothesis that cumulative changes in DNA methylation lead to sequential sex change in this species. The dataset was further interrogated to determine if the total length at which sex change occurs differs between barramundi from different geographic regions, and if these differences are associated with changes in DNA methylation. To do so, DNA methylation levels in the predicted promoter and first exon of four key sex-related genes were measured, including male-associated genes *nr5a2* and *dmrt1* and female-associated genes *cyp19a1a* and *esr1*. The data demonstrated male and female-specific patterns of DNA methylation, with males exhibiting significantly higher levels of DNA methylation in male-associated genes *nr5a2* and *dmrt1* and significantly lower levels in female-associated genes *cyp19a1a* and *esr1*. The reciprocal pattern was found in female barramundi. Furthermore, despite the relationship between increasing total length and male to female sex change, male barramundi exhibit increasingly more *male-specific* DNA methylation patterns with increases in total length. Thus, the work did not find direct support for a gradual accumulation of changes in DNA methylation leading to male to female sex change. Instead, it was concluded that methylation changes are likely to arise rapidly, upon the induction of male to female transition, rather than gradually accumulating over the lifespan of an individual. The data further revealed that region-specific differences in total length-at-sex change are reflected in differences in DNA methylation of male barramundi. For example, individuals

from the southern Gulf of Carpentaria, Australia, exhibited smaller total lengths-at-sex change and DNA methylation patterns that were significantly different from the mid-northern Gulf of Carpentaria and north Queensland east coast. These differences were not correlated to the average ambient temperatures and, as such, the environmental drivers contributing to these differences remain unknown. As a whole, the first data chapter provides a unique example of population level, epigenetically driven phenotypic plasticity in a commercially important, sex changing species.

Second, the research in this thesis examined the effect of experimental temperature treatment on DNA methylation and phenotypic sex during early development in cultured barramundi and explored the possibility of producing primary females. To do so, juvenile barramundi were temperature-treated from 10-90 days post hatch, corresponding to the period just prior to, and during the onset of sexual differentiation (the predicted temperature-sensitive period). Here fish were reared in either cold (24 °C), hot (34 °C), fluctuating (24 - 34 °C), or control (29 °C) temperatures. DNA methylation in amplicons covering the predicted promoter and partial first exons of seven sex-related genes (*amh, cyp19a1a, dmrt1, esr1, foxl2, nr5a2* and *sox9*), as well as the final exons of three of the same genes (*amh, cyp19a1a* and *dmrt1*) were quantified at 6 and 12 months post hatch. Histological examination of the gonads was also undertaken, as well as length and weight measurements of the fish recorded. Comparisons among treatments revealed a delayed effect of temperature on gonadal DNA methylation, a significant effect on fish weight, but not length, and marked differences in male gonadal development stage for cold temperature treated fish compared to all other treatments. Specifically, cold temperature treatment resulted in more male-specific patterns of DNA methylation in the gonads and an increased proportion of late spermatogenesis cell types compared to treatment with hot, fluctuating of control temperatures. This is the first investigation of the effect of temperature on juvenile sexual differentiation in a sequential hermaphrodite. Future work should focus on manipulating the timing and intensity of temperature treatments in order to confirm or preclude the possibility of primary female production in barramundi, and thus temperature-induced sex reversal in sequential hermaphrodites.

Finally, the research examined the effect of temperature treatment on DNA methylation, gene expression, body size and sexual phenotype in one-year-old, sexually differentiated (male) barramundi. Here fish were exposed to either cold (24 °C), hot (34 °C), fluctuating (24 - 34

°C), or control (28 °C) temperatures from 12 to 24 mph. Whole genome bisulphite sequencing and RNA-Seq techniques were employed, allowing the extraction of gene expression and DNA methylation levels and along the entire length and 2000 bp upstream of eight previously annotated barramundi sex genes: *amh, cyp19a1a, dmrt1, esr1, foxl2, nr5a2, sox8* and *sox9*. The results show that temperature treatment of barramundi from 12 – 24 mph causes significant alterations in gene expression, changes in patterns of DNA methylation and leads to marked differences in phenotypic sex. Notably, the previously observed effect of high temperature treatment (33 and 35 °C) on sex in this species was shown to be reproducible, and the early induction of transition from male to female was here shown to be accompanied by changes in gene expression and DNA methylation, notably in two genes essential to ovary formation; *foxl2* and *cyp19a1a*. While further interrogation of the genomic data will likely provide increased understanding of the underlying mechanisms behind the observed changes in phenotype, refinement of temperature treatments as applied here may offer an economically viable and environmentally friendly solution to sex control in *L. calcarifer.*

The data presented within this thesis collectively demonstrates that sex in barramundi is associated with substantial differences in DNA methylation of known sex genes, and that both DNA methylation and phenotypic sex can be altered by experimental temperature treatment. While sex change in barramundi cannot be attributed to a gradual accumulation of changes in DNA methylation, consistent and marked differences between males and females emphasise an epigenetic basis for sex change in this species, and likely other sequential hermaphrodites. Concurrent differences in length-at-sex change and DNA methylation patterns between barramundi from different geographic locations, in the absence of substantial genetic variation, suggests that environmental factors are influencing the timing of sex change in wild-caught *L. calcarifer*. What the major environmental factors are, however, remains unclear. Significant effects of experimental temperature treatment on DNA methylation and primary sexual differentiation in juvenile and one-year-old barramundi demonstrate that temperature may, at least in part, be an important environmental driver of sexual differentiation and sex change. While further work is required to better understand the biological mechanisms underlying temperature effects on sex change, and the species-specific molecular pathways governing sexual development in barramundi, these results provide insight into the molecular mechanisms driving differences in length-at-sex change in wild-caught barramundi and highlight the potential for temperature to be used as a method of sex control in aquaculture. In essence, this

thesis presents a first exploration of the relationships between fish length, geographic origin and the effects of temperature treatment on DNA methylation and phenotypic sex in a sequentially hermaphroditic fish.

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GLOSSARY

Terms	
Digonic	Of or denoting reproductive organs that typically produce eggs and sperm but exhibit physical separation.
DNA demethylation	The process of removal of a methyl group from nucleotides in DNA. Can be active or passive.
DNA methylation	The addition of methyl groups to specific sites on a DNA molecule. Here largely used to refer to 5-methylcytosine (5mC) specifically; which is methylation of the fifth position of cytosine.
Epigenetic	Describing heritable changes that are not the result of changes in DNA sequence.
Female	Of or denoting reproductive organs that produce only female gametes.
Female gamete	The sex cell that, during sexual reproduction, fuses with a male gamete in the process of fertilization. Female gametes are generally larger than the male gametes and are usually immotile.
Gonochoristic	Of or denoting reproductive organs that typically develop as either ovaries or testis.
Hermaphrodite	Of or denoting reproductive organs that produce both male and female gametes.
Male	Of or denoting reproductive organs that produce only male gametes.
Male gamete	The sex cell that, during sexual reproduction, fuse with female gametes in the process of fertilization. Male gametes are generally smaller than the female gametes and are usually motile.
Primary sex	Of or denoting the classification of sex into which the gonads first differentiate.
Protandrous	Of or denoting gonads that typically change from testis to ovary.
Protogynous	Of or denoting gonads that typically change from ovary to testis.
Secondary sex	Of or denoting the classification of sex into which the gonads undergo secondary differentiation towards.
Sequential hermaphrodite	Of or denoting reproductive organs that typically change from on sex to another, including protogynous or protandrous types.
Sex	A classification of organisms or parts of organisms according to the kind of gamete produced; larger nutrient-rich gametes are typically classified as female; smaller, nutrient-poor gametes are typically classified as male. An individual animal that produces both female and male gametes is typically classified as hermaphroditic.
Sex change	Of or denoting a change in the functioning of reproductive organs from one classification of sex to another, e.g. protandrous sex change involves a gonadal transition from functional testis to ovary.
Sex determination	Describes a genetic or environmental cue(s) and how it governs the sex of an individual.
Sexual differentiation	Refers to the physical realisation of sex, and largely pertains to the development of the testicular or ovarian tissues may follow on from a sex determining cue.
Sex reversal	Here defined as of or denoting a change in developmental pathway, typically by exposure to stimuli not commonly encountered in the environment in which a species has evolved (e.g. extreme temperatures), resulting in altered gonadal development from one classification of sex to another.
Simultaneous hermaphrodite	Of or denoting reproductive organs that produce viable male and female gametes at the same time.
Typical development	Describes the processes of development which occur when individuals of a given species are subject to a range of conditions that are commonly encountered in the habitat in which they have evolved and follow a sequence that is statistically more prevalent. Is in contrast to atypical development, where exposure to stimuli not commonly encountered (e.g. extreme temperatures) results in deviations from typical development and is a source of great diversity.

Abbreviations

11-KT	11-Ketotestosterone
amh	anti-Mullerian hormone
BSAS	Bisulphite amplicon sequencing
CT	Cold temperature
cyp19a1a	cytochrome P450, family 19, subfamily A, polypeptide 1a; gonadal aromatase *Cyp19a1a* is associated with ovarian differentiation in fish and generally shows low methylation and high expression in females compared to males [see Piferrer *et al.* (2019)]
dmrt1	doublesex and mab-3 related transcription factor 1 *Dmrt1* is associated with testis differentiation in fish and generally shows low methylation and high expression in males compared to females [see Piferrer *et al.* (2019)]
DNMT	DNA methyltransferase
dph	Days post hatch
E2	17β-Estradiol
ESD	Environmental sex determination
esr1	estrogen receptor alpha
foxl2	forkhead box L2
FT	Fluctuating temperature
GSD	Genetic sex determination
GxE	Genotype by environment interactions
HT	Hot temperature
mph	Months post hatch
nr5a2	nuclear receptor subfamily 5 group A member 2
PCR	Polymerase chain reaction
RNA-Seq	RNA sequencing
RT-qPCR	Reverse transcription quantitative PCR
sox8	SRY-related HMG box 8
sox9	SRY-related HMG box 9
TSD	Temperature-dependent sex determination
TSP	Temperature-sensitive period
WGBS	Whole genome bisulphite sequencing

Chapter 1

General Introduction

1.1 Summary

This chapter gives an overview of the diversity and variety in systems of sexual development and mechanisms of sex determination and sexual differentiation in fish. Throughout the chapter, many of the concepts explored and examples referred to within this thesis are introduced for the first time. After a brief introductory paragraph in section 1.2, section 1.3 provides an in-depth overview of sex determination and differentiation in fish, including the mechanisms by which sex is established and the diverse systems of sexual development employed by different fish species. Section 1.4 of this chapter briefly introduces the concept of epigenetics and contains a synthesis of the existing case studies where the most widely researched epigenetic mechanism, DNA methylation, has been linked to temperature-induced sex reversal or sequential sex change in fish. This section first discusses case studies in gonochoristic species, followed by those in hermaphroditic species excluding barramundi, *Lates calcarifer*, and ends with some concluding remarks about the current state of knowledge in the field.

1.2 Introduction

Sexual reproduction has many advantages. It enables the random assortment of two discrete genomes, thereby increasing genetic variation through recombination and enabling accelerated evolution through the process of natural selection. Additionally, sexual reproduction dramatically reduces the accumulation of recessive deleterious alleles and homozygosity thereby increasing species fitness. Despite the significant temporal and energetic costs involved in finding a reproductive partner, sexual reproduction is markedly more common than asexual reproduction and self-fertilisation (Otto, 2009). Even in hermaphrodite plants and animals, self-fertilisation is often evaded via systems of self-incompatibility, including self-sterility and sequential hermaphroditism (Ghiselin, 1969; Takayama and Isogai, 2005; Harada *et al.*, 2008). For sexual reproduction to transpire, a species must first evolve sexual dimorphism, or distinct reproductive functioning (e.g. testis and ovary). At the individual level, sex is first determined during development, initiating divergent patterns of gene expression that allow for sex-specific cells and sexually dimorphic phenotypes (Bull, 1983). At the very least, these phenotypes are present within the gonads, but are often also expressed through external primary (present at birth) and secondary (emerging during puberty) sex characteristics. Whilst sexual development is nearly universal among the eukaryotes, sex itself can be established through a variety of complex and rapidly evolving regulatory pathways (Gamble and Zarkower, 2012; Ravi *et al.*, 2014).

1.3 Genetic sex determination and differentiation in fish

Processes of sex determination and differentiation are highly variable among vertebrates and in some animals distinguishing between the two processes can be difficult. The term sex determination can be used to describe the genetic and/or environmental cue(s) that ultimately govern the sex of an individual (Devlin and Nagahama, 2002). For example, in all but a few mammals, inheritance of the Y chromosome determines that an individual will develop as male (Koopman *et al.*, 1991). In many reptiles, however, temperatures experienced during embryonic development, rather than genetic factors, provide the sex-determining cue (Bull, 1980). Sexual differentiation, on the other hand, often refers to the subsequent *physical* realisation of sex determination cues, and largely pertains to the development of the testicular or ovarian tissues that follows on from the sex-determining cue (Devlin and Nagahama, 2002). The terms sex determination and sexual differentiation are often used interchangeably, as in

many cases the two processes can partially overlap (Penman and Piferrer, 2008; Heule *et al.*, 2014). This overlap is especially evident in teleost fish.

In contrast to mammals (XX/XY) and birds (ZZ/ZW), teleost fish lack an evolutionarily stable, monofactorial genetic sex determination system and, instead, display remarkable variety in the genetic mechanisms that underlie sexual development. Teleost fish also exhibit variety in the systems of sexual development employed, with gonadal differentiation taking the form of gonochorism, hermaphroditism, and in rare cases, unisexuality (Figure 1.1). Gonochorism refers to systems in which individuals develop either testis (male), or ovary (female), and retain their sex for the remainder of their lifespan (Devlin and Nagahama, 2002). Hermaphroditism describes systems in which individuals are neither exclusively male or female, but can employ one of the following strategies: 1) simultaneous hermaphroditism where individuals develop both functional testis and ovaries at the same time and can either a) self-fertilise, or b) cross fertilise; 2) digonic hermaphrodites develop both testis and ovaries, but exhibit physical separation between the two tissues and generally undergo sexual phase change to sequentially switch from the functionality of one sex to the other; 3) sequential hermaphroditism where individuals develop either ovary or testis initially, but undergo complete sex change later in life either in the form of protogyny (female first), or protandry (male first); or 4) serial hermaphroditism where individuals can change back and forth between functional ovary and testis. Unisexuality is a rare and extreme form of gonochorism whereby populations consist entirely of a single sex (see Figure 1.1 for examples of each system). The plasticity of fish sexual phenotypes is governed by similarly plastic mechanisms of sexual development that require complex regulatory pathways governed by genetic factors (genetic sex determination; GSD), environmental cues (environmental sex determination; ESD), or an interaction between the two GxE (genotype x environment interactions; GxE; Baroiller and D'Cotta, 2001).

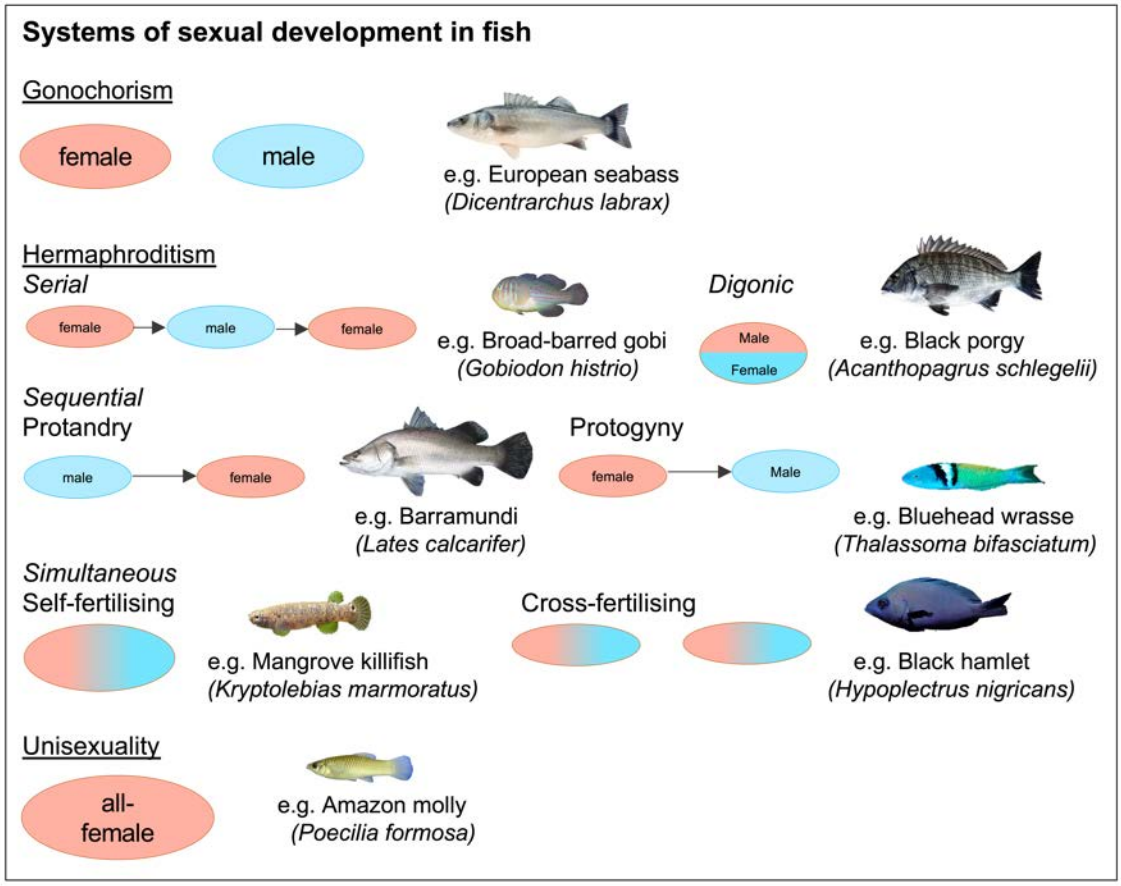

Figure 1.1 Illustration of the diverse systems of sexual development in fish. Orange ovals represent the occurrence of adult females with exclusively ovarian tissue, whereas blue ovals represent the occurrence of adult males with exclusively testicular tissue. Arrows depict where individuals can change between the two sexes (serial and sequential). Bi-colour filled ovals indicate the occurrence of both ovarian and testicular tissue in the gonads of a single individual, with gradient fill indicating synchronous functionality (simultaneous), and half-half fill indicating phase change (digonic). Examples of species employing each strategy are given to the right. Adapted from Heule *et al.* (2014).

1.3.1 Chromosomal and polygenic sex determination

Genetic sex determination can be grouped into two broad categories: chromosomal sex determination (CSD), where sex is governed by the inheritance of sex-related genes located on specific chromosomes (referred to as sex chromosomes), or polygenic sex determination (PSD) whereby sex determining genes are distributed throughout the entire chromosome complement, although major genetic effects are found in most cases (Martinez *et al.*, 2014). In fish, CSD can take the form of either male (XX/XY) or female (ZZ/ZW) heterogamety. CSD can also involve a loss of the derived sex chromosome (X0 or Z0), as in the Chilean galaxiid, *Galaxias platei* (Campos, 1972), while others demonstrate translocations or fusions of sex chromosomes with autosomes ($X_1X_1X_2X_2/X_1X_2Y$), as in the wolf fish, *Hoplias malabaricus* (Bertollo and Mestriner, 1998), or exhibit multiple, fully derived chromosomes (WXZ), as in the southern

platyfish, *Xiphophorus maculatus* (Devlin and Nagahama, 2002). In the majority of teleost fish species, however, sex chromosomes are weakly differentiated with only 7 % of species showing sex-associated chromosomal heteromorphism (Penman and Piferrer, 2008; Oliveira *et al.*, 2009). Whilst this lack of chromosomal differentiation is suggestive of a high occurrence of PSD in fish, there are very few examples in which this has been experimentally demonstrated. Examples include domesticated zebrafish, *Danio rerio* (Liew and Orban, 2014) and European seabass, *Dicentrarchus labrax* (Vandeputte *et al.*, 2007). The lack of heteromorphism between sex chromosomes in fish may instead be explained by the inability of traditional cytogenetic techniques to identify small scale differences, such as the inversions and deletions in the Y chromosome of threespine stickleback *Gasterosteus aculeatus* (Ross and Peichel, 2008), or the one single nucleotide polymorphism (SNP) variation between X and Y chromosomes in the tiger puffer fish, *Takifugu rubripes* (Kamiya *et al.*, 2012). Furthermore, whilst domesticated zebrafish are thought to exhibit PSD, sex chromosomes have been identified in wild type individuals, indicating that a shift in genetic mechanism has occurred in less than 100 generations (Wilson *et al.*, 2014). This potential for rapid evolutionary shifts, in conjunction with the documented variability in chromosomal arrangement between species, provides insight into the plasticity of sex determination and differentiation systems in fish; however, these lines of insight do not paint the full picture.

1.3.2 Sex determining and differentiating genes

Sex determining genes, whether located on specific sex chromosomes or not, can be considered as either upstream "master" switches, or downstream differentiators depending on their relative influence and positioning in sex determination and/or differentiation pathways. Whilst in mammals, sex determination is almost ubiquitously governed by a single gene, known as sex-determining region on the Y chromosome (*SRY*), teleost fish exhibit huge variety in their master sex-determining genes (Heule *et al.*, 2014). More specifically, seven master sex-determining genes have been identified and isolated to date in fish, including: 1) PG17: DM-domain gene on the Y chromosome (*dmY*) as the major testis-determining factor in the Japanese medaka, *Oryzias latipes* (XX/XY; Matsuda *et al.*, 2002; Nanda *et al.*, 2002); 2) anti-Müllerian hormone (*amhY*) in the Patagonian pejerrey, *Odentesthes hatcheri* (Hattori *et al.*, 2013) and Nile tilapia (Li *et al.*, 2015); 3) closely related anti-Müllerian hormone receptor, type II (*amhr2*) in the tiger puffer fish (Kamiya *et al.*, 2012); 4) gonadal somatic cell derived factor ($gsdf^{Y}$) in the Philippine medaka, *Oryzias luzonensis* (Myosho *et al.*, 2012); 5) sexually

dimorphic on the Y-chromosome gene (*sdY*) in the rainbow trout (Yano *et al.*, 2012); 6) SRY-related HMG-Box gene 3 (*sox3*) in the marine medaka, *Oryzias dancena* (Takehana *et al.*, 2014) and 7) doublesex and mab-3 related transcription factor 1 (*dmrt1*) in half-smooth tongue sole, *Cynoglossus semilaevis* (Chen *et al.*, 2014). These master sex-determining genes in fish are thought to have been co-opted from the network of downstream genes that in the mammalian SRY-determined and other vertebrate models are involved more specifically in sex-differentiation (Piferrer *et al.*, 2012).

Sex differentiating genes do not act as master switches, but function in combination with each other to generate alternative sexual phenotypes. As a general model for non-mammalian vertebrates, male sex-differentiation can be achieved through up-regulation of highly conserved transcription factor, *dmrt1*, which acts in combination with transcription factor *SRY-box 9 (sox9*) to promote testis formation and maintenance (Kent *et al.*, 1996; Smith *et al.*, 2009). Alternatively, female sexual differentiation is stimulated by cytochrome P450 aromatase (*cyp19a1a*) through a positive feedback loop involving the female-associated transcription factor forkhead box protein L2 (*foxl2*; Wang *et al.,* 2007; Guiguen *et al.,* 2010) . Other downstream differentiating genes, such as r-spondin 1 **(***rspo*) and wnt-signalling protein (*wnt*) in the ovary and anti-müllerian hormone (*amh)* and gonadal somatic cell derived factor (*gsdf)* in the testis, are thought to play important roles in promoting sexual differentiation and subsequent gonadal development via β-catenin and TGF-β signalling pathways, respectively (see Figure 1.2 for generalised male and female pathways).

Precisely how the downstream differentiators and, where present, master switches interact to coordinate gonadal development, particularly in the ovaries, remains a major question within the field of teleost sexual development (Nelson, 2006). Further complicating the question is that fish are not only the largest group of vertebrates, with over 33,000 named species, fish are also the only class to have undergone a third round of whole genome duplication (fish-specific genome duplication; FSGD). This genome duplication event has resulted in, for some genes, up to nine different alleles at a single locus (Meyer and Van de Peer, 2005). This large-scale genomic duplication is likely a major contributor to extensive speciation within the teleost group and have provided the genetic material necessary for the evolution of new gene functions and novel mechanisms of sexual development (Nelson, 2006; Nakamura *et al.*, 2012).

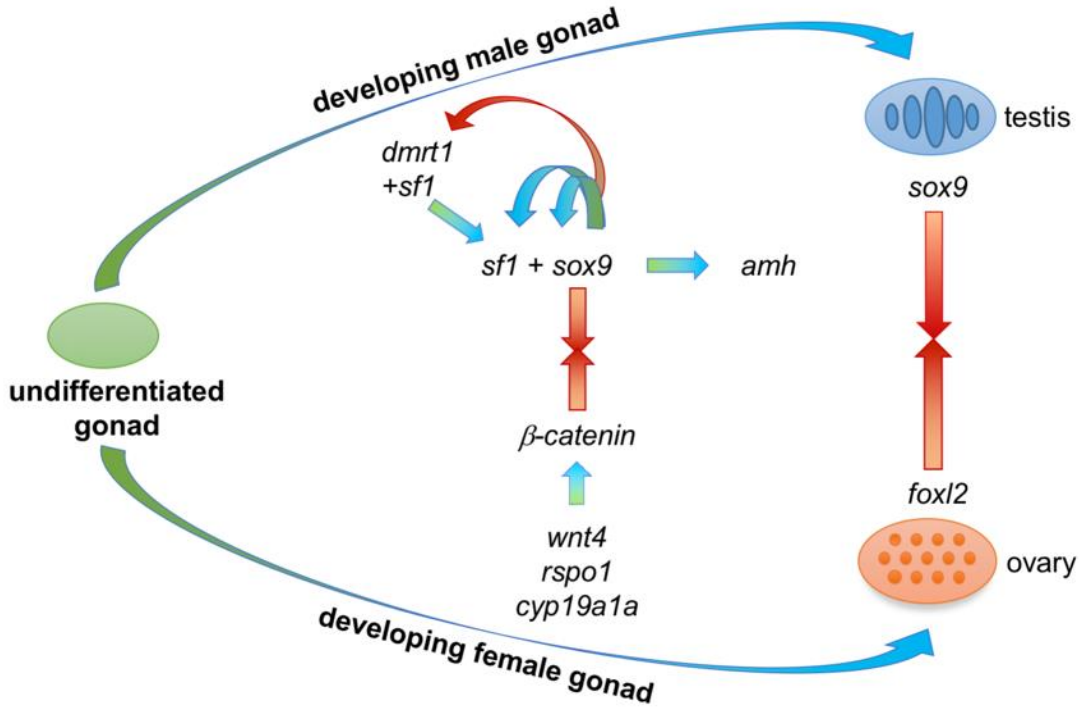

Figure 1.2 Generalised male and female pathways of mammalian gonadal sexual differentiation. Green/blue arrows represent positive regulation whereas red/orange arrows represent negative regulation. Adapted Sekido and Lovell-Badge (2009).

1.3.3 Environmental sex determination and differentiation

In contrast to genetic sex determination (GSD), true environmental sex determination (ESD) does not rely on sexual dimorphism at the genomic level (Liew and Orban, 2014). This is because the major determinant of sex is not genetic, but environmental. The best-known environmental determinant of sex is temperature (temperature-dependent sex determination; TSD), which has been well documented in many turtles and all crocodilians where egg incubation temperatures determine the development of either testes or ovary (Gilbert, 2000). For example, in the red-eared slider turtle, *Trachemys scripta*, egg incubation at temperatures of 31°C produces female offspring, and 26 °C produces male offspring (Ramsey *et al.*, 2007). Evidence for TSD also exists in fish, including popular South American aquaculture species, the Patagonian pejerrey. In this fish species, temperature treatments of 29 and 17 °C applied during development can be used to achieve all-male and all-female populations, respectively (Karube *et al.*, 2007). In most fish, however, the application of even the most extreme temperature treatments rarely produces all-male, or all-female progeny. For example, in European seabass temperature treatments of 20 and 15 °C can be applied to achieve

approximately 73 % male and 77 % female populations, respectively, but no temperatures are known to achieve 100 % offspring of either sex (Socorro *et al.*, 2007). An incomplete shift in the sex ratio towards either the male or female phenotype suggests that temperature is not the only determinant of sex in these cases (Ospina-Alvarez and Piferrer, 2008).

Incomplete shifts in sex ratio have also been observed for environmental sex determinants in fish, such as density, pH, social environment and hypoxia. In *Anguillidae* spp., a group of catadromous eels popular in Chinese aquaculture, high stocking densities produce a greater number of male eels (Davey and Jellyman, 2005). A reduction in stocking density is therefore advantageous for equal production of the sexes, however, where space is a limiting factor, periodic transfer from high to low density can also result in higher proportions of females, as well as increased growth rates (Wickström *et al.*, 1996). Acidity of culture water can also influence sex ratios. In the poeciliid green swordtail fish, *Xiphophorus helleri*, pH 6.2 and 7.8 produces all male and all female progeny, respectively (Rubin, 1985). However, in other poeciliids species such as *P. pulcher, P. subocellatus and P. taeniatus,* treatment with pH is unable to produce entirely monosex progeny (Rubin, 1985). Sensitivity to pH is also seen in cichlids. At pH 6.5, 96 % of *Apistogramma caetei* progeny develop as female (Römer and Beisenherz, 1996). In other species, social factors can also play a role. For example, isolated individual paradise fish, *Macropodus opercularis*, develop as male whereas in groups the proportion of developing females can be correlated with density (Francis and Barlow, 1993). Finally, sexual differentiation in zebrafish can be influenced by hypoxic conditions wherein 0.8 mg O_2 L^{-1} can produce largely male-biased populations (approximately 74 %), compared to control groups held at 5.8 mg O_2 L^{-1} (normoxic) whereby approximately 62 % develop as males (Shang *et al.*, 2006). Examples such as these, where less than 100 % of either sex is able to be obtained in progeny after treatment, are not thought to be true cases of ESD. Instead, the results suggest that the genotype of the individual is inhibiting complete control of the sexual differentiation pathway from environmental factors; a mechanism known as genotype by environment interactions (GxE; Figure 1.3).

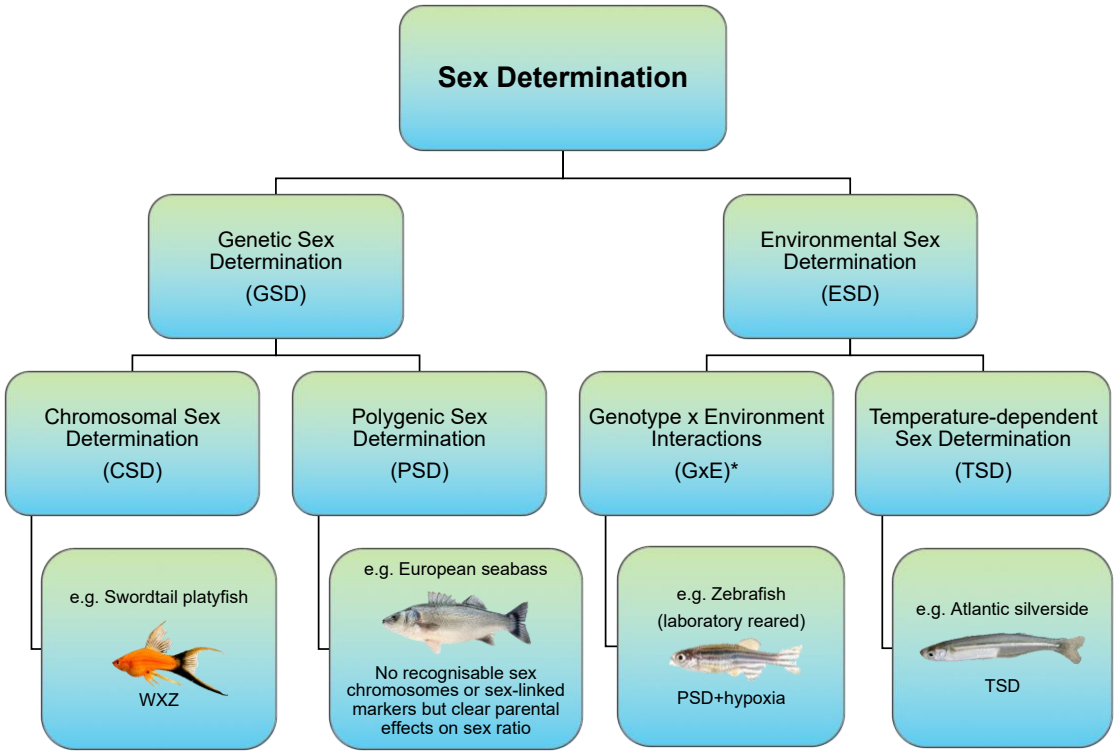

Figure 1.3 Overview of sex determining mechanisms in gonochoristic fish

1.3.4 A fine line between genetic and environmental sex determination

Traditionally, ESD and GSD have been approached as discrete categories (Figure 1.3), however, evolutionary transitions between different mechanisms and the prevalence of GxE interactions in fish has led to a more contemporary view that the two systems exist as polar ends of a continuum (Figure 1.4; Heule *et al.*, 2014; Shen and Wang, 2014). The evolutionary transition between the two ends of this continuum, GSD and ESD, can be quite rapid. For example, Atlantic silverside, *Menidia menidia*, previously thought to be a TSD species, are known to alter their sex determining mechanism in response to changing ecological pressures (Charlesworth, 1996; Sarre *et al.*, 2011). In this species, contribution of genetic cues and temperature determinants vary with latitude (Lagomarsino and Conover, 1993). At low latitudes TSD appears to be the primary determinant of sex. Here, females are produced at low temperatures that occur early in the breeding season and males are produced at higher temperatures that occur later in the breeding season (Conover and Present, 1990). The earlier production of females is thought to offer a longer growing period before the next breeding season starts and allow females to obtain a larger body size compared to the later-born, slightly younger males. Body size is thought to be more advantageous in females than males, as egg

production is more energetically expensive than sperm production (Conover and Heins, 1987). In contrast, populations of this species at higher and therefore more seasonal latitudes employ GSD. At these latitudes, the breeding season is comparatively shorter and the growing season longer, so the differences in the growth period for fish born at the start and end of the breeding season are negligible. Since the early birth of females has little advantage, sex ratios are maintained at a 1:1 level and there is no evidence for temperature sensitivity in these populations (Conover and Present, 1990). The GSD to ESD transition of Atlantic silverside illustrates the ability of individual species to undergo rapid evolutionary shifts in sex-determining mechanisms and emphasises that sensitivity to temperature can evolve (or not) based on differences in the ecological relevancy of temperature in a given species' habitat.

The idea that sensitivity to environmental factors is retained due to ecological context is not only supported by the Atlantic silverside, but has been observed in many other fish species (Baroiller *et al.*, 2009b). For example, European seabass (a temperate oceanic species) are sensitive to temperature, but not density, or pH (Saillant *et al.*, 2003). Tilapia (a large tropical freshwater cichlid) are sensitive to temperature, but not salinity, density or confinement (Baroiller *et al.*, 2009b), and *Apistogramma caetei* (a small tropical freshwater cichlid) are senstive to pH, but not temperature (Römer and Beisenherz, 1996). These fish are thought to show selective sensitivity to the precise environmental factors that fluctuate most significantly in the habitats in which they have evolved (Baroiller *et al.*, 2009b). However, it has also been suggested that such plasticity can be retained due to ecological *irrelevancy,* whereby sensitivity to certain factors that do not fluctuate in the species' habitat are retained in the genome because they are neutral (Baroiller *et al.*, 2009b). An example of this is the insensitivity to temperatures experienced in the wild, but some responsiveness to extreme laboratory derived temperatures in the otherwise GSD medaka (Shinomiya *et al.*, 2004; Sato *et al.*, 2005). A similar scenario occurs where species retain sensitivity to a broad range of factors, none of which are likely to fluctuate significantly in the environment in which a given species has evolved. This is the case in zebrafish where temperature, density, and hypoxia can all alter sex ratios in laboratory-reared fish, but are unlikely to be observed in the wild (Nusslein-Volhard and Dahm, 2002; Uchida *et al.*, 2004; Shang *et al.*, 2006; Lawrence *et al.*, 2008). Whilst species like the seabass and tilapia show sensitivity to specific, ecologically relevant environmental cues, medaka and zebrafish show either unspecific or weak sensitivity to cues that are unlikely to be encountered and may be ecologically irrelevant (Baroiller *et al.*, 2009b). These examples demonstrate that

while classifying fish in terms of GSD, ESD, and GxE can be useful, it is important to recognise that these classifications demonstrate discrete points along a continuum, and that the mechanisms of sexual development in fish often retain their plasticity and can change over time, depending on environmental (ecological or experimental) factors.

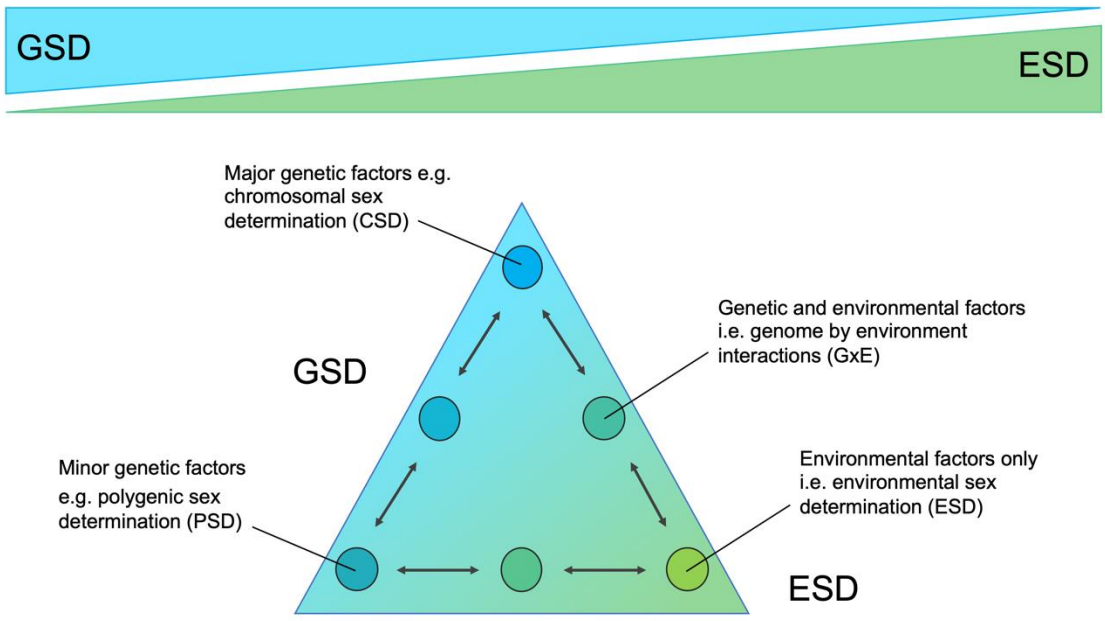

Figure 1.4 Theoretical illustration of the continuum between genetic (GSD) and environmental sex determination (ESD), emphasising the major types of sex determining mechanisms present in gonochoristic fish. Arrows indicate that evolutionary transition from one to another type of sex determination is possible. Sex determination in a given species (circles) can be explained by one or the combination of several effects. Any combination of the three main effects (chromosomal sex determination; CSD, polygenic sex determination; PSD and ESD) is theoretically possible. Adapted from Penman and Piferrer (2008).

1.3.5 Sex reversal and the labile period

Sensitivity to environmental factors in fish allows for sex reversal, a term here used to describe cases in which phenotypic sex no longer corresponds to genotypic sex following experimental manipulation. This experimental phenomenon provides insight into mechanisms of sexual differentiation and is of great importance to the development of sex control methods for aquaculture. For example, in the half-smooth tongue sole, individuals with ZW chromosomes (genotypically female) may exhibit testis formation, as well as other male-specific secondary sexual characteristics (phenotypically male) following temperature treatment (Shao *et al.*, 2014). It is not only the type (e.g. temperature, pH, hypoxia), but also the timing of treatments able to induce sex reversal that varies greatly between species. Specifically, there is a discrete

time period during development, often referred to as the labile period (also sensitive window or sensitive period; Figure 1.5), during which the gonad undergoes differentiation towards the male or female state (Baroiller *et al.*, 2009b). Often temperature, hormone, or other treatments become ineffective following this period of differentiation, once the gonads have become established and sex is stabilised (Valenzuela *et al.*, 2003; Ospina-Alvarez and Piferrer, 2008). In contrast to TSD reptiles, whose temperature sensitive window most often occurs *during* gonadal differentiation (Mrosovsky and Pieau, 1991), there appears to be a delay between timing of the labile period and the onset of sexual differentiation in fish (Baroiller and D'Cotta, 2001). In fish, temperature treatments are effective when applied *prior* to the onset of sexual differentiation, but the exact timing differs between species (Baroiller and D'Cotta, 2001). In the European seabass, for example, temperature treatments may be applied at approximately 1 - 64 days post fertilisation (dpf) to achieve high female sex ratios (Pavlidis *et al.*, 2000). In many tilapia species, however, temperature treatments should be applied at 10 - 20 dpf to ensure they are within the temperature sensitive period (Baroiller *et al.*, 2009a). Both the ecological drivers and molecular processes underlying the observed differences in the timing of the labile period between fish and reptiles, as well as between different species of fish, remain largely unresolved.

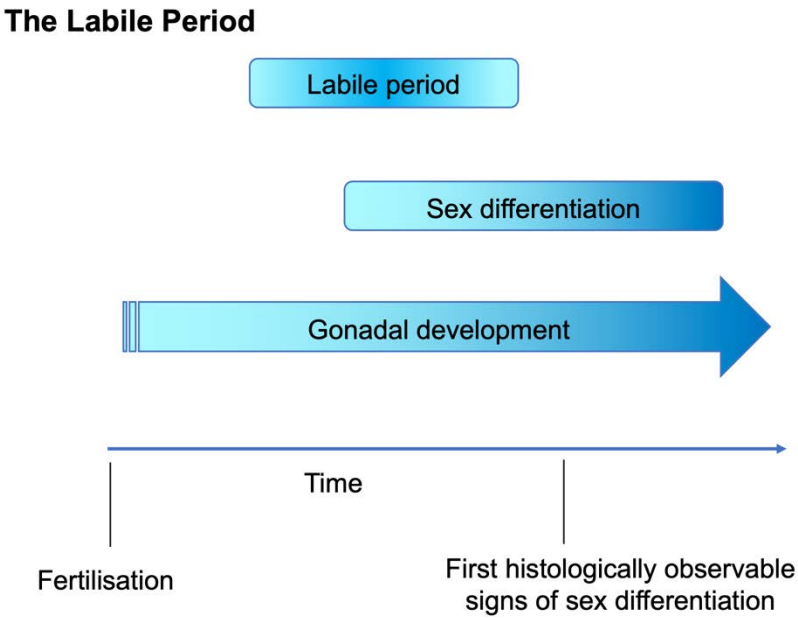

Figure 1.5 Diagrammatic representation of the labile period concept in relation to gonadal development and sexual differentiation in fish. In the diagram, the horizontal line represents development progressing with time, and includes the events of fertilization and first observable signs of sexual differentiation, for reference. Shading indicates progression of each developmental period. Adapted from (Piferrer, 2001).

1.3.6 Sequential hermaphroditism in fish

In gonochoristic species, individuals develop as either male or female and do not typically change sex at any time during their life. Sex, once established, is stable. In sex-changing fish, however, the gonads remain responsive to endogenous and/or exogenous environmental stimuli well into adulthood. The triggers, timing, and directionality of sequential sex change vary greatly between species.

Of the documented cases of sequential hermaphroditism in fish, many appear to be under social control including clownfish, grouper and wrasse (Robertson, 1972; Munday *et al.*, 2006). Protogynous systems involve female to male sex change are usually characterised by high rates of polygyny (a single male with multiple female partners). Polygynous systems result in strong social structures dominated by large males that either outperform smaller males in group-spawning, or control harems of females (Taborsky, 1994; Benvenuto *et al.*, 2017). Removing the dominant male in these groups usually induces rapid sex change in the largest female in the group, who takes up the position as the dominant male in the social hierarchy [as seen in the

bluehead wrasse, *Thalassoma bifasciatum*, Warner and Swearer (1991)]. Conversely, protandrous systems involve male to female sex change and are characterised by monogamous mating (one mating partner at a time) and their populations are typically predominated by small males and fewer, but larger and highly fecund females (Benvenuto *et al.*, 2017). For example, in the protandrous Red Sea clownfish, *Amphiprion bicinctus*, social assemblages are characterised by the presence of one large dominant female, a single mature male, and a number of smaller immature non-breeding juveniles (Fricke and Fricke, 1977). If the dominant female dies the mature male rapidly changes sex and the largest of the immature juveniles sexually differentiates into a functional male (Fricke and Fricke, 1977; Moyer and Nakazono, 1978). In socially sex changing fish, social cues are thought to be transmitted from the brain to the gonad, which stimulates hormonal changes and induce sequential sex change (Nakamura *et al.*, 1989; Godwin *et al.*, 2003).

Other protandrous fish are known to employ random or near random mating (e.g. mass spawning), which does not demand strong social structures (Warner, 1975; Shapiro, 1987). In these fish, sex change occurs upon attainment of a certain age or size, often taking place during or just after the spawning season, although the exact triggers are unknown (Wu *et al.*, 2010). For example, in barramundi, *Lates calcarifer*, male to female sex change occurs directly after spawning at approximately 60-90 cm in length and 3-8 years of age, depending on geographic location (Davis, 1982; 1984b; Guiguen *et al.*, 1994). In the protandrous white seabream, *Diplodus sargus*, sex change also occurs post-spawning season, generally in fish between 20 - 25 cm in length and 2-5 years of age, but not in all individuals (Micale and Perdichizzi, 1994; Benchalel and Kara, 2013). In the digonic black porgy, *Acanthopagrus schlegelii*, individuals function as males for the first 2 years and sex change to female at 3 years of age, during the third spawning season (Chang and Yueh, 1990; Wu *et al.*, 2010). In this species, both testis and ovarian tissue develop, but the switch from functioning testis to ovaries only occurs in 30-50 % of the population. It is likely that sex change in these species is driven by an alteration in circulating hormones that takes place concurrently with the breeding season, but specifically what triggers these changes in circulating hormones in the brain and gonad around a specific spawning season in specific individuals, is unknown.

Sex change may also occur due to a combination of both social factors (e.g. haremic species) and the attainment of a minimum body size (e.g. mass spawners). More specifically,

protogynous group spawning takes place in groups typically comprised of multiple dominant males and subordinate females. In the dusky grouper, *Epinephelus marginatus*, fish undergo female to male sex change throughout the breeding season in fish that are approximately 90 cm in length and 9-10 years of age (Marino *et al.*, 2001). In cultured orange-spotted grouper, *Epinephelus coioides*, fish undergo female to male sex change at about 65-75 cm in length; however, transferring fish that were initially stocked at low densities to high density tanks can lead to earlier production of males in this species (Quinitio *et al.*, 1997). Furthermore, rearing orange-spotted grouper in isolation can lead to primary male maturation, with approximately 39 % of experimental juveniles maturing first as males, compared with less than 5 % primary males obtained under typical culture conditions (Liu and Sadovy de Mitcheson, 2011). In the half-moon grouper, *Epinephelus rivulatus*, sex change occurs at approximately 22-35 cm in length, but was found to have a degree of social control exhibited by a threshold sex ratio that is maintained by the suppressive dominance of large males over smaller females (Mackie, 2000; Mackie, 2003). This suggests that sex change is likely dependent upon the attainment of a critical body size, but is also influenced by social structure within a spawning group (Garcia *et al.*, 2013). The degree of social structure within the spawning group of a given species is likely linked to the extent that sex control in that species is governed by social cues (Table 1.1).

Table 1.1 Summary of spawning and sex change strategies in hermaphrodite fish.

Species	Reproductive Strategy	Spawning Method	Mating is random/ non-random	Direction of sex change	Stimulus
Bluehead wrasse *Thalassoma bifasciatum*	Polygyny (multiple female partners)	Haremic	Non-random	Protogyny (♀-to-♂)	Social factors
Red Sea clownfish, *Amphiprion bicinctus*	Serial monogamy (one partner at a time)	Partnered	Non-random	Protandry (♂-to-♀)	Social factors
Barramundi aka Asian seabass *Lates calcarifer*	Polygynandry (multiple male and female partners)	Mass	Random	Protandry (♂-to-♀)	Size/age
White Seabream *Diplodus sargus*	Polygynandry (multiple male and female partners)	Mass	Random	Protandry (♂-to-♀)	Size/age
Black Porgy *Acanthopagrus schlegelii*	Polygynandry (multiple male and female partners)	Serial mass	Random	Digonic Protandry (♂-to-♀)	Size/age
Orange-spotted grouper *Epinephelus coioides*	Polygyny (multiple female partners)	Group	Non-random	Protogyny (♀-to-♂)	Size/age +social factors
Dusky grouper *Epinephelus marginatus*	Polygyny (multiple female partners)	Group	Non-random	Protogyny (♀-to-♂)	Size/age +social factors
Half-moon grouper *Epinephelus rivulatus*	Polygyny (multiple female partners)	Group	Non-random	Protogyny (♀-to-♂)	Size/age +social factors

1.3.7 Sexual differentiation in hermaphrodite fish

While sex determination in hermaphrodites is usually governed by external factors, processes of sexual differentiation and sex change appear to involve the same suite of genes that gonochoristic fish use for their sexual development as well (Wang *et al.*, 2018). In protogynous fish, sex change is characterised by a shift from an estrogenic to an androgenic environment, which is initiated by a significant drop in the female dominant sex hormone oestradiol (E2), followed by an increase in the male dominant sex hormone 11-ketotestosterone (11-KT) during the final stages (Nakamura *et al.*, 1989; Bhandari *et al.*, 2003; Todd *et al.*, 2016). Accordingly, although less convincingly [see Larson (2011)], the opposite effect is observed in protandrous sex change where androgen levels, including 11-KT, stay high throughout the sex change

process and a transition to an estrogenic environment occurs, with E2 rising in the final stages of sex change once the ovaries are formed (Figure 1.6; Nakamura *et al.*, 1989). These changes in circulating hormones in the brain and gonad, are underpinned by changes in the genes from which these hormones are derived.

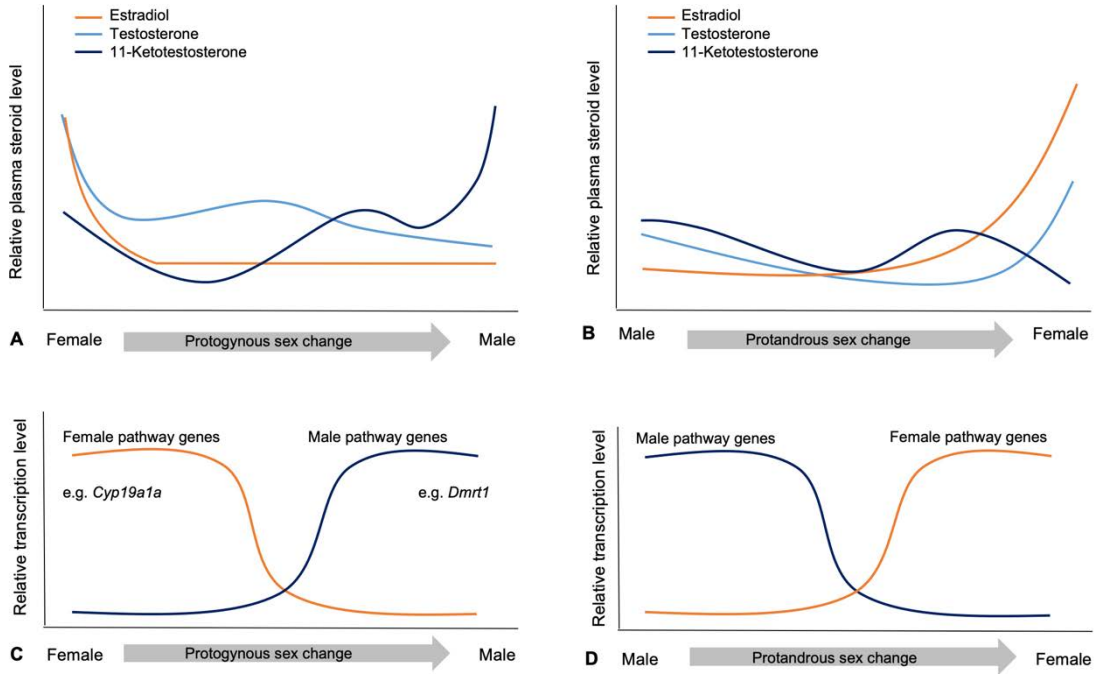

Figure 1.6 Typical shifts in steroid hormone and gene expression profiles during protogynous (left) and protandrous (right) sex change. A. Shows hormone profiles shifting from estrogen-dominated to androgen-dominated environment and B. showing the opposite pattern. C. Shows the concurrent changes in gene expression, with progressively upregulated male-associated genes and downregulated female-associated genes and D. displays the opposite effect. Adapted from Todd *et al.* (2016).

E2 levels in the gonad are strongly correlated with the highly conserved vertebrate sex determining gene *cyp19a1a*. *Cyp19a1a* encodes for gonadal aromatase, an enzyme that irreversibly converts androgens (male dominant hormones) to estrogens (female dominant hormones) and is known to play a pivotal role in female development in gonochoristic fish and is likely a trigger for sex change in sequential hermaphrodites (Guiguen *et al.*, 2010). In protogynous wrasse, the inhibition of expression in the aromatase gene leads to progressive downregulation of other female-associated genes, which results in gene expression profiles becoming increasingly male biased (Liu *et al.*, 2015). The *cyp19a1a* promoter region contains DNA binding motifs for a number of important transcription factors, such as forkhead box protein L2 (*foxl2*), Steroidogenic factor 1 (*Sf1*), SRY-box (*Sox)* proteins and Wilms Tumour 1

(*Wt1*; Piferrer *et al.,* 2005; Huang *et al.,* 2009; Guiguen *et al.,* 2010). In the Japanese flounder, *Paralichthys olivaceus*, the *cyp19a1a* promoter region also contains a binding motif for the transcription factor *dmrt1,* the dominant gene in the male sex determining pathway (Wen *et al.*, 2014). *Foxl2* is a highly conserved sex-related transcription factor that activates *cyp19a1a* expression in the gonad, resulting in E2 synthesis which, in turn, regulates *foxl2* via a positive feedback loop (Figure 1.7; Wang *et al.*, 2007). Indeed, in the protandrous clownfish, *Amphiprion bicinctus,* and black porgy, *foxl2* and *cyp19a1a* display increasing, parallel expression profiles during testis to ovary transition (Wu *et al.*, 2008; Casas *et al.*, 2016). Accordingly, during female to male sex change in the protogynous honeycomb grouper, *Epinephelus merra*, *foxl2* exhibited decreasing expression profiles, whilst male-associated gene *dmrt1* increased in expression (Bhandari *et al.*, 2003; Alam *et al.*, 2008). This is similar to the bluehead wrasse, wherein *foxl2* and *cyp19a1a* expression decreased, and *dmrt1* increased during female to male sex change; however, there was a sharp increase in expression of both *cyp19a1a* and *foxl2* in the early stages of sex change (Liu, 2016). Since increases in *dmrt1* tend to occur during late female to male sex change, it is likely that this is a consequence of decreased E2 in circulation, rather than the cause.

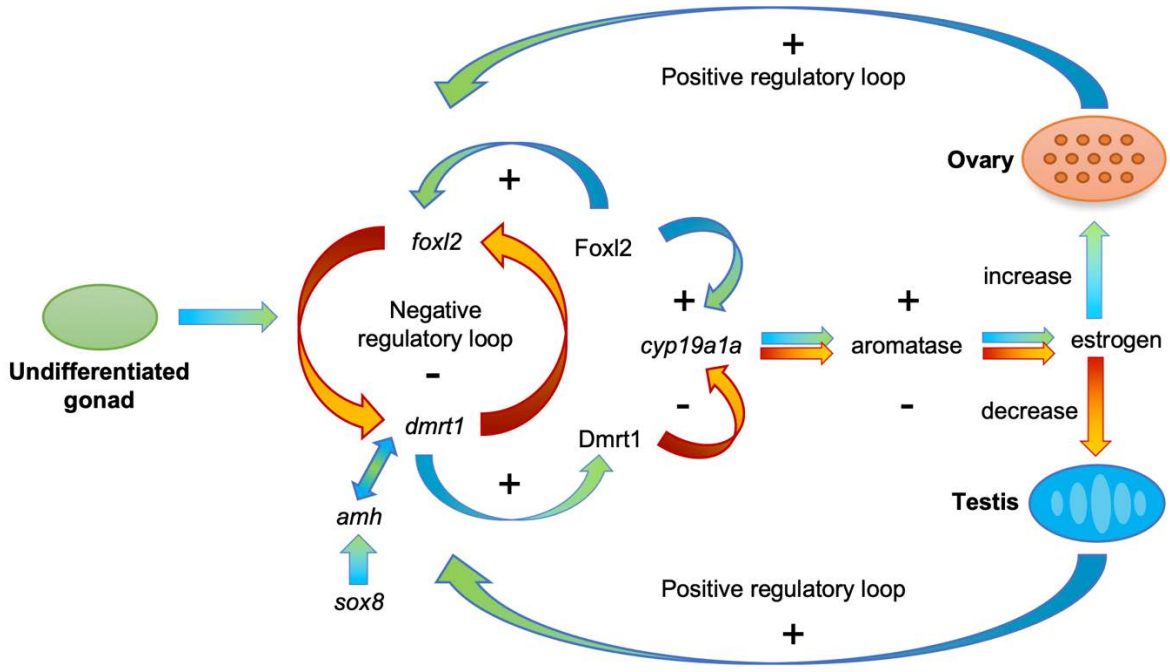

Figure 1.7 Schematic representation of proposed genetic mechanism underlying sex change in *Amphiprion bicinctus* gonad. Arrows represent the direction of gene and protein regulation in females (upper) and males (lower). Red/orange arrows and plus signs indicate up-regulation, blue/green arrows and minus signs indicate down-regulation. Adapted from Casas *et al.* (2016)

1.4 Epigenetics of sex determination and differentiation in fish

One of the most interesting and challenging questions within the field of vertebrate sex determination is: How do fish change sex? All other vertebrates are known to employ the reproductive mode of gonochorism, where sexual phenotype is typically both binary and static. Conversely, about 5 % of fish species employ hermaphroditism and can house both male and female gonads simultaneously, or undergo sequential sex change to switch between the two gonadal phenotypes. Furthermore, in gonochoristic fish a phenomenon called sex reversal may be stimulated by experimental treatments such as hormones, pH and temperature, which override their genetically encoded sex. In both cases, differences in sexual phenotype occur in the absence of differences in genotype, with both testicular and ovarian tissue developing from the same genetic template. The ability of cells and tissues to differentiate into different phenotypes based on a single genotype, particularly in response to environmental cues, is a hallmark of epigenetic control.

Epigenetics is a relatively new, but rapidly expanding field in biology that investigates changes in gene function that cannot be explained by changes in the DNA sequence (Russo *et al.*, 1996). Epigenetic changes largely act to enable or inhibit the activity of transcriptional machinery to regulate patterns of gene expression and can be influenced by environmental stimuli (Turner, 2009). The term epigenetics translates to 'above' the 'genes' and refers to changes in the structural or chemical composition of chromatin and, more specifically, the DNA and histone proteins from which chromatin is formed, rather than the sequence of DNA itself that act to modify gene regulation. Structural modifications are linked to gene expression and transcriptional regulation through their ability to affect chromatin structure at regional and genome-wide levels [through the formation of euchromatin (less condensed, accessible for transcription) or heterochromatin (more condensed, silenced)] and/or affect the accessibility of DNA to transcriptional complexes (such as DNA polymerase and transcription factors) at the local level. Epigenetic modifications can be classed into three broad categories: 1) DNA methylation; 2) histone modifications; and 3) noncoding RNAs (Figure 1.8). Given their structural nature and susceptibility to environmental stimuli, epigenetic modifications are likely the key mechanism allowing for external factors to stimulate sex reversal and/or sex change in fish, in the absence of genetic change (Piferrer, 2013).

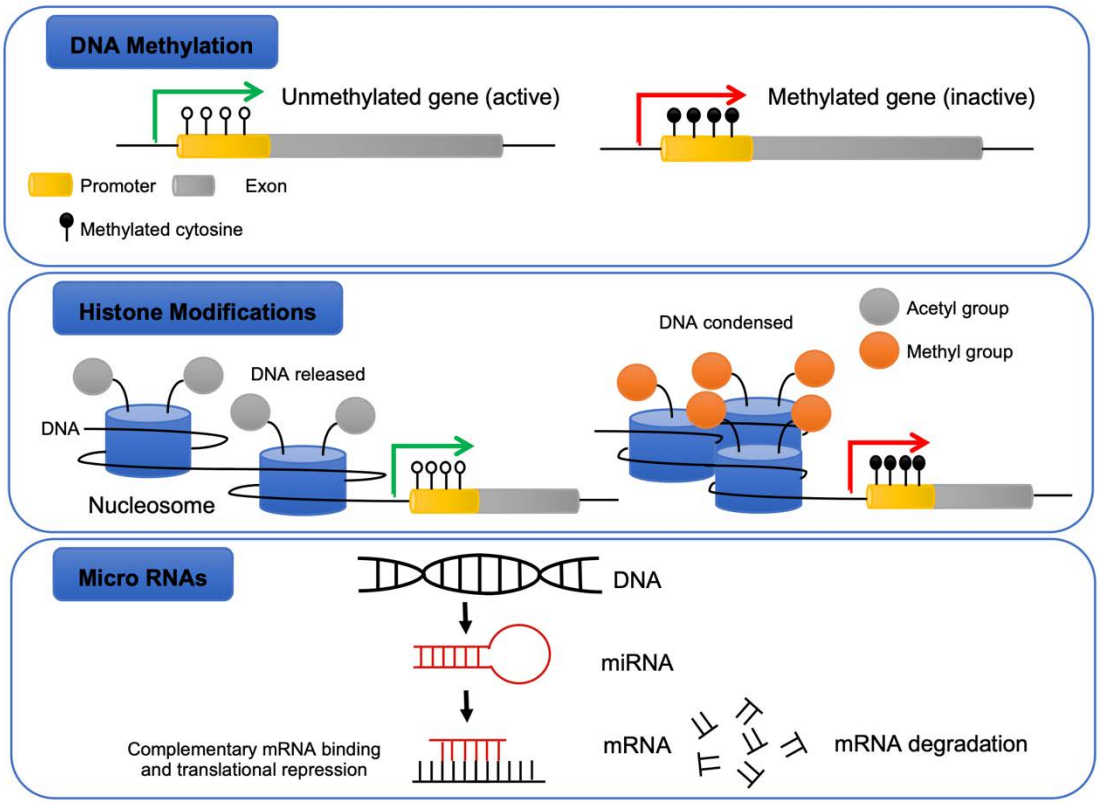

Figure 1.8 Three broad classes of epigenetic modification: 1) DNA methylation can lead to transcriptional silencing by making the gene inaccessible to transcriptional machinery (top panel); 2) Histone modifications involve a variety of post-translational modifications to a histone's N-terminal tails, including methylation and acetylation, which can lead to the active transcription of DNA around loosely packed histones, or repressed transcription of DNA around tightly packed histones (middle panel); 3) Non-coding RNAs can regulate gene expression through post-transcriptional silencing of target genes, for example miRNAs undergo sequence-specific base pairing resulting in target degradation and/or inhibition of translation (bottom panel). Adapted from D'addario *et al.* (2013).

1.4.1 DNA methylation

DNA methylation involves the replacement of the fifth carbon of cytosine with a methyl group almost exclusively at CpG dinucleotides, where cytosine (C) is phosphate-bonded (p) to guanidine (G). CpG's can be differentially methylated to allow tissue-specific or sexually dimorphic gene expression through the activation or repression of the associated downstream gene (Gardiner-Garden and Frommer, 1987). This is because, at the genomic level, the methylated CpGs limit the accessibility of the target gene by transcriptional machinery which inhibits gene expression (Figure 1.9).

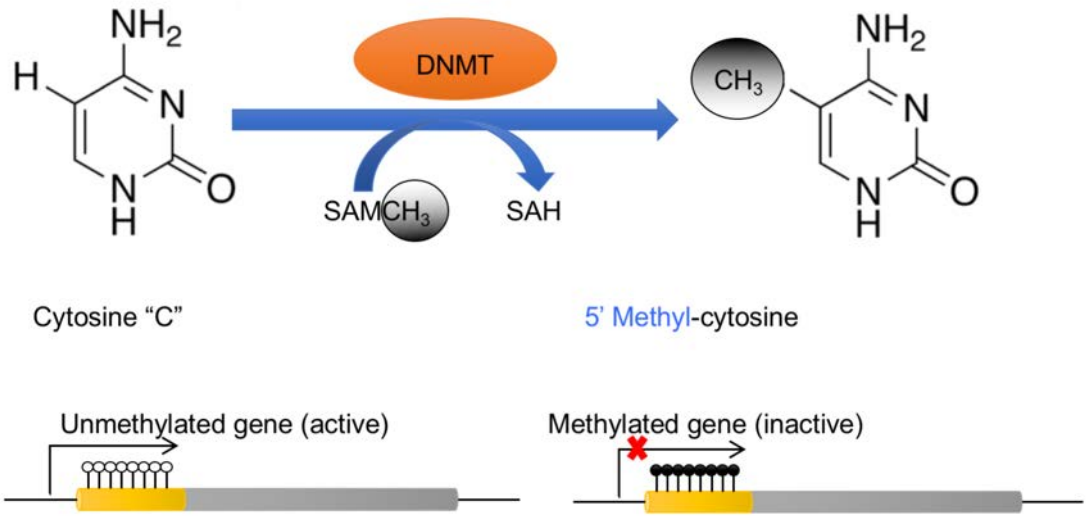

Figure 1.9 Schematic representation of 5mC DNA methylation, which converts cytosine to 5′methyl-cytosine via the actions of DNA methyltransferases (DNMTs). SAM = S-adenosylmethionine; SAH = S-adenosylhomocysteine. Yellow represents predicted promoter region, where it is predicted that methylation is most critical to inhibition of transcription. Grey represents the genes coding sequence. Adapted from (Zakhari, 2013)

DNA methylation was first proposed by Gorelick (2003) as the mechanism allowing organisms with homomorphic sex chromosomes (i.e. chromosomes that are indistinguishable by karyotype data alone) to alter their gender in response to small environmental changes, as occurs in environmental sex determination. Support for this hypothesis is building rapidly. While it is likely that all three epigenetic mechanisms work in combination, methods of analysis for DNA methylation specifically are the most well established and the resultant data provide some of the clearest examples of how epigenetics bridges the gap between phenotypic sex and environmental change. Relevant cases in fish, particularly in relation to temperature-induced sex reversal and/or hermaphroditic sexual development, are discussed below.

1.4.2 Case studies in gonochoristic species

In gonochoristic vertebrates, sex is typically determined genetically, either via specific sex chromosomes (chromosomal sex determination; CSD), or via sex-related genes spread throughout the chromosome complement (polygenic sex determination; PSD). However, sex can also be determined solely by environmental factors, such as temperature (temperature-dependent sex determination; TSD), or a combination of both genetic and environmental

factors (genotype by environment interactions; GxE; see section 1.3 and Figure 1.3). GxE interactions are not only of biological interest, but are of significant economic importance in many aquacultured species, as understanding the effects of culture environment on sex ratios offers greater reproductive control and can enable biased production of the better performing sex. Over the last decade, research into temperature-mediated sex reversal (a form of GxE) of many cultured species has revealed that changes in phenotypic sex are often accompanied by changes in DNA methylation of key sex genes (Table 1.2).

Table 1.2 Summary of case studies in fish sexual development on temperature, DNA methylation and gene expression for *dmrt1* and *cyp19a1a* in teleost fish. NS = not significant and NR = not reported. NB: analysis methods differ between studies and not all gene targets were studied in full.

Species	Sexual System	Temp. effects?	Gene target/s	DNA methylation	Gene expression	Citation
Gonochoristic species						
European sea bass *Dicentrarchus labrax*	Polygenic SD with GxE	HT = ♀-to-♂ sex reversal LT = 15°C HT = 21°C	*cyp19a1a* *dmrt1*	↑ with ♀-to-♂ sex reversal NR	↓ with ♀-to-♂ sex reversal ↑ with ♀-to-♂ sex reversal	Navarro-Martin *et al.* (2011) Diaz & Piferrer (2015)
Half-smooth tongue sole *Cynoglossus semilaevis*	Chromosomal SD (ZW) with GxE	HT = ♀-to-♂ sex reversal LT = 22°C HT = 28°C	*cyp19a1a* *dmrt1*	↑ with ♀-to-♂ sex reversal ↓ with ♀-to-♂ sex reversal	↑ with ♀-to-♂ sex reversal ↑ with ♀-to-♂ sex reversal	Shao *et al.* (2014)
Japanese flounder *Paralichthys olivaceus*	Chromosomal SD (XY) with GxE	NR	*cyp19a1a* *dmrt1*	↓ ovary vs. testis ↑ ovary vs. testis	↑ ovary vs. testis ↓ ovary vs. testis	Wen *et al.* (2014)
Nile tilapia *Oreochromis niloticus*	Chromosomal SD (XY) with GxE	HT = ♀-to-♂ sex reversal LT = 28°C HT = 36°C	*cyp19a1a* *dmrt1*	↑ with ♀-to-♂ sex reversal ↓ with♀-to-♂sex reversal*	↓ with ♀-to-♂ sex reversal ↑ with♀-to-♂ sex reversal	Wang *et al.* (2019)
Hermaphrodite species						
Ricefield eel *Monopterus albus*	Sequential protogyny (♀-to-♂sex change)	NR	*cyp19a1a*	↑ with ♀-to-♂ sex change	↓ with ♀-to-♂ sex change	Zhang *et al.* (2013a)

			dmrt1a, b	NR	↑ with ♀-to-♂ sex change	
			dmrt1d	NR	No change with ♀-to-♂ sex change	
Bluehead wrasse *Thalassoma bifasciatum*	Sequential protogyny (♀-to-♂ sex change)	NR	*cyp19a1a*	↑ with ♀-to-♂ sex change	↓ with ♀-to-♂ sex change	Todd *et al.* (2019)
			dmrt1	↓ with ♀-to-♂ sex change	↑ with ♀-to-♂ sex change	
Black Porgy *Acanthopagrus schlegelii*	Digonic protandry (♂-to-♀ sex maturation)	NR	*cyp19a1a*	↓ with ♂-to-♀ sex change	↑ with ♂-to- ♀sex change	Wu *et al.* (2016)
			dmrt1	NR	↓ with ♂-to- ♀sex change	Wu *et al.* (2012)
Mangrove killifish *Kryptolebias marmoratus*	Simultaneous (♂and ♀)	LT = ⚮ -to- ♂ sex reversal LT = 18°C HT = 25°C	*cyp19a1a*	↓ High temperature (HT) testis and ovotestis ↑ LT testis and ovotestis	↓↓ HT testis ↓ LT testis ↑LT ovotestis (NS)	Ellison *et al.* (2015)
			dmrt1	↑ ovotestis vs. testis (untreated)	NR NR	
				↑ ovotestis vs. testis (untreated)		

1.4.2.1 The European seabass

The European seabass, *Dicentrarchus labrax,* is a popular marine food fish with a polygenic sex determination system influenced by temperature (Vandeputte *et al.*, 2007). While rearing seabass at high temperatures (> 17 °C) leads to faster growth, it also leads to a greater percentage of male offspring (~ 75 - 95 %). Male seabass are unfavourable to aquaculture due to slower comparative growth rate and precocious sexual maturation (Saillant *et al.*, 2001). Using this species as an experimental model, Navarro-Martin et al. (2011) were able to show firstly, that the promoter of gonadal *cyp19a1a* had double the DNA methylation in males

compared to females. Secondly, the study revealed that exposure of juveniles to high temperatures (21 °C) during the thermosensitive period (0 - 60 dpf) increased DNA methylation levels, inducing testis development and increasing the male to female sex ratio. Methylation of *cyp19a1a* is thought to inhibit *cyp19a1a* expression and subsequent production of the aromatase enzyme, inhibiting catalysis of the conversion of androgens into estrogen in these fish (Navarro-Martín *et al.*, 2011). This was the first demonstration that an epigenetic mechanism was mediating temperature effects on sex ratios in any vertebrate. Further analysis by the same research group later revealed that while expression of *cyp19a1a* is consistently different between seabass from temperature treatments and persists long after the treatment period, gene expression in a closely related gene, *cyp19a1b,* showed no difference (Díaz and Piferrer, 2015). The key male-associated gene *dmrt1* showed the expected, opposite gene expression pattern (higher expression in males, lower in females), but actual methylation patterns of *dmrt1* were unreported (Díaz and Piferrer, 2015). While analysis of DNA methylation patterns in additional genes, particularly *dmrt1*, would be advantageous, the relationship between temperature, sex, DNA methylation and gene expression in European seabass *cyp19a1a* provided the first clue as to the mechanism by which temperature may be operating to alter sex ratios in fish.

1.4.2.2 The half-smooth tongue sole

The half-smooth tongue sole, *Cynoglossus semilaevis,* is an east Asian food fish of high economic value that possesses a ZZ/ZW chromosomal sex determining system. Female tongue sole grow faster and 2-4x larger than males, generating great interest in understanding this species' sex determination system and enable the production of a greater proportion of female stock (Song *et al.*, 2012). Under common culture conditions (22 °C) approximately 14 % of genetic ZW females develop as phenotypic males. Under high temperatures (28 °C), however, approximately 73 % of ZW females develop as phenotypic males (Chen *et al.*, 2014). Shao et al., (2014) found that global DNA methylation patterns in the testis of temperature-induced males and genetic males were similar, but clearly distinguishable from ovary, producing the second known example of how epigenetics was linked to sexual differentiation in vertebrates. Specifically, exposure to high temperature during embryonic development decreased DNA methylation and increased expression of the key testis-determining gene *dmrt1* and the related gene *gsdf,* promoting male development in ZW fish (Shao *et al.*, 2014). Notably, not all genes examined demonstrated an inverse relationship between methylation and expression. For

example, despite an increase in methylation of *cyp19a1a* in the testis, an increase in *cyp19a1a* expression was also observed (Shao *et al.*, 2014). This is in contrast to the European seabass where an increase in methylation of *cyp19a1a* led to a decrease in expression and subsequent testis development (Navarro-Martín *et al.*, 2011). Instead, testis development in ZW (genetically female) tongue-sole arose from demethylation and subsequent activation in expression of *dmrt1* (Graves, 2014).

1.4.2.3 The Japanese flounder

The Japanese flounder, *Paralichthys olivaceus*, is a species of critical importance to Chinese mariculture with an XX/XY sex determination system. As in the European seabass and the tongue sole, female flounder grow faster than males, and as such, Japanese flounder have been the subject of numerous experiments to produce temperature treated sex reversed (XX) males and enable faster growth (Kitano *et al.*, 1999). In this species, it has been shown that *dmrt1* expression is 70 x higher in the testis than ovary, and *cyp19a1a* expression 40 x higher in the ovaries than the testis (Wen *et al.*, 2014). These differences in gene expression were accompanied by strongly sexually dimorphic patterns of DNA methylation, which were higher in ovary *dmrt1* (~ 60:0 %, ovary to testes) and higher in testes *cyp19a1a* (~ 100:70 %, testes to ovary; Wen et al., 2014). Furthermore, during ovarian development DNA methylation of *cyp19a1a* and its regulatory transcription factor *foxl2* are inversely related to gene expression, resulting in increased circulating estrogen (Si *et al.*, 2016). These results suggest that DNA methylation is assisting the suppression of testis-associated genes in ovary and ovary-associated genes in testis. It would be interesting to examine whether these methylation patterns, which appear to be consistently inversely correlated to the expression quantities of their target genes, are influenced by temperature treatments during differentiation.

1.4.2.4 Nile tilapia

Nile tilapia, *Oreochromis niloticus*, are a freshwater cichlid of African origin that have been extensively translocated throughout the tropics and are one of the world's most important food fish species (Fitzsimmons, 2000). *O. niloticus* possesses an XX/XY chromosomal sex determining system that can be manipulated with high temperature (36 °C) treatment during early development to produce a greater proportion of males, which typically demonstrate faster growth rate compared to females (Baras *et al.*, 2001). In temperature-induced XX male tilapia, global patterns of DNA methylation and gene expression are highly similar to genetic (XY)

males and distinct from genetic (XX) females (Wang *et al.*, 2019). For the 361 genes that were differentially expressed between XX males and XX females in Nile tilapia, a general inverse correlation between CpG methylation and gene expression was observed, particularly when methylation occurred in the first exon and intron of the gene (Wang *et al.*, 2019). Specifically, methylation levels were lower in temperature-induced males and XX males compared to XX females between specific regions of *dmrt1,* but not along the entire length of the gene, whereas methylation levels in *cyp19a1a* were generally higher in XY males and XX males compared to XX females (Wang *et al.*, 2017; Wang *et al.*, 2019). Accordingly, a reciprocal relationship was observed in gene expression, with *dmrt1* and *cyp19a1a* exhibiting higher and lower expression in XY and XX males compared to XX females, respectively (Wang *et al.*, 2017; Wang *et al.*, 2019). This case study in tilapia provides an example of how temperature-induced, inverse patterns of methylation and gene expression in both *cyp19a1a,* as similar to the European seabass, and *dmrt1,* as similar to the tongue sole, accompany sex reversal. However, because differences in methylation levels between XX male and XX female Nile tilapia occurred only in specific regions of the *dmrt1* gene, but not on average for the whole gene sequence, this example highlights the importance of full-length sequence and single base pair resolution data for detecting methylation differences in other species.

1.4.3 Case studies in hermaphroditic fish species

Unlike in gonochoristic fish, where sex is generally determined during a discrete period in development, hermaphrodites may function as both sexes, change sex once, or change sex multiple times throughout their lifespan (simultaneous, serial and sequential hermaphroditism; see Figure 1.1). Despite the unique ability of hermaphrodite fish to exhibit alterations in form and function of the gonads during adult life, the biological mechanisms underpinning sex change appear to be highly similar to those employed by gonochoristic fish undergoing sex reversal during early development. This is most convincingly observed when alterations in sexual phenotype are accompanied by changes in DNA methylation and gene expression, but also with some evidence to suggest that temperature may play a similar role (Table 1.2).

1.4.3.1 The ricefield eel

The ricefield eel, *Monopterus albus*, is a protogynous hermaphrodite and one of the most economically important freshwater fishes in China. Large-scale propagation in this species is

hindered by a shortage of mature male broodstock, increasing the interest in understanding what governs female to male sex change in the ricefield eel (Yuan *et al.*, 2011). Using *M. albus* as an experimental model, Zhang *et al.* (2013a) demonstrated a continual decrease in gonadal *cyp19a1a* DNA methylation and gene expression levels during female to male sex change. To further validate the hypothesis that DNA methylation plays a key role in sequential sex change in this species, a DNA methylation inhibitor was used to reverse protogynous sex change of the ricefield eel, demonstrating a functional relationship between sex change and DNA methylation (Zhang *et al.*, 2013a). In the ricefield eel, alternative splicing of exons during transcription results in four isoforms of *dmrt1* (*dmrt1a-d*), each of which is differentially co-expressed within the gonad and encodes for a different sized protein (Huang *et al.*, 2005). For example, while expression of *dmrt1a* and *dmrt1b* increases throughout ovary to testis transition, expression of *dmrt1d* does not significantly change (Huang *et al.*, 2005). Although methylation patterns of *dmrt1* throughout the sex change process in this species are unreported, these findings caution that gene expression results found in other fish may be biased by the presence of unidentified isoforms.

1.4.3.2 Bluehead wrasse

The Bluehead wrasse, *Thalassoma bifasciatum*, is found throughout the Caribbean and exhibits socially controlled, protogynous sex change (Warner and Swearer, 1991). Removal of the dominant male in harems of smaller females will cause the largest female to undergo female to male sex change, resulting in changes in body colouration and behaviour, as well as gonadal morphology and function (Warner and Swearer, 1991; Semsar and Godwin, 2004). More recently, it has been discovered that these phenotypic changes are accompanied by a progressive accumulation of DNA methylation in the gonads (Todd *et al.*, 2019). Specifically, *cyp19a1a* became increasingly methylated and decreasingly expressed, and *dmrt1* became reciprocally methylated and expressed during male to female sex change (Todd *et al.*, 2019). The authors suggest that the transition from ovary to testes in sex changing fish does not occur by direct trans-differentiation as once thought (see Devlin and Nagahama, 2002), but instead via an epigenetically altered, intermediate state not dissimilar to that of primordial germ cells typically formed during early embryonic development (Todd *et al.*, 2019). Studies on the bluehead wrasse have also unveiled evidence that stress is a regulator of sex change in protogynous hermaphrodites, whereby cortisol mediates methylation of *cyp19a1a* during female to male sex change (Todd *et al.*, 2019). Stress has been similarly implicated in

masculinisation in other fish and reptile species (Deveson *et al.*, 2017; Ribas *et al.*, 2017). Evidence from the bluehead wrasse reveals a pattern of opposing methylation and expression in *dmrt1* and *cyp19a1a* similar to that known in temperature treated, sex reversed (XX or ZW) males of gonochoristic species. Furthermore, insights from the wrasse suggest that increasing methylation and decreasing expression of *cyp19a1a* during female to male sex change, as previously observed in the ricefield eel, may be universal to protogynous hermaphrodites.

1.4.3.3 Black porgy

The black porgy, *Acanthopagrus schlegelii*, is a digonic hermaphrodite, with testes and ovaries existing simultaneously, but physically separated by connective tissue and temporally separated in functionality (Huang *et al.*, 2002). The species exhibits sexual phase protandry with only the testes functioning during the first two reproductive cycles (male phase) and the ovaries becoming functional thereafter (female phase; Wu *et al.*, 2005). In the black porgy, *dmrt1* is thought to play a role in the development and maintenance of functional males, with expression levels of *dmrt1* found to be much higher in testis than ovary, and RNA interference designed to inhibit *dmrt1* mRNA causes male to female sex change (Wu *et al.*, 2012). Wu et al., (2016) found that methylation of *cyp19a1a* is higher in testis than in ovary (~ 80:60 % in testes to ovary), and during digonic sexual phase change, DNA methylation of this gene decreases and gene expression increases as fish become functional females. Furthermore, removal of the testicular portion of the gonad results in decreased levels of *cyp19a1a* promoter methylation and promotes the formation of precocious females; however, treatment with estrogen did not have the same effect on methylation and newly formed ovary reverted back to testis upon treatment termination (Wu *et al.*, 2016). This suggests that despite treatment with estrogen, ovarian *cyp19a1a* expression remains repressed by DNA methylation and that male to female sexual phase alternation is instead determined by growth or degeneration of the testis (Wu *et al.*, 2016). The authors suggest that testis degeneration in the porgy is regulated by gonadotrophin signalling, similarly to how testis formation is stimulated by gonadotrophin treatment in protogynous ricefield eel (see Yeung *et al.*, 1993) and bluehead wrasse (see Koulish and Kramer, 1989). While the digonic gonads and protandrous nature of the black porgy are a unique example of hermaphroditism and gonadal development, it appears that the mechanisms employed to achieve sexual fate in this species are not dissimilar, albeit directionally opposite, to those observed in the development of genetically female males in gonochoristic fish and protogynous sex change in hermaphrodites.

1.4.3.4 Mangrove killifish

The mangrove killifish, *Kryptolebias marmoratus*, is a marine-brackish species best known for their air-breathing ability and being one of just two known vertebrates to employ simultaneous hermaphroditism (Wright, 2012). While largely adopting a self-fertilisation strategy (46-98 %, depending on the population), mangrove killifish can develop as either hermaphrodites, or as males, allowing occasional outcrossing and increased genetic diversity (Tatarenkov *et al.*, 2009). Recent evidence suggests that sex (male or hermaphrodite) in killifish is regulated by DNA methylation and mediated by temperatures experienced during development (Ellison *et al.*, 2015). Ellison *et al.* (2015) found that proportion of male offspring increased when egg incubation temperature was decreased, with 0 - 7 % males produced at 25 °C, and 50 - 85 % males produced at 18 °C, depending on the genotype. Furthermore, these differences were best attributed to methylation status of the gonad with DNA methylation of *cyp19a1a, dmrt1, sox9a, foxl2* and *GnRHR* (Gonadotropin Releasing Hormone Receptor) significantly differentially methylated in males compared to hermaphrodites. Validation of the results using alternate methods confirmed the observed differences in methylation of *cyp19a1a* between males and hermaphrodites, but showed no significant differences in gene expression between treatment groups (Ellison *et al.*, 2015). While further investigation is necessary to better understand the relationship between low temperature treatment, methylation and gene expression in killifish, these results are the first to suggest that the mechanism by which temperature treatment alters sex ratios in gonochoristic species may also extend to simultaneous hermaphrodite species.

1.4.4 Conclusion

As once hypothesised nearly two decades ago by Gorelick (2003), these case studies in fish ascertain that DNA methylation is indeed a key mechanism allowing for changes in phenotypic sex in response to environmental change and in the absence of genetic change. While it is likely that the suite of genes, the timing, onset, environmental triggers and relative role DNA methylation has in altering sexual phenotype is different between species, it appears that epigenetic control of conserved sex genes *cyp19a1a* and, to a lesser degree *dmrt1*, is widespread throughout the teleost infraclass. Increases in the understanding of how differential DNA methylation affects phenotypic sex will be assisted by the use of full length, single base pair resolution methylation data to identify specific gene regions or CpG sites whose

differential methylation is correlated with changes in gene expression. Furthermore, the presence of multiple gene isoforms cautions the interpretation of results from gene expression assays that are insensitive to splice variants, or for data deficient species where isoforms are yet to be identified. While temperature effects are largely unreported in hermaphrodite fish, insights from the killifish suggest that temperature-mediation of sexual development is not restricted to gonochoristic fish. It will be interesting to see if temperature effects exert similar DNA methylation changes in sequential hermaphrodites. Finally, while the black porgy is digonic, but exhibits epigenetically mediated male to female phase change, it is unknown if DNA methylation plays a similar role in male to female sex change in protandrous sequential hermaphrodites. Examination of DNA methylation patterns during protandrous sex change would provide a reciprocal comparison to protogynous sex change exemplified by studies in the ricefield eel and bluehead wrasse. Whilst we are some way away from a complete understanding of the interaction between temperature, DNA methylation and phenotypic sex in fish, insights gained in this field are instrumental to the development of sex control methods for aquaculture, understanding the potential impact of changes in climate on sex ratios in wild populations, and more broadly provide unique insight into the epigenetic regulation of sexual differentiation in non-mammalian vertebrates.

1.5 The need for sex control in aquaculture

Sex control is one of the most important and highly targeted areas of aquaculture research due to the influence of sex and reproduction on husbandry management, productivity and economics. Without the ability to regulate sexual development, including sexual differentiation, maturation, and reproduction, farmers have little control over breeding processes. Arguably, sex and reproductive control have been the primary facilitators of large-scale industrial production in all aquaculture species that have become global commodities thus far. In those species which are yet to reach industrial scale production, elucidation of processes of sexual development and methods of sex control remain key areas of research.

Several broad goals in aquaculture can be achieved through increased understanding of sexual development and the attainment of methods of sex control. These include: 1) the prevention of precocious maturation and uncontrolled reproduction; 2) the desire to farm monosex populations due to differences in growth rate and/or economic value of the sexes and 3) increasing stability of mating systems. The relative importance of each of these goals depends upon the reproductive biology and culture system of the species of interest. Some relevant examples of each are explored below.

Precocious maturation occurs in several farmed species including Nile tilapia (Mires, 1995) and Atlantic salmon, which have a tendency to sexually mature and reproduce before attaining a suitable body size for harvest. Due to the diversion of energy into reproduction, this precocious maturation leads to slowed growth and reduces product size at harvest. Furthermore, deterioration in flesh quality is often observed specifically in female Atlantic salmon as they reach sexual maturity, resulting in differences in economic value between males and females (Piferrer *et al.*, 2009). In Nile tilapia, female and male triploids are reproductively sterile which prevents precocious maturation and leads to significantly larger individuals at harvest (Brämick *et al.*, 1995).

The desire to farm monosex populations may be provoked by sex-specific growth rates. For example, male Nile tilapia, grow faster and have lower feed conversion rates than females (Ridha, 2011). As a result, farmers have adopted both manual (e.g. hand sexing and selective removal) and/or various technological approaches to produce monosex populations for culture. Examples of technological approaches for the production of monosex populations include

hormonal manipulation in Atlantic cod, *Gadus morhua* (Lin *et al.*, 2012), hybridisation in Tilapia, *O. aurea x O. niloticus* (Pruginin *et al.*, 1975), chromosomal ploidy in Rainbow Trout, *Oncorhynchus mykiss* (Chourrout and Quillet, 1982), and maker assisted selection in Turbot, *Scophthalmus maximus* (Martínez *et al.*, 2009).

In sex-changing species, optimal, stable sex ratios are difficult to maintain. For example, Barramundi, is a mass-spawning, protandrous (male to female) hermaphrodite farmed throughout South-East Asia and Australia. Female barramundi mature several years later than males, which inhibits same generation selection practices (Robinson *et al.*, 2010). Furthermore, in captivity, barramundi can exhibit precocious sexual maturation and sex change, maturing as males and sex changing to females earlier and at smaller size ranges (Guiguen *et al.*, 1994; Schipp, 1996). Both of these attributes dictate that sex ratios are difficult to stabilise, necessitating sex control methods to both encourage female development and maintain functional males (Pankhurst, 1998; Allan and Stickney, 2000). Reliable methods to predict and control sex change in barramundi are yet to be established.

The importance of sex in aquaculture production and large potential for economic gain offers opportunities for research to investigate processes of sexual development and allow for the discovery or improvement of methods of sex control in farmed fish.

Chapter 2

Sex-specific differences in DNA methylation are associated with region-specific variability in total length at sex change in wild-caught Australian barramundi, *Lates calcarifer*

2.1 Summary of results

In Chapter 2 the role of DNA methylation in sequential sex change in Australian barramundi, *Lates calcarifer*, is explored. Fish total length, phenotypic sex and gonadal DNA methylation levels of four key sex-related genes (*cyp19a1a, esr1, dmrt1* and *nr5a2*) were analysed in wild-caught individuals from three geographic regions in Queensland. The major results include:

1. DNA methylation levels in amplicons covering the partial promoter and first exon of *cyp19a1a, esr1, dmrt1* and *nr5a2* were significantly different between male and female barramundi ($p < 0.001$).
    a. Male-specific methylation patterns were characterised by high methylation in female-associated genes *cyp19a1a* and *esr1* [*Mdn* (IQR) = 0.91 (0.16) and 0.83 (0.19), respectively] and low methylation in male-associated genes *dmrt1* and *nr5a2* [*Mdn* (IQR)= 0.04 (0.04) and 0.22 (0.22), respectively].
    b. Reciprocally, female-specific methylation patterns were characterised by comparatively low methylation in female-associated genes *cyp19a1a* and *esr1* (*Mdn* = 0.59 (0.27) and 0.59 (0.26), respectively) and comparatively high methylation in male-associated *dmrt1* and *nr5a2* (*Mdn* = 0.11 (0.10) and 0.52 (0.23), respectively).
2. DNA methylation differences between the sexes become increasingly divergent with increasing total length and, as such, it does not appear that gradual changes in DNA methylation over time lead to male to female sex change in this species. For example, in north Qld east coast barramundi:
    a. Male fish become more male-specific in their DNA methylation patterns with increasing total length [i.e. DNA methylation increases in *cyp19a1a* ($p < 0.001$) and *esr1* ($p < 0.001$) and decreases in dmrt1 ($p < 0.001$), nr5a2 ($p < 0.001$)]
    b. Female fish become more female-specific in their DNA methylation patterns with increasing total length in female associated amplicons [i.e. DNA methylation decreases in *cyp19a1a* ($p < 0.001$), *esr1* ($p < 0.01$)], but exhibited greater variation in DNA methylation overall.

    Similar results were found for Gulf of Carpentaria (GoC) barramundi.
3. The total length at which sex change occurs (total length-at-sex change) in barramundi differs between regions, with barramundi from the mid-northern GoC demonstrating a higher frequency of females at smaller total lengths than fish from the north Qld east

coast (b = 2.1296, z(13464) = 25.943, $p < 0.001$) and southern GoC (b = 0.2818, z(13464) = 3.289, $p < 0.01$)

4. Differences in the total length-at-sex change in the mid-northern GoC barramundi are accompanied by differences in DNA methylation in male fish for all four amplicons examined ($p < 0.01$)
5. Differences in DNA methylation and total length-at-sex change identified in barramundi from the mid-northern GoC compared to southern GoC and north Qld east coast were not correlated with ambient environmental temperature, as temperatures within the GoC were more similar to each other compared to temperatures experienced by north Qld east coast regions (i.e. the differences were not concurrent).

The results of this chapter highlight that DNA methylation differs between the sexes, reveals that these sex-specific differences becoming increasingly binary with increasing fish length and uncovers that region-specific differences in total length-at-sex change are accompanied by differences in DNA methylation in barramundi. The work provides a unique example of how differential DNA methylation of trait-of-interest (sex-related) genes are associated with phenotypic plasticity (differences in total length-at-sex change).

2.2 Introduction

The ability of individual genotypes to produce different phenotypes in response to environmental change is an important driver of species resilience, and is known as phenotypic plasticity (Pigliucci, 2005; Hu and Barrett, 2017). In contrast to adaptive evolution, which is underpinned by random genetic mutation alone, phenotypic plasticity is often mediated by epigenetic changes (Richards *et al.*, 2010; Duncan *et al.*, 2014). Epigenetic changes can be defined as mitotically and/or meiotically heritable changes that occur to the structure of the DNA, rather than the sequence, resulting in a change in phenotype in the absence of a change in genotype. They occur within the lifetime of an individual, in response to environmental stimuli, and can offer higher and more dynamic rates of change than genetic mutation alone (Zhang *et al.*, 2013b). In eukaryotes, the best characterised epigenetic modification is DNA methylation, which involves the replacement of the fifth carbon of cytosine with a methyl group, typically at CpG dinucleotides (Jones, 2012). In all wild fish populations studied to date, variation in DNA methylation has been found in excess of genetic variation (see Hu and Barrett, 2017 and references therein). As such, epigenetic variation can provide explanation for cases in which classical quantitative genetics, based on sequence variation alone, has been unable to explain rapid phenotypic responses to environmental change (Bossdorf *et al.*, 2008).

Barramundi, *Lates calcarifer*, are an apex predator in tropical near-shore marine and freshwater ecosystems throughout the Indo-West Pacific from the Arabian gulf, across south-east Asia to northern Australia and Papua New Guinea (Grey, 1987). In Australia, the species supports important recreational, commercial and indigenous fisheries. Barramundi are a protandrous hermaphrodite, maturing first as male before sex changing to female upon the attainment of a minimum body size or age (Moore, 1979; Davis, 1982; Guiguen *et al.*, 1994). Sex change presents a unique challenge for fisheries management, necessitating both upper and lower legal catch size limits and increasing the complexity of population models needed to accurately assess stock status (Benvenuto *et al.*, 2017; Campbell *et al.*, 2017). Adding to this complexity are differences in the total length (anterior to posterior) at which sex change occurs in barramundi from different geographic regions. For example, *L. calcarifer* from the Gulf of Carpentaria (GoC) sex change at smaller total lengths than those in the northern Territory (NT) and on the east coast of Queensland (Qld). More specifically, in the GoC most barramundi change sex at total lengths between 68 - 90 cm, compared to 84 - 97 cm in the NT and 85 - 100 cm on the Qld east coast (Davis, 1982; QFMA, 1991). Furthermore, within the GoC,

populations at latitudes north of 13°S exhibit sexual precociousness, maturing early and sex changing on average at 46 cm, compared to 78 cm in the southern GoC (Davis, 1984b). These differences in sexual maturation and change are accompanied by differences in growth rate, whereby fish from the northern GoC exhibit slower growth rates compared to other regions (Davis, 1982; 1984b). This slowed growth, known as stunting, is thought to occur due to the channelling of energy into gonadal growth at the expense of somatic growth and, together with sexual precociousness, the phenomenon was previously thought to be underpinned by genetic factors (Davis, 1984b). Indeed, subpopulations of barramundi in the GoC are genetically differentiated from those of the Qld east coast (Shaklee and Salini, 1985) and differentiated within the GoC, at and north of 13°S where previous differences in growth rate and sexual precociousness were identified (Figure 2.1; Keenan, 1994; Jerry, 2014). However, genetically distinct populations, or major stocks, only exist more broadly between western Australia and the Qld east coast, with the GoC and NT representing a central region of admixture (Loughnan *et al.*, 2019). This suggests that differences in growth rate, sexual maturation and sex change between regions may be underpinned by epigenetic changes, rather than genetic factors alone, and are thus likely to be influenced by environment. Current fisheries modelling for barramundi relies on assumptions of unchanging sex ratio over time and temporally representative data collected by Davis (1982; 1984a) on maturity and fecundity at length (Campbell *et al.*, 2017). As such, identifying the environmental drivers of sex change and potential molecular indicators of sexual maturation status would enable increased accuracy in fisheries modelling and refined management for this species.

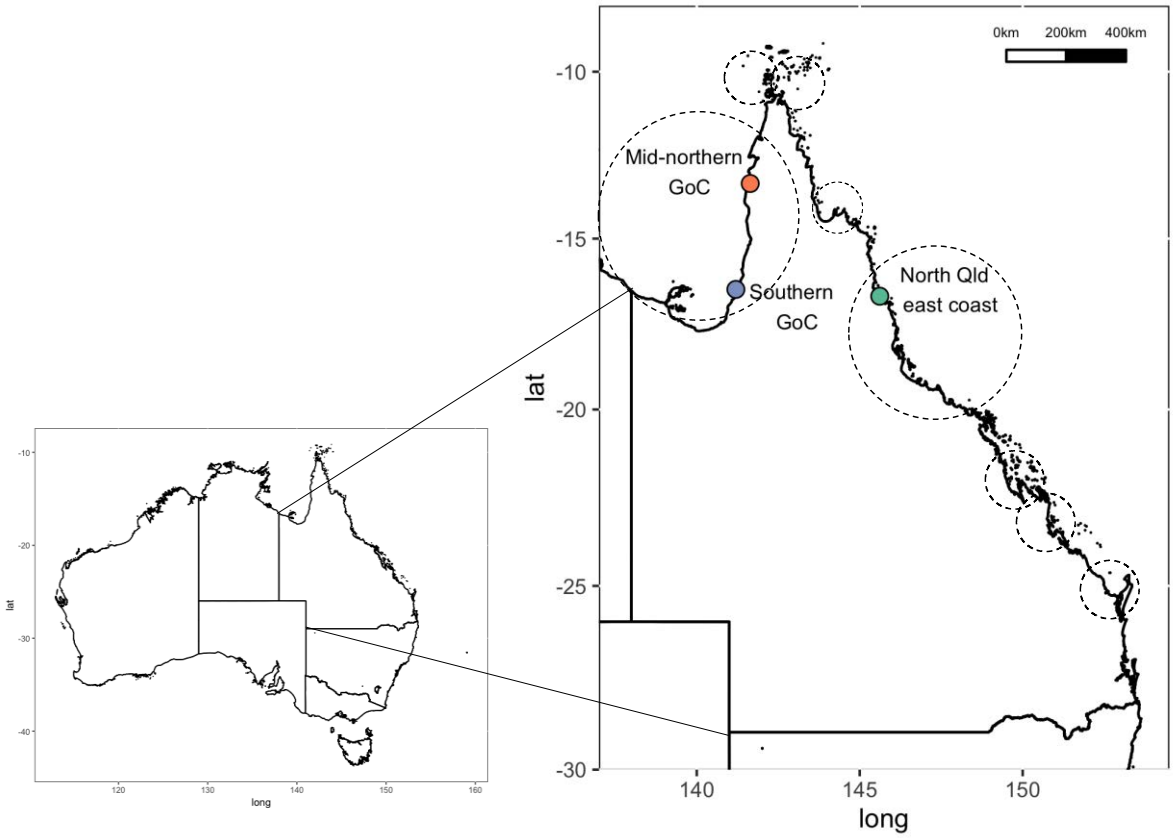

Figure 2.1 Map of Queensland, Australia showing sampling locations (coloured markers) as well as distribution of the genetically distinguishable subpopulations of barramundi, *Lates calcarifer*, identified by Jerry (2014; dashed circles).

Recent research has revealed that male and female barramundi exhibit differential DNA methylation and expression of several conserved vertebrate sex determining genes suggesting that sex change is, at least in part, attributed to changes in DNA methylation (Domingos *et al.*, 2018). Significantly higher methylation in *dmrt1* [doublesex and mab-3 (DM)-related transcription factor 1] and *nr5a2* (Nuclear Receptor Subfamily 5 Group A Member 2) as well as lower methylation in *cyp19a1a* (cytochrome P450, family 19, subfamily A, polypeptide 1a) occurred in females compared to males (Domingos *et al.*, 2018). While this research provided a comparison of binary male and female phenotypes In barramundi, previous work in the ricefield eel, *Monopterus albus*, additionally examined DNA methylation patterns throughout the sex change process including early, middle and late intersex stages (Zhang *et al.*, 2013a). The authors revealed a gradual increase in methylation of the female-associated sex gene *cyp19a1a* during female to male sex change, accompanied by a reciprocal change in the gene's expression (Zhang *et al.*, 2013a). Prior to sex change, female ricefield eels exhibit approximately 50 % methylation in this gene, suggesting that there is an accumulation of DNA methylation before the onset of sex change. In barramundi, it is not known whether the

observed sex-specific differences in methylation levels accumulate with fish growth, or if changes occur instantaneously upon transition from male to female. Furthermore, in wild populations of Atlantic salmon, *Salmo salar*, a species whose sexual maturation state is mediated by DNA methylation (Kijas *et al.*, 2019), high levels of epigenetic variation are thought to enable the precocious maturation of small 'sneaker' males in otherwise genetically similar fish (Morán and Pérez-Figueroa, 2011). While precocious maturation and sex change is similarly exhibited in GoC populations of barramundi, it is not known if this sexual precociousness is underpinned by variation in DNA methylation, which may be acting in combination with genetic variability to lead to changes in sexual phenotype.

Here, patterns of DNA methylation within the promoter and first exon four key sex-related genes *dmrt1*, *nr5a2*, *cyp19a1a* and *esr1* (estrogen receptor alpha) in wild-caught *L. calcarifer* were quantified to: 1) determine the association between DNA methylation patterns and total length in barramundi and investigate the hypothesis that changes in DNA methylation accumulate with fish growth to become more female-specific; 2) enable comparisons of DNA methylation levels between north Qld east coast and two GoC populations and investigate the hypothesis that differences in total length-at-sex change are associated with differences in DNA methylation levels, and finally; 3) to explore evidence that temperature is a potential environmental contributor to epigenetic variation in fish. This chapter presents the first investigation of population-specific epigenetic markers for a protandrous hermaphrodite and provides an important *in situ* example of how epigenetics underpins fundamental population demographics and adaptability to environmental change.

2.3 Methods

2.3.1 Animal collection and sampling design

Total length, sex and age data were collected as part of the Queensland Government, Department of Agriculture and Fisheries (DAF) barramundi Long Term Monitoring Program (LTMP) from the year 2000 onwards, and gonadal tissue samples were collected throughout the 2015 and 2016 fishery open seasons (February through October) and stored in RNAlater (Thermo Fisher Scientific). Sex was determined macroscopically, whereby male and female gonads were identified by their distinct colour and texture (Queensland, 2010). Fish in gonadal transition were not identified in this sample set as their identification is not possible without histological examination (Davis, 1982). Total length was measured from the anterior-most part

of the fish's snout to the posterior tip of the caudal fin while compressed dorso-ventrally, to the nearest 10 mm (Queensland, 2010). Age was determined by sectioned otolith examination including increment counts, edge classification and periodicity and timing of opaque zone transformation (Stuart and McKillup, 2002). Due to differences in the total length at which sex change occurs (total length-at-sex change, herein) identified within our GoC samples, the southern GoC sub-population was split into 'southern GoC' at ~ 16°30'S and 'mid-northern GoC' at ~ 13°30'S (Figure 2.1). For the LTMP's Qld east coast population, only the 'north Qld east coast' region at ~ 16°30'S was included due to insufficient sample size for fish below this latitude (Figure 2.1). Samples collected were fisheries regulation capture dependent and restricted to minimum and maximum size limits of 58 and 120 cm total length, respectively. Samples were obtained from commercial, charter operator and recreational harvests, and therefore did not require collection permits or animal ethics approval as fish were taken by recreational and commercial fishers as part of usual fishing practice.

2.3.2 Genomic DNA extraction

Gonadal tissue was removed from storage in RNAlater (Thermo Fisher Scientific), washed once in PBS and dried with a KimWipe (Kimtech Science) before immediate immersion into DNA extraction buffer [100 nM Tris-HCl, 1.4 M NaCl, 20 nM EDTA, 2 % Cetyl trimethylammonium bromide (CTAB), 2 % polyvinylpyrrolidone (PVP)]. Genomic DNA (gDNA) was extracted following the CTAB protocol (Doyle and Doyle, 1987), including overnight digestion with Proteinase-K followed by the addition of a phenol:chloroform:isoamyl alcohol (25:24:1) step to assist with the removal of proteins. Quantity and purity of gDNA was assessed using an ND-1000 spectrophotometer (Nanodrop technology) based on absorbance at 260 nm and 260/280 nm ratio, and integrity was assessed by visualisation on a 0.8 % agarose gel with lambda DNA standards at 50, 20, 10 and 5 ng/µl and a 1 kb Plus DNA ladder (Thermo Fisher Scientific).

2.3.3 Bisulphite conversion of gDNA and amplicon specific PCR

To analyse DNA methylation levels of the promoter region and/or first exons of *cyp19a1a, esr1, dmrt1* and *nr5a2* a targeted bisulphite amplicon sequencing (BSAS) approach, adapted from Masser *et al.* (2013), was applied. Following the manufacturer's instructions, 500 ng of extracted gDNA was subject to bisulphite treatment using EZ DNA Methylation-Gold™

(Zymo Research). Gene-specific primers were designed (Table 2.1) using MethPrimer (Li and Dahiya, 2002) with the following Illumina adapter overhang nucleotide sequences added; Forward overhang: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[Gene specific sequence]; Reverse overhang: 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[Gene specific sequence]. PCR amplification of these regions was achieved using Platinum® Taq DNA Polymerase (Thermo Fisher Scientific) following the manufacturer's instructions. Reaction conditions were as follows: 95 °C for 2 min followed by 40 rounds of 95 °C for 30 s, 57.5 °C for 35 s, 72 °C for 40 s, with a final extension of 72 °C for 10 min. PCR products were purified using Sera-Mag SpeedBeads (GE Healthcare) prepared following Faircloth and Glenn (2014) and quantified using QuantiFluor (Promega) fluorometric nucleic acid quantitation and measured on an EnSpire Multimode plate reader (PerkinElmer).

Table 2.1 *Lates calcarifer* sex-related genes and primer sequences investigated for bisulphite amplicon next-generation sequencing (BSAS).

Gene	Accession	Primer name	Primer sequence (5' - 3') [1]
dmrt1	KR232516.1	Dmrt1_BS_P_F2(2)	FO–AAATTAAGTGTAGTAGAGTGATGTTAT
		D1_BS_CDS_R1	RO–AAACACTAACAATCCCTCCAATTAC
cyp19a1a	KR492506.1	CYP19-BS-F	FO–TGGTTGTTTATAAAGGGGAAGTTT
		CYP19-BS-R	RO–CCAACAACAAACAAACAAATAACATA
esr1	KR492509.1	ESR1_BS_PE1_F1	FO-TGTGTTGTGATGTTGTTTAGGTAGAG
		ESR1_BS_PE1_R1	RO-TTCCAAAAAATCCACAATAACTACC
nr5a2	KR492512.1	sf1-BS-F2	FO–GGAAAAGAGATTGTTTAGTATAGTAATAGA
		sf1-BS-R2	RO–TAAAAACACTAACCTTACAACTCTC

1 FO (5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG) and RO (5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG) are Illumina's forward overhang (FO) and reverse overhang (RO) adapter sequences added to locus-specific primer sequences.

2.3.4 NGS library preparation and DNA methylation quantification

Dual indexed libraries were generated using a Nextera XT Index Kit following the manufacturer's protocol (Illumina). Purified PCR products were indexed using limited cycle number PCR under the following reaction conditions: 95 °C 5 min followed by 12 rounds of 95 °C for 30 s, 58 °C for 35 s, 72 °C for 40 s, with a final extension of 72 °C for 10 minutes. Indexed amplicons were purified and quantified as described in section 2.3.3, normalised by molarity to 4 nM and pooled into a final library. The final library was size, and quality checked

on an Aligent TapeStation (Aligent Technologies) and quantified on a Qubit 3.0 Fluorometer (Invitrogen) for subsequent molarity determination. Libraries were then diluted to 8 pM, spiked with 20 % PhiX and loaded onto a 600 cycle V3 reagent cartridge for sequencing on an Illumina MiSeq (Illumina). FASTQ files were imported into Geneious Version 10.2.6 (Kearse *et al.*, 2012). Paired reads were merged using BB merge paired Read Merger Version 37.25 (Bushnell *et al.*, 2017), sequences were then trimmed based on an error probability limit of 0.05 with a maximum of one ambiguity and resultant reads were aligned to *in-silico* bisulphite converted reference sequences using the Geneious in-built read mapper end to end read mapping and a minimum mapping quality of 30. Variants were detected using a minimum coverage of 500 to identify C-T variants in CpG positions, which correspond to the percentage of cytosine methylation, using a minimum coverage of 500 reads.

2.3.5 Temperature data

Air temperature data for north Queensland east coast (Barron, Mossman, Daintree, Johnstone rivers), mid-northern GoC (Coen, Archer, Watson rivers) and southern GoC (Gilbert river) catch locations were derived from an Australia-wide climate model constructed from observational records (i.e. raw meteorological data) of the Australian Bureau of Meteorology (BOM) to generate a dataset that is spatially and temporally complete. The modelled data are referred to as SILO (see Jeffrey *et al.*, 2001; Jeffery, 2006), and were downloaded from https://www.longpaddock.qld.gov.au/silo/. SILO data have been used in numerous analyses as proxies for climate data (e.g. Chamberlayne *et al.*, 2020). Air temperature can be representative of patterns in estuarine water temperature, both within and between years, provided that the aquatic ecosystems in question are relatively shallow and well mixed (e.g. Kienzle *et al.*, 2016).

2.3.6 Statistical analyses

All statistical analyses were carried out using R (version 2.15.2); beta regression analyses were performed using algorithms implemented in the R package *betareg* (Cribari-Neto and Zeileis, 2009; Team, 2013). Since methylation data generated by BSAS are proportional (bound between 0 and 1), beta regression was used to fit the data on the logit scale (Ferrari and Cribari-Neto, 2004; Seow *et al.*, 2012). For each gene, DNA methylation levels were initially modelled adjusting for sex, region, CpG site, total length and/or age with the inclusion of all possible three-way interaction terms. To avoid multicollinearity between fish age and total length,

variance inflation factor (VIF; Appendix A), Bayesian information criterion (BIC; Appendix B) and pseudo-adjusted coefficients of determination (R^2; data not shown) values were calculated. While VIF did not indicate a problem with multicollinearity and BIC indicated that including both age and size produced the preferred model, regression analysis revealed a significant correlation between age and total length ($p < 0.001$; Appendix C). With the exception of *dmrt1*, all BIC values for models including total length (but not age) were more preferable to those including age (but not total length). Therefore, based on the regression results and BIC values, total length was retained in the final model, and age was excluded (Appendix B). A chi-square test of goodness-of-fit based on deviance was performed to determine the effect of each factor in the model and identify interaction terms that were non-significant for all amplicons, which were subsequently dropped from the model. The resulting model, used for all amplicons included six interaction terms, five of which included sex. Given that sex had significant interactions with all other factors (Appendix D), the data was subset by sex (male and female) to enable greater resolution in the resulting beta regression models Appendix E). To account for multiple testing, a Benjamini-Hochberg FDR correction was applied (Benjamini and Hochberg, 1995). The final model for each sex within each gene predicted methylation based on the three remaining factor variables (CpG, region and total length) and included one significant interaction term (region: total length). The final models explained 20 - 80 % of the total variance in methylation, depending on the gene and sex (Appendix E). Post-hoc evaluation of the differences in the mean response between geographic regions (holding other predictors at their means) was performed through linear hypothesis testing (*linearHypothesis* function, *car* package; Fox et al., 2013). Binomial GLMs for fitting length frequency distributions were performed using general linear models (*glm* function, stats base package) using DAF barramundi LTMP data years 2000 to 2016. Growth curves were fitted using the von Bertalanffy growth model (Von Bertalanffy, 1938) fitted to the observed length-at-age data from the same data set used for the GLMs excluding ages greater than 20 years. A function for the typical VGBF was constructed using the *vbFuns* function from the FSA package and starting values were obtained using *vbStarts*. The parameters of the VGBF were estimated using Levenberg-Marquardt least squares non-linear regression (Moré, 1978) as implemented in the *nlsLM* function from the minpack.lm package (Elzhov *et al.*, 2010).

2.4 Results

2.4.1 Methylation patterns in male and female barramundi are highly sex-specific

Analysis of the gonads of wild-caught Australian barramundi revealed that males and females exhibit highly divergent patterns of methylation in all four sex-related genes examined (Figure 2.2, Table 2.2, Figure 2.3, Figure 2.4). Specifically, higher methylation levels in female-associated genes *cyp19a1a* and *esr1* in male fish, and higher methylation levels in male-associated genes *dmrt1* and *nr5a2* in female fish (Figure 2.2 and Table 2.2). On average, female-associated genes *cyp19a1a* and *esr1* were 33 % and 24 % more methylated in male fish, and male-associated genes *dmrt1* and *nr5a2* were 7 % and 29 % less methylated in male compared to female fish (Figure 2.2 and Table 2.2). Thus, male-specific methylation patterns were characterised by high methylation in *cyp19a1a* and *esr1* and low methylation in *dmrt1* and *nr5a2*. Reciprocally, female-specific methylation patterns were characterised by comparatively low methylation in *cyp19a1a* and *esr1* and comparatively high methylation in *dmrt1* and *nr5a2* (Figure 2.2 and Table 2.2).

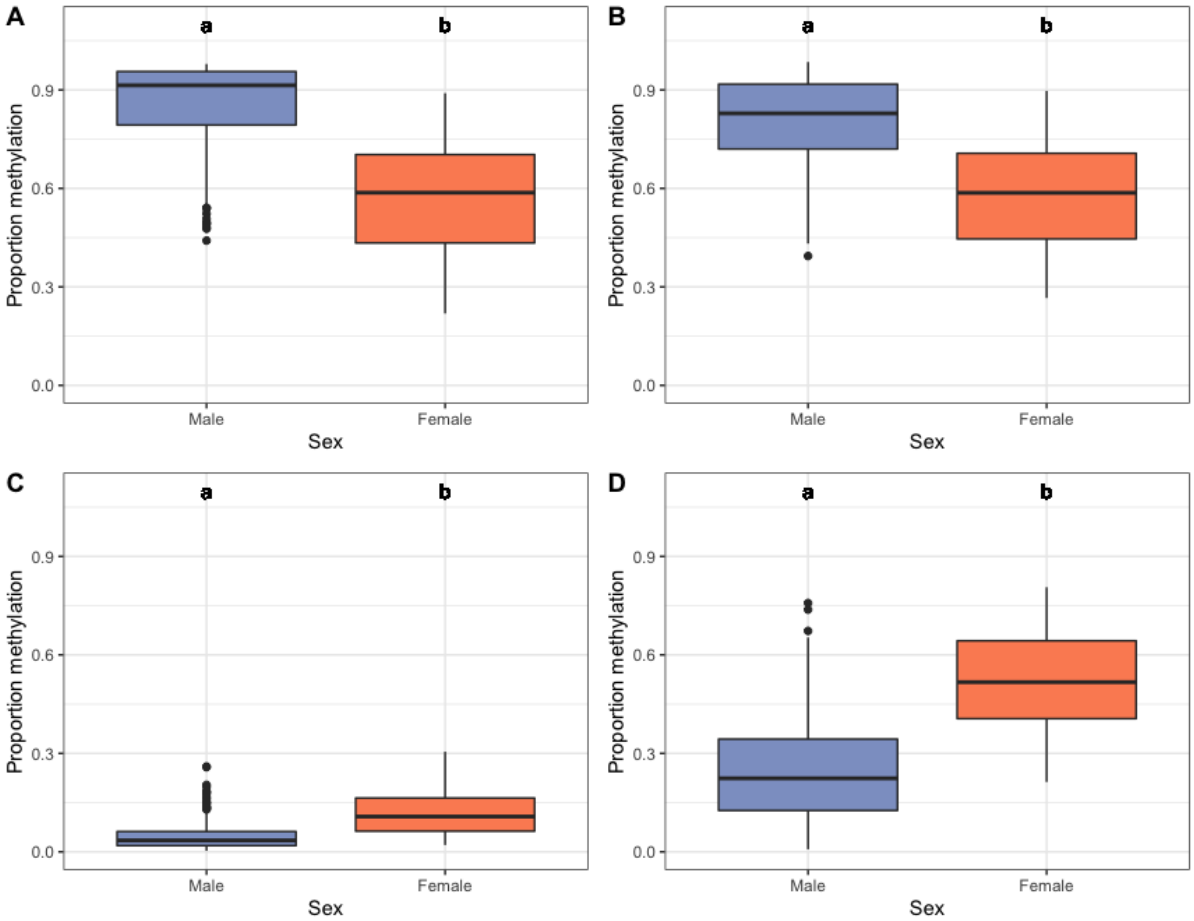

Figure 2.2 Comparison of DNA methylation levels between male and female barramundi, *Lates calcarifer,* from Queensland, Australia by amplicon demonstrating high methylation of female-associated genes A. *cyp19a1a* and B. *esr1* and low methylation of male-associated genes C. *dmrt1* and D. *nr5a2* in male fish (blue), and the reciprocal pattern in female fish (orange). Letters denote significant differences between males and female resulting from Mann-Whitney test ($p < 0.001$).

Table 2.2 Comparison of DNA methylation levels between male and female barramundi, *Lates calcarifer*, from Queensland, Australia by amplicon. IQR = interquartile range.

Amplicon	***cyp19a1a***	***dmrt1***	***esr1***	***nr5a2***
Mann Whitney U (W)	10833	108650	19870	113500
Z	-18.89506	-1.63E+01	-15.63031	-18.03144
r	3.636356	3.13321	3.008054	3.470152
Male median methylation (IQR)	0.91 (0.16)	0.04 (0.04)	0.83 (0.19)	0.22 (0.22)
Female median methylation (IQR)	0.59 (0.27)	0.11 (0.10)	0.59 (0.26)	0.52 (0.23)
Difference (male-female median methylation)	0.33	-0.07	0.24	-0.29
***p* value**	6.26e-80	6.77e-60	2.26e-55	5.52e-73

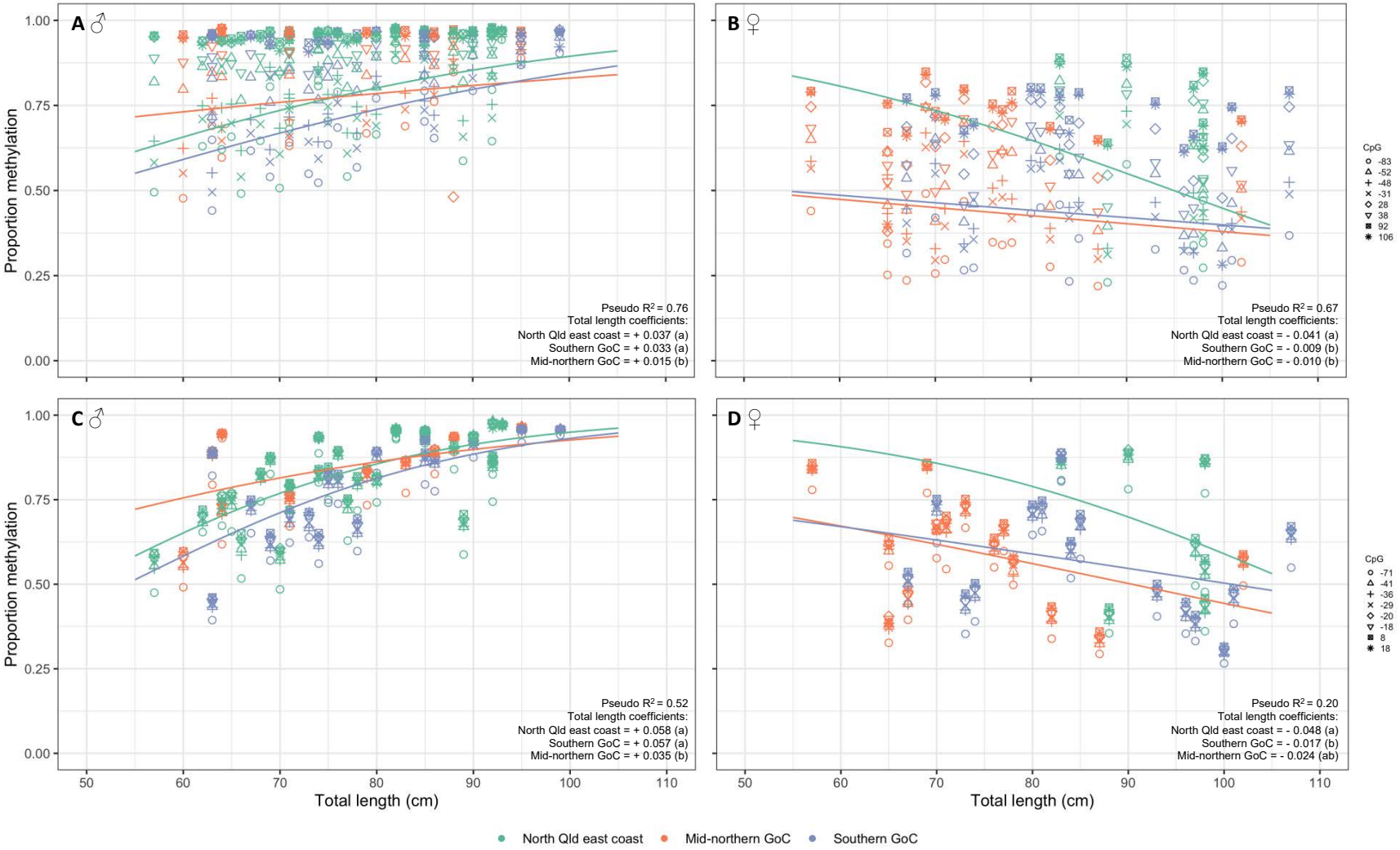

Figure 2.3 Proportion of methylation explained by *Lates calcarifer* total length and CpG position (indicated by symbol) for amplicons of female-associated genes: A. *cyp19a1a* in males, B. *cyp19a1a* in females, C. *esr1* in males and D. *esr1* in females. Fitted curves correspond to beta regression with logit link for three regions in Queensland, Australia (indicated by colour). Curves were evaluated at varying lengths with the CpG whose intercept value was closest to the average intercept.

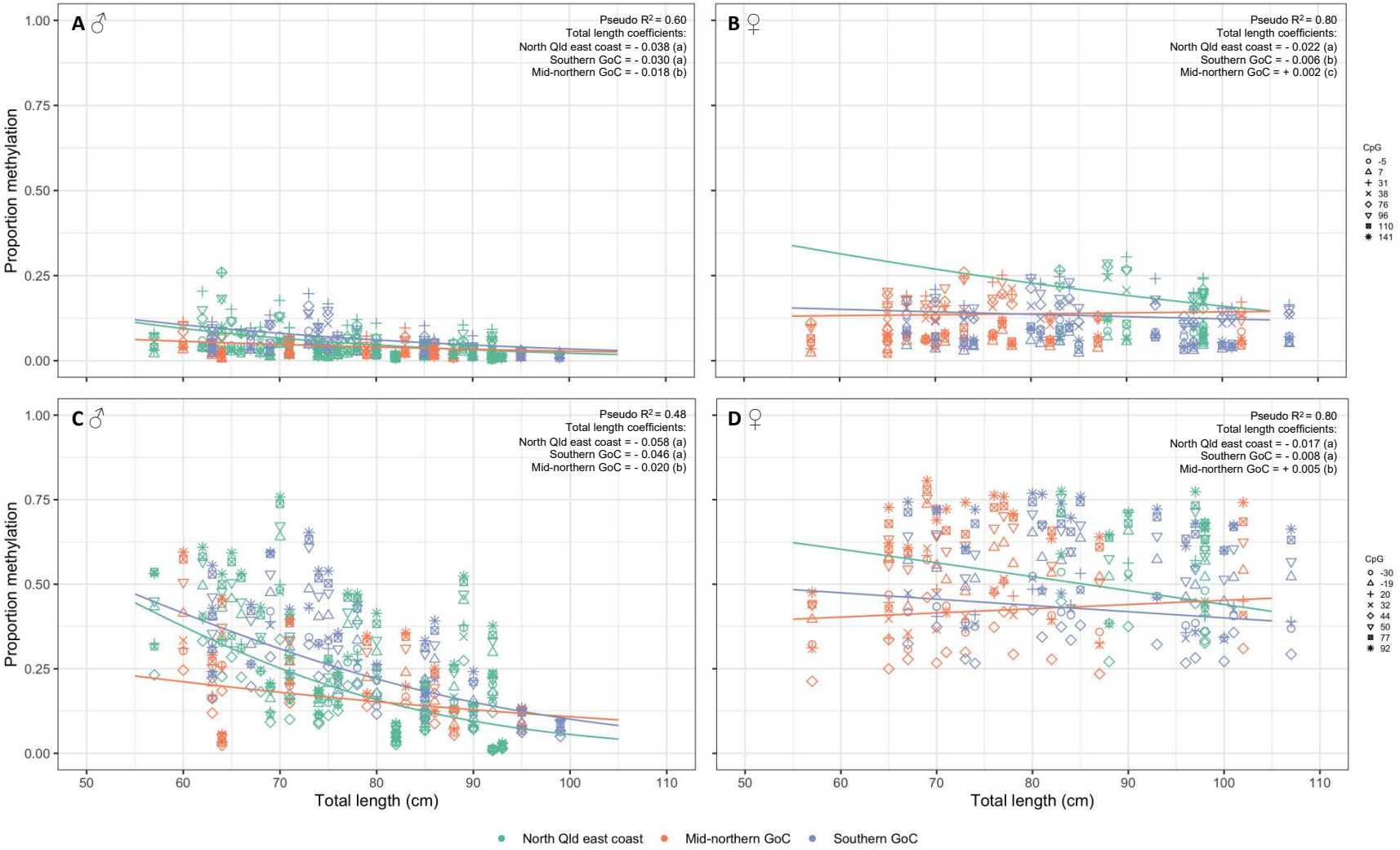

Figure 2.4 Proportion of methylation explained by *Lates calcarifer* total length and CpG position (indicated by symbol) for amplicons of male-associated genes: A. *dmrt1* in males, B. *dmrt1* in females, C. *nr5a2* in males and D. *nr5a2* females. Fitted curves correspond to beta regression with logit link for three regions in Queensland, Australia (indicated by colour). Curves were evaluated at varying lengths with the CpG with the intercept whose value was closest to the average intercept.

2.4.2 Methylation patterns in male barramundi become more male-specific with increasing length

Beta regression modelling revealed that while growth-associated changes in methylation do occur in barramundi, in male individuals they typically lead to an increase in male-specific, rather than female-specific, methylation patterns. For example, methylation of female associated amplicons *cyp19a1a* and *esr1* increased with increasing length in male fish and methylation of male associated amplicons *dmrt1* and *nr5a2* decreased with increasing length (Figure 2.3 and Figure 2.4). This result was in opposition to the expected increase in methylation of male associated amplicons with increasing length initially hypothesised to lead to male-female sex change in large barramundi.

2.4.3 Methylation patterns in female barramundi with increasing length are unlikely to contribute to sex change

For female barramundi, methylation patterns in female associated amplicons became more female-specific with increasing total length for fish from all geographic regions, whereby in male associated amplicons, methylation patterns became more female specific for female fish from the mid-northern GoC only. These lines of evidence do not provide support for the hypothesis that cumulative changes in methylation lead to male-female sex change. Specifically, for female associated amplicons *cyp19a1a* and *esr1* methylation increased with increasing length in female fish from all regions, leading to more female-specific patterns of methylation with increasing length (Figure 2.3). In the male associated amplicons *dmrt1* and *nr5a2*, the direction of the relationship between methylation and total length was region dependent. For example, in female fish from the mid-northern GoC, methylation of *dmrt1* and *nr5a2* increased with increases in total length, whereas in female fish from north Qld east coast and southern GoC methylation decreased with total length (Figure 2.3). This led to more female-specific patterns of methylation with increases in total length in fish from the mid-northern GoC, but more male-specific patterns of methylation with increasing length in fish from the north Qld east coast and southern GoC. Despite the observed increase in female specific methylation patterns in female associated amplicons for females from all regions, and in male associated amplicons in females from the mid-northern GoC, this cumulative change in methylation occurred beyond the point of sex change (i.e. in females), rather than preceding

it (i.e. in males) and therefore does not provide support for cumulative changes in methylation leading to male-female sex change in this species.

2.4.4 Mid-northern GoC barramundi demonstrate differential DNA methylation, growth rate and total length at which sex change occurs

In the mid-northern GoC, male barramundi demonstrated differential methylation patterns and a smaller average total length-at-sex change compared to male fish from both other regions. Individual hypothesis testing revealed that male fish from the mid-northern GoC had significantly different methylation levels compared to male barramundi from the north Qld east coast and southern GoC, which did not significantly different from each other (Table 2.3). Specifically, male barramundi from the mid-northern GoC demonstrated more male-specific patterns of methylation in smaller size classes, becoming more female-specific compared to the southern GoC and north Qld east coast in larger size classes indicated by a smaller total length: region + total length coefficient for all genes (Figure 2.3, Figure 2.4 Appendix E). Binomial GLM demonstrated the established relationship between total length and sex, with total length significantly predicting sex ($b = 0.0992$, $z(13464) = 36.559$, $p < 0.001$). The model further demonstrated that region was also a significant predictor, with barramundi from the mid-northern GoC being more strongly differentiated from east coast Qld ($b = 2.1296$, $z(13464) = 25.943$, $p < 0.001$) than fish from the southern GoC ($b = 0.2818$, $z(13464) = 3.289$, $p < 0.01$). Plotting the model reveals a clear shift towards females of smaller size classes in fish from the mid-northern gulf, indicating that barramundi from the mid-northern GoC also demonstrate a smaller total length-at-sex change than fish from the southern GoC and Qld east coast, in addition to exhibiting differential methylation patterns (Figure 2.5). Data modelled using binomial GLM are also presented as length-frequency distributions in Appendix F. Von Bertalanffy growth modelling also revealed that barramundi from the mid-northern GoC exhibit the slowest growth trajectory for each of the three regions (Figure 2.6).

Table 2.3 Comparison of DNA methylation in amplicons covering partial sex gene and promoter regions in barramundi, *Lates calcarifer*, between three geographic regions in Queensland, Australia. Results are derived from linear hypothesis testing to compare regression coefficients for each region specified in the comparison column based on the beta regression model specified in text and *p* values are corrected based on false discovery rate (FDR) methods of Benjamini & Hochberg (1995). Results show that regression coefficients and therefore methylation levels are significantly different in the mid-northern gulf compared to both other study regions in male barramundi for all four amplicons investigated and female barramundi for *nr5a2*. Df = degrees of freedom.

Sex	Amplicon	Residual df	Df	Comparison (null hypothesis)	Comparison coefficients	Chi-square statistic	FDR adjusted *p* value
Male	*cyp19a1a*	426	1	southern GoC = mid-northern GoC	-0.0522 = 1.6965	14.2776	5e-04***
				southern GoC = north Qld east coast	-1.7487 = -1.6965	0.0176	0.9332
				mid-northern GoC = north Qld east coast	1.7487 = 0.0522	15.7142	3e-04***
	dmrt1	426	1	southern GoC = mid-northern GoC	-0.4018 = -1.757	8.0728	0.0077**
				southern GoC = north Qld east coast	1.3553 = 1.757	1.1102	0.3504
				mid-northern GoC = north Qld east coast	-1.3553 = 0.4018	15.6529	3e-04***
	esr1	426	1	southern GoC = mid-northern GoC	-0.2309 = 1.8542	13.5347	7e-04***
				southern GoC = north Qld east coast	-2.0851 = -1.8542	0.2343	0.6855
				mid-northern GoC = north Qld east coast	2.0851 = 0.2309	12.8918	9e-04***
	nr5a2	426	1	southern GoC = mid-northern GoC	-0.5556 = -3.0923	18.5584	1e-04***
				southern GoC = north Qld east coast	2.5367 = 3.0923	1.257	0.3312
				mid-northern GoC = north Qld east coast	-2.5367 = 0.5556	32.5645	0***
Female	*cyp19a1a*	274	1	southern GoC = mid-northern GoC	-3.4138 = -3.4093	1.00E-04	0.9908
				southern GoC = north Qld east coast	-0.0045 = 3.4093	11.6048	0.0013**
				mid-northern GoC = north Qld east coast	0.0045 = 3.4138	11.5778	0.0013**
	dmrt1	274	1	southern GoC = mid-northern GoC	-1.9043 = -2.5568	6.2843	0.0195*
				southern GoC = north Qld east coast	0.6525 = 2.5568	11.548	0.0013**
				mid-northern GoC = north Qld east coast	-0.6525 = 1.9043	20.8876	1e-04***
	esr1	274	1	southern GoC = mid-northern GoC	-3.388 = -3.0063	0.4252	0.5878
				southern GoC = north Qld east coast	-0.3817 = 3.0063	5.0145	0.0377*
				mid-northern GoC = north Qld east coast	0.3817 = 3.388	3.9449	0.0664.
	nr5a2	274	1	southern GoC = mid-northern GoC	-1.0598 = -2.1089	17.516	2e-04***
				southern GoC = north Qld east coast	1.049 = 2.1089	2.9392	0.1153
				mid-northern GoC = north Qld east coast	-1.049 = 1.0598	11.6397	0.0013**

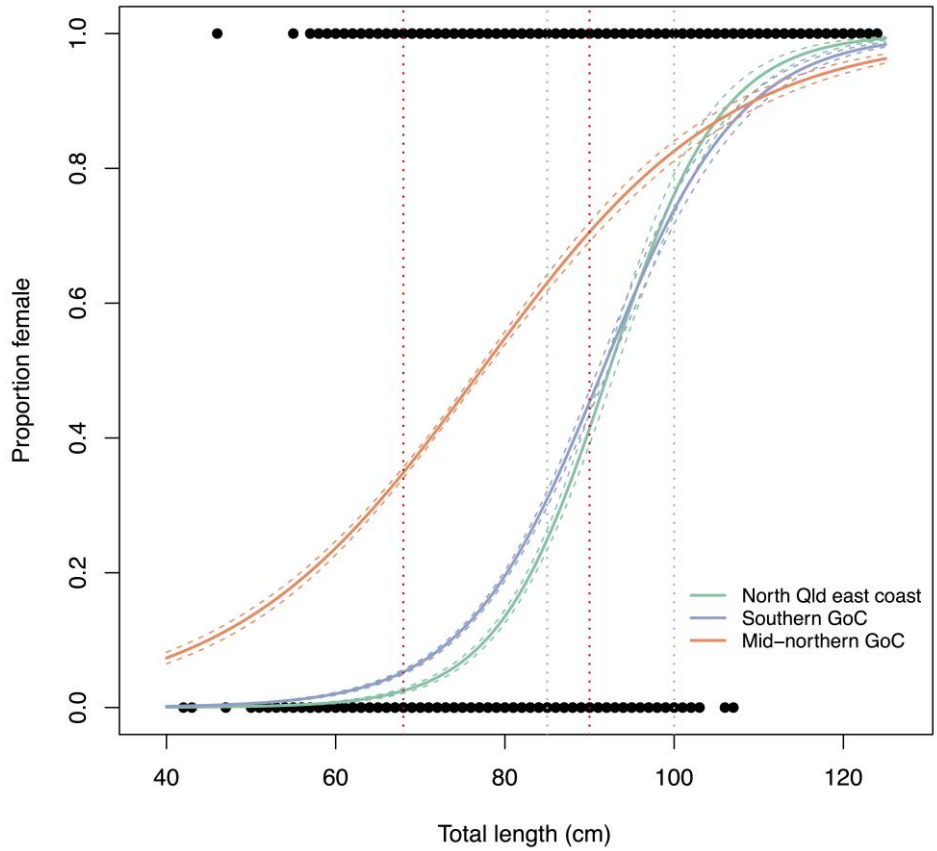

Figure 2.5 Fitted binomial GLM (solid lines) with standard error (dashed lines) showing predicted proportion of female barramundi, *Lates calcarifer*, at a given length for north Qld east coast (n=1999; green), mid-northern Gulf of Carpentaria (GoC; n=4900; orange) and southern GoC (n=4871; blue) for fish collected between 2000 and 2016. Red and green dotted vertical lines indicate previously reported size ranges for sex change in the northern GoC (68 - 90 cm) and Qld east coast (85 – 100 cm), respectively, as discussed in text.

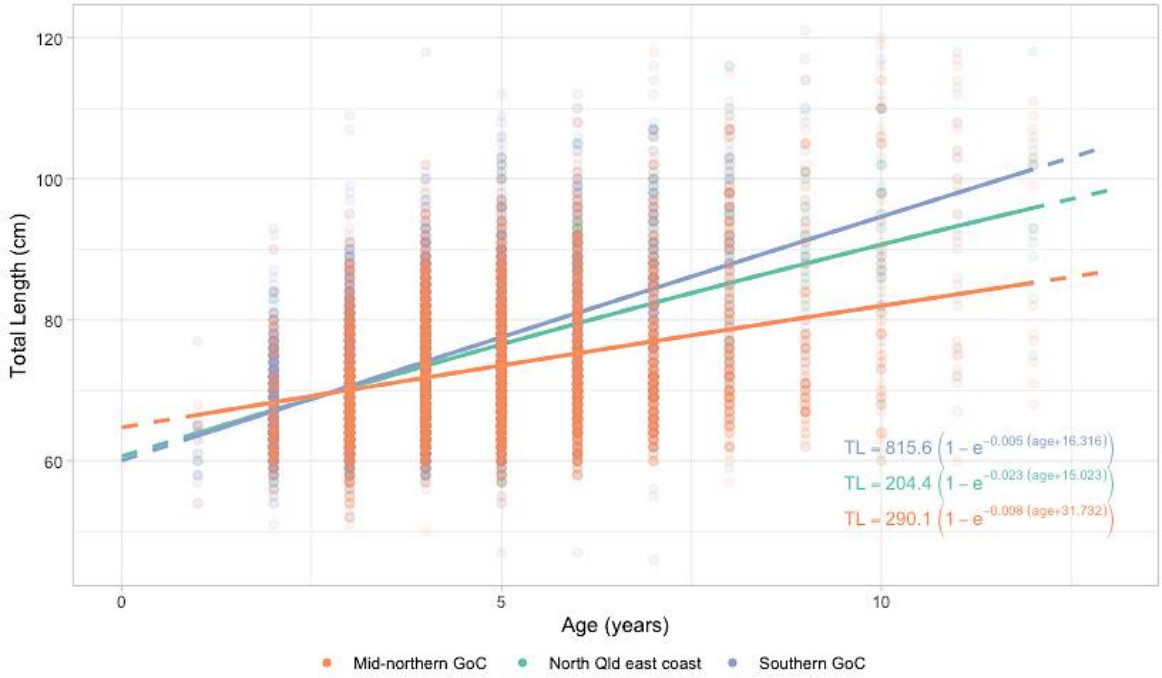

Figure 2.6 Fitted von Bertalanffy growth (solid lines) overlaid with raw data (dots) showing growth rate for *Lates calcarifer* collected from three regions in Queensland (Qld), Australia from 2000-2015. The equation for each region is written in the form of $TL = L_\infty(1\text{-}e^{-K(t\text{-}t0)})$ Where TL is the predicted length at age t years, L_∞ is the asymptotic mean length, K is the growth constant and t_0 the theoretical age at zero length. Individuals older than 12 years were omitted from the analyses.

2.4.5 Region-specific relationships between methylation and total length are more variable in female barramundi

In female barramundi, patterns of methylation were more variable between both amplicon and region comparisons compared to male fish. For example, methylation patterns in female associated amplicons *cyp19a1a* and *esr1* in female fish from the north Qld east coast were greater overall, and changes with length were of larger effect size than female fish from the mid-northern and southern GoC (Figure 2.3). While the observed negative relationship between methylation and total length in *cyp19a1a* and *esr1* in female barramundi was consistent between fish from all regions, the effect was significantly more pronounced in females from the north Qld east coast compared to the mid-northern and southern GoC (Figure 2.3). Female barramundi from the north Qld east coast region also had significantly greater methylation levels in *cyp19a1a* and *esr1* than fish from the mid-northern and southern GoC of the same length (Figure 2.3 and Appendix E). Individual hypothesis testing revealed significant differences between north Qld populations and both GoC populations for *cyp19a1a*, but only between north Qld and the southern gulf for *esr1* (Table 2.3). While methylation levels in male associated amplicons *nr5a2* and *dmrt1* were also higher in female fish from the north Qld east coast, the relationship between length and methylation was not significantly different between the southern GoC and north Qld east coast for *nr5a2* (Figure 2.4 and Table 2.3). Additionally, while the relationship between length and methylation was significantly different in all three regions for *dmrt1* in females, it is worth noting that methylation of *dmrt1* did not exceed 30 % in any fish. Thus differences in methylation levels between fish, while statistically significant, were of small effect size (Figure 2.3, Figure 2.4 and Appendix E). Overall, methylation levels were different in females from the north Qld east coast, but the direction or the relationship between methylation and total length was opposite in female fish from the mid-northern GoC compared to both other regions (Figure 2.3 and Figure 2.4). Specifically, for *dmrt1* and *nr5a2* in female fish, fish from the mid-northern GoC showed a decreasing pattern of methylation with increasing total length, whereas fish from the southern GoC and north Qld east coast showed an increasing pattern of methylation with increasing total length (Figure 2.4). Overall, while female barramundi from the north Qld east coast tend towards higher levels of methylation than females within the GoC, female barramundi within the mid-northern GoC exhibit a directionally divergent relationship between methylation and total length for male associated amplicons *nr5a2* and *dmrt1*.

2.4.6 Temperature

Analysis of variance in temperature data revealed small but significant differences in average minimum temperatures [F (2, 49677) =13.73, $p < 0.001$] and large and differences in maximum [F (2, 49677) =11481, $p < 0.001$] temperatures. Most notably, comparisons between the three regions specifically revealed that the average maximum temperature between the southern and mid-northern GoC did not significantly differ from each other ($p > 0.05$, Figure 2.7).

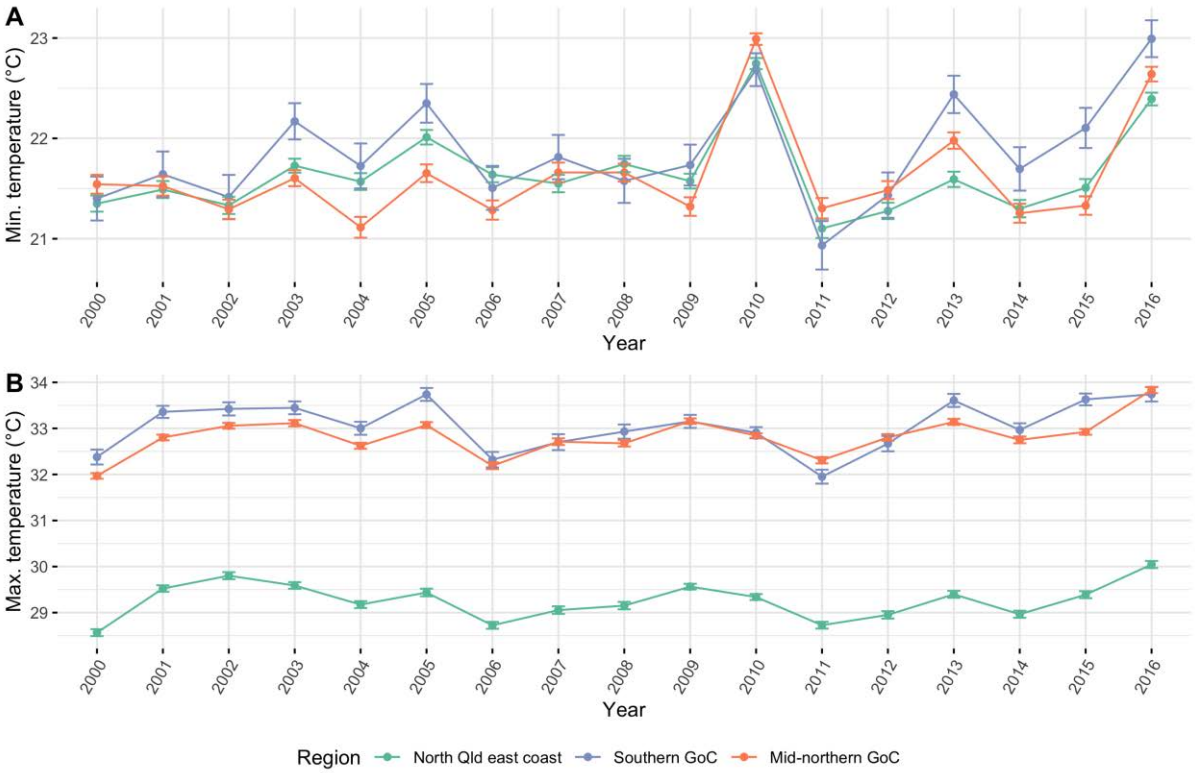

Figure 2.7 Average calendar year temperatures measured for north Queensland east coast (Barron, Mossman, Daintree, Johnstone rivers; green), mid-northern GoC (Coen, Archer and Watson rivers; orange) and southern GoC (Gilbert river; grey) barramundi catch locations. Data are represented as mean point with standard error bars.

2.5 Discussion

Here, results are presented for amplicons from three genes (*cyp19a1a, dmrt1* and *nr5a2*) initially identified by Domingos *et al.* (2018) as exhibiting male- and female-specific methylation in *L. calcarifer* plus an additional amplicon (*esr1*). The work additionally includes wild-caught samples from wider geographic range including individuals from genetically diverse populations accompanied by both length and age data. These attributes allowed for novel analysis of 1) an association between DNA methylation patterns, total length and sex in barramundi to investigate the hypothesis that gradual changes in DNA methylation accumulate with fish growth to become more female-specific; 2) comparisons of these patterns in barramundi from different regions to investigate the hypothesis that differences in total length-at-sex change are associated with differences in DNA methylation levels and 3) temperature as a potential environmental contributor to epigenetic variation in barramundi. Unequivocal evidence was not found to support the hypothesis that sex change is underpinned by a gradual accumulation of DNA methylation changes associated with increasing size in barramundi. Instead, highly specific male and female patterns of methylation were identified, with male and female *L. calcarifer* becoming more male-specific and female-specific in their methylation patterns with increasing length, respectively. Comparison of fish total length, DNA methylation and temperature between geographic regions revealed significant differences in total length-at-sex change between barramundi collected within the GoC, and that these differences were reflected in differences in DNA methylation levels but were not correlated with changes in the environmental temperature metric analysed here.

2.5.1 Sex-specific differences in methylation are likely to arise rapidly rather than gradually

Considering barramundi are protandrous hermaphrodites whose male to female sex change is closely related to the attainment of a minimum total length or associated body weight, our initial hypothesis was that methylation patterns in sex-associated genes would become more female-specific with increasing fish length, eventually leading to male to female sex change. Methylation analysis from fish of varying lengths, however, did not support this unequivocally. While in female barramundi, methylation patterns became more female-specific with increasing total length for female-associated genes in barramundi from all regions investigated (as well as male-associated genes in barramundi from the mid-northern GoC), sex change cannot be attributed to increased female-specific methylation patterns as this trend was only

observed in female fish that had already undergone the sex change process. Furthermore, a trend towards increasing female-specific methylation with increasing length was not observed for male barramundi from any region, or for any genes. As such, it does not appear that gradual changes in methylation over time is what leads to male-female sex change in *L. calcarifer*. Instead, it is likely that female-specific patterns of methylation arise rapidly during or just prior to sex change, rather than gradually over time with fish growth. Indeed, sex change in barramundi has been reported to occur in less than 17 days (Guiguen *et al.*, 1994). These previously observed, rapid changes in gonad morphology may provide explanation for the sizable and significant differences in gonadal methylation levels between male and female barramundi described here. Due to the short timeframe in which sex change occurs, transitional barramundi are rare [Moore (1979) reported a frequency of 1.4 %] and detection requires destructive sampling followed by histological examination of the gonad (Davis, 1982). As such, investigation of transitional gonads in wild fish is laborious for this species. Despite this, examination of methylation changes in barramundi actively undergoing sex change, as has been done in the protogynous ricefield eel (Zhang *et al.*, 2013a) and bluehead wrasse (Todd *et al.*, 2019), would provide further insight into how female-specific methylation patterns arise in *L. calcarifer* and other protandrous species.

2.5.2 Evidence for increased masculinisation in large male barramundi

Our data revealed that methylation in male barramundi becomes more male-specific with increases in total length. Male- and female-specific patterns of methylation in barramundi were first observed by Domingos *et al.* (2018) and were accompanied by changes in gene expression. While expression data is not available for the individuals used in this study, as samples obtained were not suitable for RNA extraction, it is expected that the observed changes in methylation would be negatively correlated with expression and lead to subsequent masculinization. A negative correlation between methylation and expression of *dmrt1*, *cyp19a1a*, *esr1* and/or *nr5a2* has been previously observed in protogynous bluehead wrasse (Todd *et al.,* 2019; *cyp19a1a* and *dmrt1*), protogynous ricefield eel (Zhang *et al.,* 2013a; *cyp19a1a*), digonic black porgy (Wu *et al.,* 2016; *cyp19a1a* and *dmrt1*) and gonochoristic Nile tilapia (Chen *et al.,* 2017; *cyp19a1a* and *esr1*). While this inverse correlation was previously observed for *dmrt1* and *nr5a2*, but not *cyp19a1a* in barramundi, it is likely that a direct relationship between methylation and expression in *L. calcarifer cyp19a1a* is complicated by the presence of multiple transcript variants and as such, may be better analysed by RNA-Seq methods than

traditional RT-qPCR (Domingos *et al.*, 2018). Indeed, in Chapter 4 it is found that full-length *cyp19a1a* expression occurs most prominently on female fish (Figure 4.15). Masculinisation in large barramundi, as predicted by an increase in male-specific methylation patterns in male fish, suggests that not all *L. calcarifer* undergo male to female sex change (as was first suggested by Moore 1979). Previous work in experimental barramundi has shown that while the average size of males is smaller than that of females of the same age, overlapping size distributions between the sexes still exist (Lim *et al.*, 1986; Guiguen *et al.*, 1994; Yue *et al.*, 2012). Collectively, these results suggest that there are factors in addition to size alone that lead to sex change in this species. For example, there may be social factors involved in the maintenance of male and/or development of female barramundi [as first proposed by Guiguen *et al.* (1994)]. In the case of protogynous half-moon grouper, *Epinephelus rivulatus,* sex change occurs at total lengths of approximately 22-35 cm and the suppressive dominance of large males over smaller females maintains a threshold female to male sex ratio (Mackie, 2000; Mackie, 2003). The effect of density and sex ratios on sex change in barramundi is yet to be investigated. Due to the diadromous nature of barramundi, smaller, predominantly male fish live further upstream whereas larger more commonly female fish frequent estuaries (Crook *et al.*, 2017). While increased salinity associated with weather changes and estuaries more generally has been investigated as an additional, potential driver of sex change, the results revealed no significant effect (Athauda and Anderson, 2014). The causes of male maintenance and possible masculinisation in large male barramundi remain unknown but warrant further investigation.

2.5.3 Evidence for feminisation in large female barramundi

In female barramundi, decreasing methylation of female associated amplicons *cyp19a1a* and *esr1* was observed with increases in length, suggesting an increase in female-associated in large female barramundi. While not linked to male to female sex change, which had already occurred in these individuals (assuming no primary females occur), these differences may indicate changes in reproductive capacity. For example, in Nile tilapia, lower methylation of *cyp19a1a* and *esr1* leads to increased gene expression (Chen *et al.*, 2017) and a subsequent increase in levels of circulating aromatase protein (Wang *et al.*, 2017). A similar increase in levels of circulating aromatase protein, as well as 17β-estradiol (E2) are known to occur in experimentally-derived female barramundi and may be associated with changes in methylation of these two genes as well (Athauda *et al.*, 2012). Furthermore, in this study the pseudo r^2 for

esr1 was the lowest out of all the models, indicating increased variability in methylation may be due to factors not measured here. E2 levels in fish are known to stimulate the production of vitellogenin (the precursor of egg yolk proteins), which subsequently leads to transcriptional activation of *esr1* (Devlin and Nagahama, 2002; Sawaguchi *et al.*, 2005; Katsu *et al.*, 2008; Todd *et al.*, 2016). In barramundi, spawning is linked to seasonal temperature cycles, preceding and coinciding with the summer monsoon in Australia (Garrett *et al.*, 1987). The observed variability in methylation of *esr1* in female barramundi may indicate changes in circulating steroid hormones that occur with changes in reproductive capacity with spawning, and temporal factors may more comprehensively explain the methylation variability observed in this gene. Overall, the general decrease in methylation of *cyp19a1a* and *esr1* with increasing length observed in female barramundi within this study may be a reflection of increasing circulation of aromatase and E2 proteins, and a subsequent increase in reproductive capacity in large female barramundi. Indeed, there is a strong relationship between increases in total length and fecundity in this species (Moore, 1982).

2.5.4 Region-specific differences in total length-at-sex change are reflected in differences in methylation of male fish

While previous reports have shown differences in total length-at-sex change between barramundi from the Qld east coast and GoC as a whole (QFMA, 1991) further analysis of the data as shown here reveal regional similarities in length-at-sex change between the north Qld east coast and southern GoC, both occurring at around 16°30'S (see Figure 2.1 and Figure 2.6). However, note that their temperature regimes are very different despite common latitudes (Figure 2.7). Furthermore, while an abrupt difference in length-at-sex change was previously observed in GoC fish north of 13 °S, significant size differences within regions of the GoC *below* this latitude were found, suggesting that variation in length-at-sex change is more clinal than previously thought (Davis, 1984b). Similarly, it is also unlikely that the variation in length-at-sex change of females is due to genetic factors since fish from both the southern and mid-northern GoC, as described here, originate from a single genetic subpopulation and stock (Shaklee and Salini, 1985; Keenan, 1994; Jerry *et al.*, 2013) but were epigenetically distinct. Other population and/or habitat-specific methylation patterns have been observed in fish species such as Atlantic salmon, *S. salar* (Morán and Pérez-Figueroa, 2011), *Chrosomus eos-neogaeus* hybrid fish (Massicotte *et al.*, 2011; Massicotte and Angers, 2012), tessellated darter, *Etheostoma olmstedi*, (Smith *et al.*, 2016) and threespine stickleback, *Gasterosteus aculeatus*

(Smith *et al.*, 2015; Trucchi *et al.*, 2016). Notably, in *C. eosneogaeus,* a clonal diploid fish, epigenetic variation occurs in the absence of genetic variation, but is consistent with significant variation in environmental pH, suggesting that identical genotypes respond to environmental cues (here, pH) via differences DNA methylation (Massicotte and Angers, 2012). Similarly, in Atlantic salmon the gonads present high levels of variation in DNA methylation in absence of genetic variation, which is thought to lead to early sexual maturation in some individuals of this species, although the two have not yet been directly linked (Morán and Pérez-Figueroa, 2011). Both of the latter examples demonstrate population-level epigenetic variation in wild fish species and provide support for the role of epigenetic processes in generating phenotypic plasticity. The work presented here additionally demonstrates that differential DNA methylation of sex-related genes may be associated with disparity in length-at-sex change between regions. As such, the work is the first to show that region-specific differential DNA methylation of trait-of-interest related genes is associated with phenotypic plasticity.

2.5.5 Differences in total length at which sex change occurs are not correlated with average ambient temperature

While the environmental causes of the differences in length-at-sex change between barramundi from different regions are unclear, there is some evidence to suggest that temperature may play a role (Athauda *et al.*, 2012). In barramundi, maturation of the gonads and subsequent spawning is intimately linked with seasonal changes in temperature and salinity that occur at the end of the dry season, just prior to the arrival of the southwest monsoon in northern Australia (Grey, 1987; Pusey *et al.*, 2004). Synchronicity with monsoonal weather allows juveniles to take advantage of increased rainfall, habitat and freshwater flow (Davis, 1985; Robins *et al.*, 2006). Sex change in adult barramundi commences just prior to spawning, as the testes ripen for a final time, and finishes shortly after (Davis, 1982). Given the intimate association between gonad maturation, spawning and changes in temperature associated with the monsoon, it is thought that temperature may additionally trigger sex change (Athauda *et al.*, 2012). Despite this, our analysis of ambient temperature between regions indicated that greater temperature differences exist between the east coast and both regions of the GoC rather than between regions of the GoC, where size differences exist. As such, it may be that there are more fine scale temperature fluctuations and/or a combination of environmental contributors, such as salinity or flow, leading to the observed differences in DNA methylation and smaller size-at-sex change in barramundi from the mid-northern GoC observed here. Furthermore, while

increases in total length typically lead to sex change in barramundi, it is also known that reduced growth rate, or stunting, is linked to precocious maturation and early sex change in this species, previously only observed for the far northern GoC stock (Davis and Kirkwood, 1984), but here observed for the mid-northern GoC population as well. While suboptimal temperatures are a key contributor to stunting (Wootton, 1998; Lee *et al.*, 2010), reduced growth rate in fish has also been attributed to high population density (Pickering, 1981; Wedemeyer and McLeay, 1981; Wedemeyer, 1997; Das *et al.*, 2016) and poor nutrition (Ali *et al.*, 2003; Abdel-Hakim *et al.*, 2009). Populations of barramundi in the mid-northern GoC are therefore be maturing and sex changing at smaller total lengths, possibly due to stunting, caused by unfavourable environmental conditions other than temperature. Further research is required to investigate the environmental correlates that could be contributing to the observed differences in length-at-sex change and growth rate in mid-northern GoC barramundi.

Exogenous environmental effects on sex change have also been investigated in cultured barramundi, revealing that maturation of the gonads is influenced by changes in temperature but not salinity (Athauda *et al.*, 2012; Athauda and Anderson, 2014). In these experiments, elevated temperatures of 31 and 34 °C, similar to that experienced in the Gulf of Carpentaria (Figure 2.7), resulted in the early induction of sex change (Athauda *et al.*, 2012). However, it remains to be determined if the observed increase in aromatase and subsequent male to female sex change is underpinned by changes in DNA methylation of *cyp19a1a and/*or other sex genes, and if hermaphrodite fish show similar mechanisms of temperature sensitivity to gonochoristic fish. In gonochoristic fish, temperature effects on DNA methylation and sex have been well documented, particularly in two key genes essential to ovarian and testis development; *cyp19a1a* and *dmrt1,* respectively (see Chapter 1, Table 1.1 for examples). While region-specific, environmental temperature differences could lead to differential methylation of *cyp19a1a, dmrt1* and other genes in barramundi, as has been observed in cultured barramundi, our analysis did not support this. Our analysis indicated that greater temperature differences exist between the east coast and both regions of the GoC rather than between regions of the GoC, where differences in DNA methylation of *cyp19a1a, esr1, nr5a2* and *dmrt1* were observed. The use of fine scale temperature data and additional parameters such as rainfall and flow, as well as an investigation of evidence for precocious maturity would likely shed greater light on the environmental causes of the differences in size at sex change and DNA methylation between geographic regions observed here.

2.6 Conclusion

Here evidence is presented for concurrent epigenetic and phenotypic variation in wild populations of barramundi in Queensland, Australia. It was found that male and female barramundi show markedly different relationships between DNA methylation and total length, with male methylation patterns becoming more male-specific at larger length despite the protandrous nature of this fish. Barramundi from the mid-northern GoC exhibited a smaller total length-at-sex change and differential DNA methylation patterns compared to barramundi from the southern GoC and north Qld east coast. Notably, greater variance in total length-at-sex change and DNA methylation occurred within the GoC despite the mid-northern and southern GoC being from a single and separate genetic stock than the north Qld east coast and experiencing more comparable average environmental temperatures. While the environmental drivers of this variation are yet to be identified, this research provides a unique example of population level, epigenetically driven phenotypic plasticity in a commercially important, sex changing species.

Chapter 3

Delayed effect of low rearing temperature on methylation in the gonads of developing juvenile barramundi, *Lates calcarifer*

3.1 Summary of results

Throughout Chapter 3, the effects of experimental temperature treatment on cultured juvenile barramundi were examined. To do so, temperature treatments of 29 °C (Control), 24 °C (cold temperature; CT), 24 - 34 °C (fluctuating temperature; FT), 34 °C (hot temperature; HT) were applied to developing barramundi from 10-90 days post hatch and the effects on DNA methylation (BSAS), gene expression (RT-qPCR), length and weight as well as gonadal development (histology) were analysed. The major results include:

1. No significant or substantial differences in DNA methylation were observed at 6 mph.
2. Significant differences in DNA methylation of amplicons covering partial promoter and first exon (PE1) of *amh, esr1,* and *nr5a2* in CT-treated barramundi at 12 mph;
    a. *amh* PE1 [$M(SD)_{CT}$ = 0.747 (0.1206), $M(SD)_{Control}$ = 0.6829 (0.1114), $p < 0.05$],
    b. *esr1* PE1 [$M(SD)_{CT}$= 0.8184 (0.0784), $M(SD)_{Control}$ = 0.6932 (0.1025), $p < 0.001$] and
    c. *nr5a2* PE1 [$M(SD)_{CT}$ = 0.2889 (0.1474), $M(SD)_{Control}$ = 0.3964 (0.1304), $p < 0.01$].
3. DNA methylation of these amplicons in CT-treated fish was more similar to, and in some cases not significantly different from, adult male barramundi;
    a. *amh* PE1 [$M(SD)_{adult\ male}$ = 0.76 (0.16), $M(SD)_{CT}$ = 0.747 (0.12), NSD]
    b. *esr1* PE1 [$M(SD)_{adult\ male}$ = 0.82 (0.12), $M(SD)_{CT}$= 0.82 (0.08), NSD]
    c. *nr5a2* PE1 [$M(SD)_{adult\ male}$ = 0.23 (0.16), $M(SD)_{CT}$ = 0.29 (0.15), $p <$ 0,001].
4. High expression (as ΔCt) of the male sex determining gene *dmrt1* in CT-treated barramundi
    a. $M(SD)_{Control}$ = 0.0111 (0.0038), $M(SD)_{CT}$ = 0.0183 (0.0092), $M(SD)_{FT}$ = 0.0114 (0.0057), $M(SD)_{HT}$ = 0.0125 (0.0038), NSD.
5. Reduced length and weight at 6 but not 12 mph, and a faster growth rate between 6 and 12 mph in CT-treated fish.
    a. Length (cm)
        i. 6 mph [$M(SD)_{CT}$ = 20.60 (1.88), $M(SD)_{Control}$ = 22.16 (2.25), $p < 0.05$]
        ii. 12 mph [$M(SD)_{CT}$ = 35.25 (2.66), $M(SD)_{Control}$ = 34.50 (2.29), NSD]
    b. Weight (g)

i. 6 mph [$M(SD)_{CT}$ = 96.38 (26.76), $M(SD)_{Control}$ = 127.65 (44.66), $p < 0.05$]
ii. 12 mph [$M(SD)_{CT}$ = 618.4 (166.59), $M(SD)_{Control}$ = 538.7 (112.07), NSD]

c. Von Bertalanffy growth as $TL = L_{\infty}(1\text{-}e^{-K(t-t0)})$ where K is the growth constant
i. CT: $TL = 38.2(1\text{-}e^{-0.298\sim(age-3.403)})$
ii. Control: $TL = 39.4(1\text{-}e^{-0.209\sim(age-2.049)})$
iii. FT: $TL = 45.2(1\text{-}e^{-0.172\sim(age-2.484)})$
iv. HT: $TL = 41.1(1\text{-}e^{-0.250\sim(age-2.928)})$

6. Advanced male development in CT-treated fish compared to hot, control and fluctuating treatments, indicated by a greater proportion of late-stage spermatogenesis cells (spermatocytes and spermatids; Figure 3.12).

This chapter constitutes the first examination of the effects of temperature on primary sexual differentiation in a sequential hermaphrodite fish. The results demonstrate that juvenile barramundi are amenable to cold temperature treatment via changes in DNA methylation and gonadal phenotype and offer a potential, currently unavailable method of sex control for barramundi aquaculture.

3.2 Introduction

Sexual differentiation in fish can be highly amenable to temperature manipulations. In many gonochoristic fish species, reversal of the genetically determined sex (e.g. XX or ZW) can be achieved through ecological or experimental alterations in temperature applied at a specific period during development. This period is known as the thermosensitive period (TSP) and is particularly evident in the larval stages of fish (Baroiller and D'Cotta, 2001; Conover, 2004). A large body of research is dedicated to sex reversing fish through temperature manipulation, for both theoretical advancement and aquaculture production (Martinez *et al.*, 2014; Budd *et al.*, 2015). Understanding the effects of culture environment on sex ratios in aquaculture can offer greater reproductive control and can enable the biased production of the better preforming sex. For example, in Nile tilapia, *Oreochromis niloticus*, male fish typically demonstrate faster growth rates than female fish and high temperature treatment has been suggested as a way to generate a greater proportion of male offspring (Baras *et al.*, 2001). In European seabass, *Dicentrarchus labrax,* high temperature treatments also lead to a greater percentage of male offspring, however, males demonstrate a slower growth rate compared to females, so the interest lies in production of females rather than males, in this species (Saillant *et al.*, 2001). Over the last decade, research into the mechanism by which temperature exerts an effect on sexual phenotype in fish, and other ectotherms, has revealed an epigenetic modification called DNA methylation as a potentially universal underlying mechanism.

DNA methylation involves the replacement of the fifth carbon of cytosine with a methyl group, almost exclusively at CpG dinucleotides; where cytosine's (C) are phosphate-bonded (p) to guanidine's (G). CpG sites can be differentially methylated to allow tissue-specific or sexually dimorphic gene expression through the activation or repression of the associated downstream gene (Gardiner-Garden and Frommer, 1987). This is because, at the genomic level, the methylated CpG sites limit the accessibility of the target gene by transcriptional machinery which, in turn, inhibits gene expression. DNA methylation was first proposed by Gorelick (2003) as the mechanism allowing organisms with homomorphic sex chromosomes (i.e. chromosomes that are indistinguishable by karyotype data alone) to alter their gender in response to small environmental changes, such as temperature. While it is likely that DNA methylation works in combination with other epigenetic mechanisms (micro RNAs and histone modifications), methods of analysis for DNA methylation are the most well established and provide an effective tool to study epigenetic changes during fish sexual development.

Temperature-induced shifts in sex ratio of many cultured fish are accompanied by changes in DNA methylation of key sex genes. For example, in the European sea bass, Navarro-Martín *et al.* (2011) showed that temperature-induced DNA methylation of cytochrome P450 aromatase (*cyp19a1a*; known as gonadal aromatase) resulted in testis development in genetically female fish. The group further went on to show that while the temperature treatment was applied during the pre-established temperature sensitive period (10-60 days post hatch; dph), treatment effects were still evident at three months post hatch (Díaz and Piferrer, 2015). Even where strict genetic sex determining systems are in place, such as the ZW chromosomes of the half-smooth tongue sole, *Cynoglossus semilaevis*, high temperature incubation during the TSP is able to induce female to male sex reversal. In the tongue sole, sex-reversed males develop due to temperature-induced inhibition of DNA methylation of the key testis-promoting gene doublesex mab-3 related transcription factor 1 (*dmrt1*; Shao et al., 2014). Male fish that have been sex-reversed are genetically female (ZW chromosomes) but phenotypically male (testis development). Similarly, in Nile tilapia, high temperature treatment leads to the development of genetically female (XX chromosomes) but phenotypically male fish, specifically via changes in DNA methylation and expression of both *cyp19a1a* and *dmrt1* (Wang *et al.*, 2019). These results, among others, suggest that DNA methylation is the underlying mechanism by which temperature induces sex reversal in gonochoristic fish species.

DNA methylation has also been shown to guide the process of sex change in hermaphrodite fish. For example, in the protogynous (female-first) ricefield eel, *Monopterus albus*, female to male sex change is accompanied by increased methylation and decreased expression of *cyp19a1a* (Zhang *et al.*, 2013a). Similarly, in the protogynous bluehead wrasse, *Thalassoma bifasciatum,* the transition from female to male is triggered by changes in DNA methylation and subsequent gene expression of *cyp19a1a* and *dmrt1* (Todd *et al.*, 2019). In the opposite direction, male to female sexual phase change in the digonic black porgy, *Acanthopagrus schlegelii,* is associated with changes in methylation and expression of these same genes (Wu *et al.*, 2012). Black porgy are considered digonic, as they possess both testicular and ovarian tissue, but function first as male and then undergo sexual phase change to functional females (Lee *et al.*, 2008). The effect of temperature on sex in hermaphrodites has received little attention, but one recent investigation in the androdioecious (consisting of males and simultaneous hermaphrodites) mangrove killifish, *Kryptolebias marmoratus,* showed that the proportion of male to hermaphrodite offspring increased with cold egg incubation

temperatures, and was associated with changes in methylation of *cyp19a1a* and *dmrt1,* as well as other known sex genes*;* SRY-box 9a (*sox9a*)*,* forkhead box protein L2 (*foxl2*) and Gonadotropin Releasing Hormone Receptor (*GnRHR*; Ellison *et al.,* 2015) . These results in killifish suggest that the mechanism by which temperature treatment alters sex ratios in gonochoristic species may also be common to hermaphrodite fish.

To date, no temperature related sex reversal techniques have been reported in a sequential hermaphrodite. Barramundi, *Lates calcarifer*, also known as the Asian seabass, is a protandrous sequential hermaphrodite and a popular food fish for which the availability of genomic and transcriptomic resources is rapidly building (Kuznetsova *et al.*, 2014; Ravi *et al.*, 2014; Vij *et al.*, 2016; Domingos *et al.*, 2018). This euryhaline, catadromous fish typically matures first as male at two to four years of age and undergoes sequential sex change to female between six and eight years, although in rare cases primary female development may occur (Davis, 1982; Guiguen *et al.*, 1994). Sex change shows a strong correlation with fish total length, occurring at about 80-90 cm, depending on the population (Moore, 1979; Davis, 1982). Sequential sex change in this species presents significant challenges for selective breeding. This is because large expensive infrastructure required to hold females limits the capacity of hatcheries to carry sufficient female broodstock to warrant the conduct of selection and once established, protandry and therefore asynchronous maturation of males and females prevents same generation selection practices, reducing overall genetic gain. The environmental factors influencing sex change in barramundi have not been identified and there is currently no intervention option available for hatchery managers to control or speed up the sex change process (Robinson *et al.*, 2010). As such, this research aims to investigate whether or not exposure to high, low, or fluctuating temperatures during development leads to altered patterns of DNA methylation in *L. calcarifer* and the possibility of primary sex reversal in hermaphrodite fish, as has been demonstrated in cultured gonochoristic species.

3.3 Methods

3.3.1 Animals, rearing conditions and temperature treatments

Larvae from a single spawning event were obtained from an on-site commercial hatchery (Townsville, QLD, Australia). Spawning procedures, handling methods and rearing conditions followed standard intensive barramundi hatchery practices in Australia (Schipp *et al.*, 2007), including a 12 h light and 12 h dark photoperiod and periodic grading after 18 dph to avoid

cannibalism (Dhert *et al.*, 1992). To examine the effect of temperature on the developing gonad in *L. calcarifer* a temperature treatment was applied throughout the predicted TSP; from 10-90 dph. This period coincides with the initial stages and just prior to the onset of gonadal development in control (29 ˚C) barramundi which occurred from approximately 44 to 140 dph (Banh *et al.*, 2017). Treatments of approximately 24 ˚C (cold temperature; CT), 34 ˚C (hot temperature; HT) and 29 ˚C (control) were selected to allow for adequate treatment intensity without compromising growth efficiency or survival (Williams *et al.*, 2006; Katersky and Carter, 2007). A fluctuating treatment (FT) of 24 - 34 ˚C was also applied to simulate accelerated seasonal temperature fluctuations that may be associated with maturation in wild populations of *L. calcarifer* (Davis, 1985). To execute these treatments, at two dph approximately 500,000 *L. calcarifer* larvae were divided into 12 500 L tanks held at 29 ˚C. Larvae were acclimatised 0.5 to 1 ˚C per day over a five-day period (6-10 dph) to 25 (CT initial temperature), 29 (control), 33 ˚C (HT initial temperature), or cycling though 24 – 34˚C (FT). Each of the four temperature treatments consisted of three tank replicates and the temperature of each tank was measured twice daily to ensure that the treatments were as close as practical to their target temperatures (Figure 3.1). At day 30, the 25 and 33 ˚C tanks were altered to their final experimental temperatures of 24 and 34 ˚C for the CT and HT treatments, respectively. Throughout the temperature treatment period, the three tanks subject to FT treatment were increased by 1 ˚C per day with a one week hold at 33 ˚C and then lowered at the same rate with a one week hold at 25 ˚C for a total of three cycles (Figure 3.1). At 90 dph, fish were re-acclimatised 1 ˚C per day over a five-day period (90-95 dph) to 29 ˚C, and at 100 dph fish from replicate tanks of each treatment were pooled into four 3000 L tanks for on rearing at 29˚C (Figure 3.1). Following pooling at 100 dph, rearing conditions for all treatments were identical. Research was conducted under James Cook University animal ethics approval Permit Number A2014.

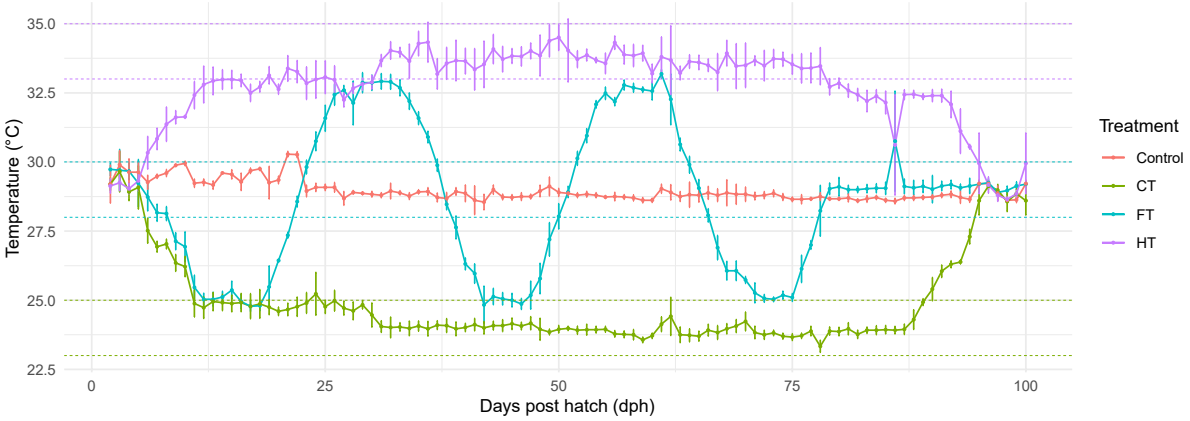

Figure 3.1 Recorded temperatures for each of four experimental treatments applied to developing *Lates calcarifer* from 0-100 days post hatch. Temperature readings were collected twice daily, and data represent means ±SD for both recordings on each of the three tank replicates. Dotted lines represent final target temperatures ±1 °C. Control: 29 °C, CT: cold temperature; 24 °C, FT: fluctuating temperature 24 – 34 °C, HT: hot temperature; 34 °C.

3.3.2 Sampling

Gonadal sampling occurred at 6 and 12 months post hatch (mph), as this was the earliest time at which the gonad tissue was able to be reliably extracted without contamination from surrounding tissue and in adequate portions for gDNA and RNA extractions from individuals. For these samplings, n=10 juvenile *L. calcarifer* from each temperature treatment were euthanised using AQUI-S, and gonadal tissue removed and immersed in RNAlater (Thermo Fisher Scientific). Length and weight were recorded for each individual. At 12 mph, a portion of the gonads was fixed in 10% neutral buffered formalin containing 4% formalin for at least 24 h for histological examination.

3.3.3 DNA/RNA extraction

Total RNA was extracted from homogenised gonad tissue using TRIzol® (ThermoFisher 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, 0.8M disodium citrate (Sambrook and Russell, 2001)] was 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 carried out on an ND-1000 spectrophotometer

(Nanodrop technology) based on absorbance at 260 nm and 260/280 nm ratio. RNA integrity was analysed using a Qsep100 (Bioptic).

Because validation of epigenetic changes occurs through gene expression, and gene expression can vary along the gradient of a developing gonad (Strüssmann and Ito, 2005) RNA and DNA were extracted from a single gonadal tissue homogenate. For this, the interphase and organic phase from the TRIzol extraction was preserved and the DNA precipitated in 100% ethanol as the manufacturer's instructions. The resulting pellet, followed the CTAB protocol (Doyle and Doyle, 1987), including an overnight digestion with proteinase K. DNA quantification was carried out as per RNA and integrity was assessed by visualisation on a 0.8% agarose gel with lambda DNA standards at 50, 20, 10 and 5 ng/µl.

3.3.4 Gene expression analyses by real-time quantitative PCR (RT-qPCR)

In order to study the difference in gene expression among different treatments, RT-qPCR was performed for *dmrt1* and *ubq* using previously published primers, as the expression of this gene is a key indicator for male development in other teleost species (De Santis *et al.*, 2011; Herpin and Schartl, 2011; Ravi *et al.*, 2014; Cui *et al.*, 2017). Analysis of female-associated gene *cyp19a1a* was not performed due to insufficient expression to generate a standard curve applicable to the male individuals in this experiment. To use as template for the RT-qPCR reactions, 5 µg of total RNA from fish at 12 mph was reverse transcribed using the iScript Advanced Complementary DNA (cDNA) Synthesis Kit following the manufacturer's instructions (Bio-Rad) and diluted 1:10. RT-qPCR reactions (15 µl final volume) contained 7.5 µl SsoFast Supermix (Bio-Rad), 0.4 µM each forward and reverse primer, 1.3 µl RNase/DNase-free water and 5 µl template cDNA. Reactions were run using the Rotor-Gene 3000 (QIAGEN) thermocycler under the following conditions: 95 ˚C for 30 sec followed by 45 cycles of 95 ˚C for 5 sec and 58 ˚C (*dmrt1*) or 61 ˚C (*ubq*) for 15 sec. A final melt step (58-95 ˚C in 0.5 ˚C increments per cycle, each 5 sec each) was included to identify the presence of primer dimers and to analyse the specificity of the reaction and a standard curve was created to determine appropriate sample concentration. Ten gonadal samples of each treatment and non-template controls were run in triplicate.

3.3.5 Histology

Formalin-fixed samples were subjected 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 CelSens Microscope Digital Camera System (Olympus) and used to determine the stage of gonadal development. Following microscopic observations, developmental stages were assessed according to Guiguen *et al.* (1994).

3.3.6 Bisulphite conversion of gDNA and amplicon specific PCR

While there are a vast number of genes known to be involved in sexual differentiation in fish, differential DNA methylation has been specifically implicated in sexual development and/or temperature manipulations for gonadal aromatase *cyp19a1a*, *dmrt1*, anti-Müllerian hormone (*amh*), nuclear receptor subfamily 5 group A member 2 (*nr5a2*), estrogen receptor 1 (*esr1*), *foxl2* and *sox9* (Zhang *et al.*, 2013a; Parrott *et al.*, 2014; Wen *et al.*, 2014; Si *et al.*, 2016; Wu *et al.*, 2016; Chen *et al.*, 2017; Domingos *et al.*, 2018; Laing *et al.*, 2018). As such, these genes were chosen for examination in temperature treated *L. calcarifer*, largely focusing on the promoter regions and partial first exons as methylation in these regions are often best correlated with gene expression (Anastasiadi *et al.*, 2018). To analyse DNA methylation of the target regions, a bisulphite amplicon sequencing (BSAS) approach adapted from Masser *et al.* (2013) was employed as described in Chapter 2 section 2.3.3, using 1 µg of extracted gDNA as input, employing additional gene-specific primers and associated annealing temperatures as listed in Table 3.1.

3.3.7 NGS library preparation and DNA methylation Quantification

Dual indexed libraries were generated as described in Chapter 2, section 2.3.4 with the exception of an additional eight rounds to the first-stage PCR (total 20 rounds), and a dilution of the final libraries to 6 pM.

3.3.8 Data analysis and statistics

All statistical analyses were carried out using RStudio v1.2.1335 (Allaire, 2012) using algorithms implemented in their associated packages including Student's t-test and analysis of variance from *stats* v3.4.2 (R base package) as well as pair-wise comparisons using Tukey's

Honest Significant Difference, Bonferroni corrections and compact letter displays in *multcomp* v1.4-8 (Hothorn *et al.*, 2016). All heat maps and box plots were drawn using ggplot2 v3.1.0 (Wickham, 2016) and gene structures were modelled using genoPlotR (Guy *et al.*, 2010). To enable assessment of the observed DNA methylation levels in temperature treated juveniles compared to the expected DNA methylation levels for adult male and female barramundi, data obtained in Chapter 2 and Domingos *et al.* (2018) are included in the analysis and presented in the figures. The Von Bertalanffy growth model (Von Bertalanffy, 1938) was fitted to the observed length-at-age data as measured at 6, 9 and 12 mph. A function for the typical VGBF was constructed using the *vbFuns* function from the *FSA* package and starting values were obtained using *vbStarts*, specifying to estimate theoretical age at zero length using the mean length of the first age with more than one data point as a known quantity (Ogle and Ogle, 2017). The parameters of the VGBF were estimated using Levenberg-Marquardt least squares non-linear regression (Moré, 1978) as implemented in the *nls* function from the *FSA* package.

Table 3.1 *Lates calcarifer* sex-related genes and primer sequences investigated for bisulphite amplicon next-generation sequencing (BSAS).

Gene	Description	Amplicon name	Primer name	Gene specific sequence	Product size (bp)	Annealing (°C)	No. CpGs
amh	promoter and exon 1	amh_PE1	amh-F	FO-TGGTGTGTGTTTGAATTAGAAAATT	408	57.5	6
			amh-R	RO-CCATAAAAAACATAAAAAACCACAC			
	exon 7 of 7	amh_E7	amh_BS_E7_F2	FO-GTATGTGATAGGAGAAAGTTAGTAT	349	50	20
			amh_BS_E7_R2	RO-CTCTCAATATAAATATTAAACAAAAC			
cyp19a1a	promoter and exon 1	cyp19a1a_PE1	CYP19-BS-F	FO-TGGTTGTTTATAAAGGGGAAGTTT	334	57.5	8
			CYP19-BS-R	RO-CCAACAACAAACAAACAAATAACATA			
	exon 8 of 9	cyp19a1a_E8	Cyp19a_BS_E8_F1	FO-TTGTAGGTGAGAGATAGTTTTAGAA	299	50	8
			Cyp19a_BS_E8_R1/2	RO-TCCAAACTAAATTCATTTAATTTAC			
dmrt1	promoter	dmrt1_P	D1-BS-P*-F1	FO-TTGATTAGGATTTGTGTTTTAAAGT	338	57.5	5
			D1-BS-P*-R1	RO-TAAAACCTATTATTTCATATAAACATATTT			
	promoter and exon 1	dmrt1_PE1	dmrt1_BS_P_F2(2)	FO-AAAATTAAGTGTAGTAGAGTGATGTTAT	498	57.5	17
			D1_BS_CDS_R1	RO-AAACACTAACAATCCCTCCAATTAC			
	exon 1	dmrt1_E1	dmrt1_BS_P_F1(2)	FO-GTTGGGTGTTTTTTTATTTTTTTTG	270	57.5	8
			D1_BS_CDS_R1	RO-AAACACTAACAATCCCTCCAATTAC			
	exon 5 of 5	dmrt1_E5	dmrt1_BS_E5_F1/2	FO-TGTGTTTTTTGTTTTATTTTTGTAG	253	50	14
			dmrt1_BS_E5_R2	RO-TCCTTAACTTCATTATTTAATTATATC			
esr1	promoter and exon 1	esr1_PE1	ESR1_BS_PE1_F1	FO-TGTGTTGTGATGTTGTTTAGGTAGAG	275	57.5	8
			ESR1_BS_PE1_R1	RO-TTCCAAAAAATCCACAATAACTACC			
foxl2	exon 1 of 1 part 1	foxl2_E1pt1	foxl2_BS_E1_F1/2	FO-GTAATGATGGTTATTTATTAAAATT	336	50	14
			foxl2_BS_E1_R1	RO-TTTTTATTTTTCTCATAAAAAAAA			
	exon 1 of 1 part 2	foxl2_E1pt2	F2-BS-CDS-F1	FO-AGTTTGTGAGGATATGTTTGAGAAG	375	50	18
			F2-BS-CDS-R1	RO-CCATACTCTACACCCTAAAATAAAAATTAT			
	promoter	foxl2_P	F2-BS-P-F1	FO-AAAGGGTTGGGTTTATTGATTTATAA	322	50	9
			F2-BS-P-R1	RO-ATCCAAATACCAACAAACAAAACTT			
sf-1	promoter	nr5a2_P	sf1-BS-F1	FO-TTTTGTGTGTTTTTATTTGTTTGTG	369	57.5	10
			sf1-BS-R1	RO-TTCTTTCTCAATTCTTTTAAACTTTTAAAT			
	nr5a2 promoter exon 1	nr5a2_PE1	sf1-BS-F2	FO-GGAAAAGAGATTGTTTAGTATAGTAATAGA	261	57.5	8
			sf1-BS-R2	RO-TAAAAACACTAACCTTACAACTCTC			

sox9	*sox9 promoter exon 1*	*sox9_PE1*	*S9-BS-F2*	*FO-ATTTAGTTTTGTTAGTTAAGTTGTG*	*411*	*50*	*19*
			S9-BS-R2	*RO-TACAAACAAAAAACTTTTCTTCTTC*			

[1] FO (5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG) and RO (5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG) are Illumina's forward overhang (FO) and reverse overhang (RO) adapter sequences added to locus-specific primer sequences

3.4 Results

Quantification of DNA methylation by BSAS methods revealed variation in amplicon methylation levels between treatments, between amplicons among different genes and between different amplicons covering different regions of the same gene. Detailed descriptions of amplicon methylation levels are described below, first discussing 5'-positioned amplicons (predominantly spanning the partial promoter and first exon) followed by 3'-positioned amplicons (occurring in the final or second last exon). Following this, RT-qPCR, fish length and weight and histology results are reported. Mean methylation values and pairwise comparisons data presented in heat maps here are detailed in Appendix G and Appendix H.

3.4.1 5' Amplicons

In the *amh* PE1 amplicon, there were no significant differences in DNA methylation among the four temperature treatments at 6 mph (Figure 3.2A). There were also no significant differences between methylation levels in the *amh* PE1 amplicon for 6 mph fish and adult females, both of which demonstrated the lowest levels of methylation for this amplicon. At 12 mph, CT treated fish had significantly higher DNA methylation compared to the FT and control treatments. With the exception of the FT treatment, all 12 mph fish had significantly higher methylation than 6 mph fish and female fish, and significantly lower methylation than adult males, except in CT treated fish which were not significantly different from adult males (Figure 3.2A).

In the *cyp19a1a* PE1 amplicon, there were no significant differences in DNA methylation between treatments within the 6 mph or 12 mph timepoints, but at 12 mph, CT treated fish exhibited higher, but not significantly higher methylation than the other treatments (Figure 3.3A). Methylation levels in the *cyp19a1a* PE1 amplicon at 6 months were significantly higher than females and significantly lower than adult males; but by 12 months all treatments had similar methylation levels to adult males, with the exception of the CT treatment which had significantly higher methylation in *cyp19a1a* PE1 than adult males (Figure 3.3A).

In the *esr1* PE1 amplicon, there were no significant differences in DNA methylation between treatments at 6 months, but at 12 months CT treated fish had significantly higher methylation than all other treatments (Figure 3.4A). All fish at 6 mph had significantly lower methylation than both adult males and females. At 12 mph control and HT and FT treated fish were not

significantly different from adult females, but CT treated fish were not significantly different from adult males (Figure 3.4A).

At 6 mph, no variation in DNA methylation between treatments was observed in *foxl2* P (Figure 3.5A), but there was a small but significant difference between CT and HT treatments compared to FT-treated fish in *foxl2* E1.2 (Figure 3.5C). Additionally, at 12 mph there were significant differences in *foxl2* E1.1 between the CT and HT treatments (Figure 3.5B). In both cases, CT treated fish exhibited lower methylation. Neither *foxl2* E1.1 or *foxl2* E1.2 exhibit significant differences between adult males and females, however in the *foxl2* P amplicon, males exhibited significantly higher methylation compared to females and all 6 mph fish exhibited significantly lower methylation than both adult males and females. Notably, average methylation of *foxl2* did not exceed 20% for any fish in any amplicon (Figure 3.5).

In the *dmrt1* P amplicon, there were no significant differences in DNA methylation between treatments at 6 months; however, CT and HT treated fish had the highest methylation levels and were most similar to adult males (Figure 3.6A). For this amplicon, differences between adult males and females were not significant, but there were significant differences between adult females and FT and control fish at 6 mph (Figure 3.6A). Similarly, in the *dmrt1* PE1 amplicon, there were no significant differences in DNA methylation between treatments within the 6 or 12 mph timepoints (Figure 3.6B). Methylation levels in the *dmrt1* PE1 amplicon were significantly different from adult females at all timepoints, but not significantly different from adult males at 6 mph for the control and CT treatments or at 12 months for the control treatment (Figure 3.6B).

In the *nr5a2* P amplicon, there were no significant differences in DNA methylation levels among temperature treatments within the 6 or 12 mph timepoints (Figure 3.7A). Methylation levels in CT and FT treated fish at 12 mph were significantly different from all fish at 6 mph as well as adult males and females. For this gene promoter, average methylation did not exceed 3.43% (Figure 3.7A). In the *nr5a2* PE1 amplicon, there were no significant differences in DNA methylation between temperature treatments within 6 mph fish, however, at 12 mph CT treated fish had significantly lower methylation than the controls (Figure 3.7B). With the exception of HT treated fish, methylation levels of fish at 6 mph were not statistically different from female fish, but significantly higher than all treated (non-control) 12 mph groups. Furthermore,

all 6 mph and 12 mph treatments had significantly higher methylation levels than adult males, which had the lowest methylation for this gene (Figure 3.7B).

For the *sox9* PE1 amplicon, FT treated fish had significantly lower DNA methylation levels than CT and HT treated fish at 6 mph compared to control and HT treated fish at 12 mph (Figure 3.8A). Similar to *foxl2*, average methylation did not exceed 20% for this amplicon and there were no significant differences between adult males and females in this amplicon (Figure 3.5 and Figure 3.8).

3.4.2 3' Amplicons

In addition to the promoter and first exon sequences, 3' exons were also sequenced in *amh* (exon 7 of 7; Figure 3.2B), *cyp19a1a* (exon 8 of 9; Figure 3.3B) and *dmrt1* (exon 5 of 5; Figure 3.6C) at 12 mph. Methylation in all three 3' amplicons was disproportionately high compared to their corresponding 5' promoter and first exon spanning amplicons [*amh*: $t(474.15) = -41.804$, $p < 0.001$, *cyp19a1a*: $t(1045.1) = -20.446$, $p < 0.001$, *dmrt1*: $t(1872.2) = -442.07$, $p < 0.001$]. There were no significant differences between treatments within the *dmrt1* E5 (Figure 3.6C) *cyp19a1a* E8 amplicons (Figure 3.2B), or *amh* E7 amplicon (Figure 3.3B).

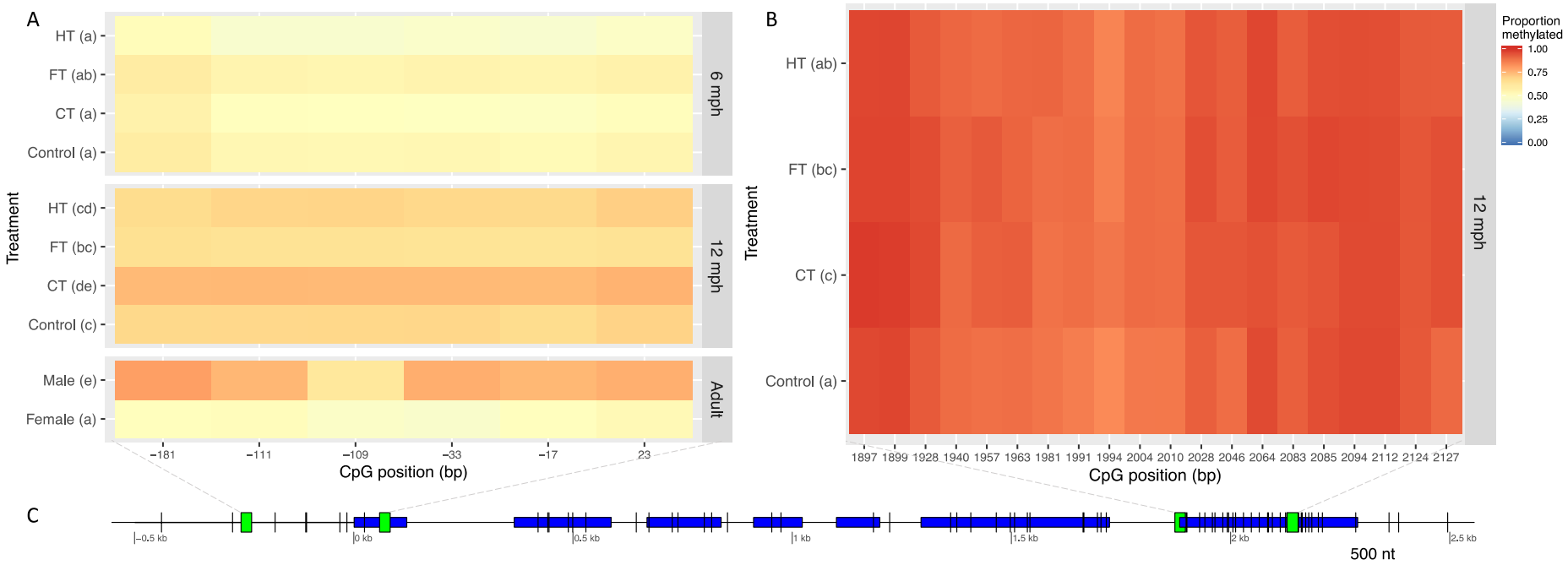

Figure 3.2 A. DNA methylation in the promoter of *amh* in *Lates calcarifer* at 6 and 12 months post hatch (mph) following treatment from 10-90 days post hatch obtained using bisulphite amplicon sequencing. Methylation levels for adult males and females are shown for reference. B. DNA methylation in the sixth (final) exon of *amh* in *L. calcarifer* months post hatch mph following the same treatment. Bracketed letters denote pair-wise comparisons of whole gene averages using Tukey's Honest Significant Difference and Bonferroni correction following ANOVA of transformed data. C. Structure of *amh* gene showing exons (blue boxes), introns (black line), primer positions (green boxes) and CpG sites (black vertical lines). Control: 29 °C, CT: cold temperature; 24 °C, FT: fluctuating temperature 24 - 34 °C, HT: hot temperature; 34 °C.

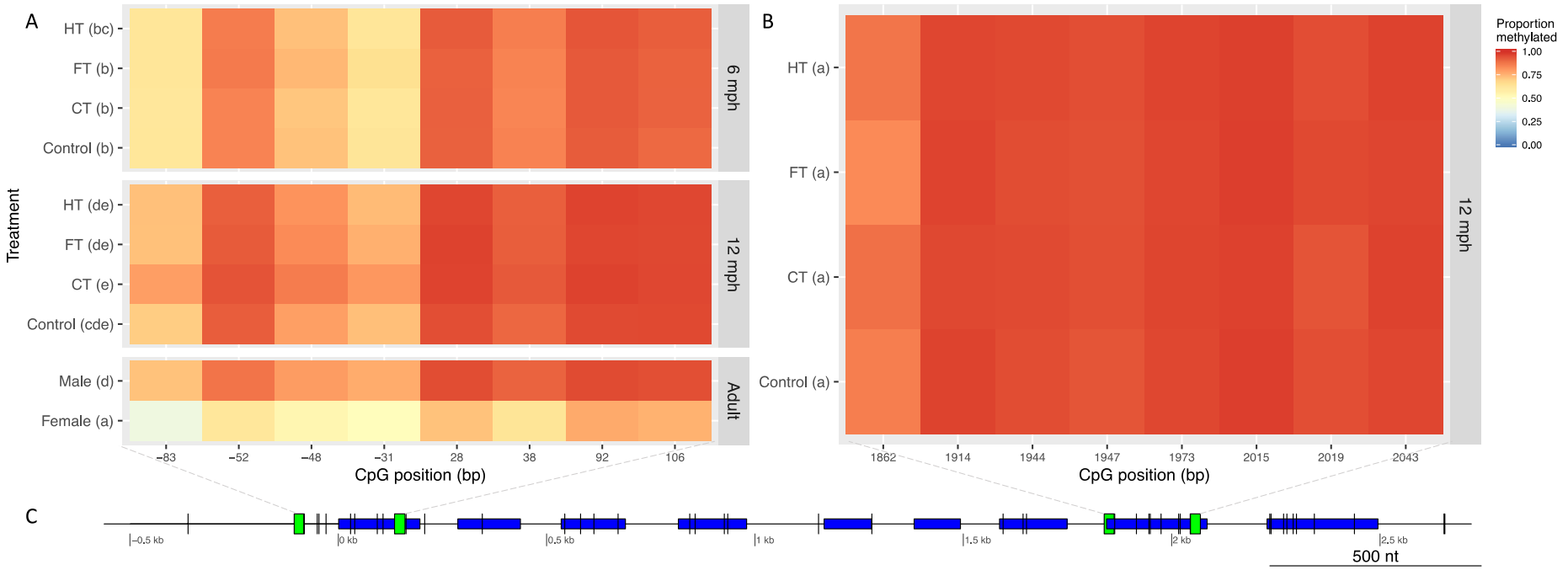

Figure 3.3 A. DNA methylation in the promoter of *cyp19a1a* in *Lates calcarifer* at 6 and 12 months post hatch (mph) following treatment from 10-90 days post hatch obtained using bisulphite amplicon sequencing. Methylation levels for adult males and females are shown for reference. B. DNA methylation in the eighth exon of *cyp19a1a* in *L. calcarifer* months post hatch mph following the same treatment. Bracketed letters denote pair-wise comparisons of whole gene averages using Tukey's Honest Significant Difference and Bonferroni correction following ANOVA of transformed data. C. Structure of *cyp19a1a* gene showing exons (blue boxes), introns (black line), primer positions (green boxes) and CpG sites (black vertical lines). Control: 29 °C, CT: cold temperature; 24 °C, FT: fluctuating temperature 24 - 34 °C, HT: hot temperature; 34 °C.

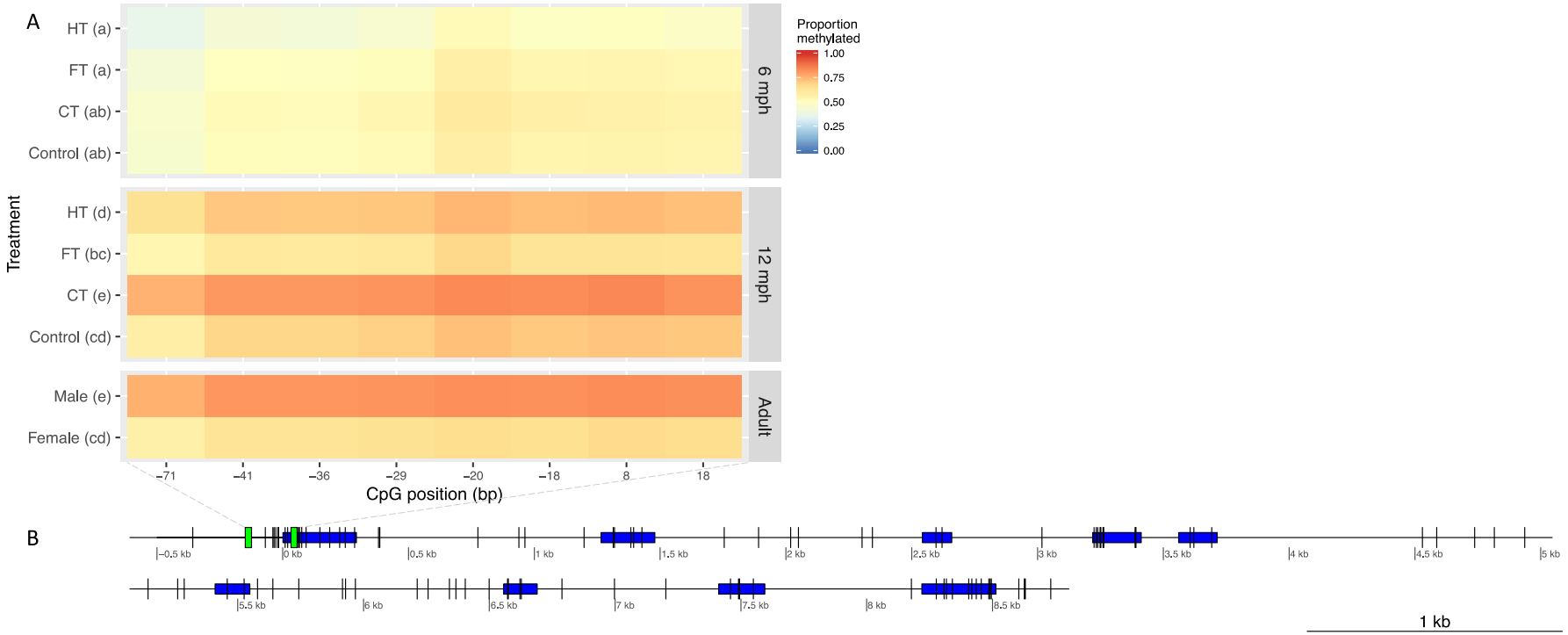

Figure 3.4 A. DNA methylation in the promoter of *esr1* in *Lates calcarifer* at 6 and 12 months post hatch (mph) following treatment from 10-90 days post hatch obtained using bisulphite amplicon sequencing. Methylation levels for adult males and females are shown for reference. Bracketed letters denote pair-wise comparisons of whole gene averages using Tukey's Honest Significant Difference and Bonferroni correction following ANOVA of transformed data. C. Structure of the *esr1* gene showing exons (blue boxes), introns (black line), primer positions (green boxes) and CpG sites (black vertical lines). Control: 29 °C, CT: cold temperature; 24 °C, FT: fluctuating temperature 24 - 34 °C, HT: hot temperature; 34 °C.

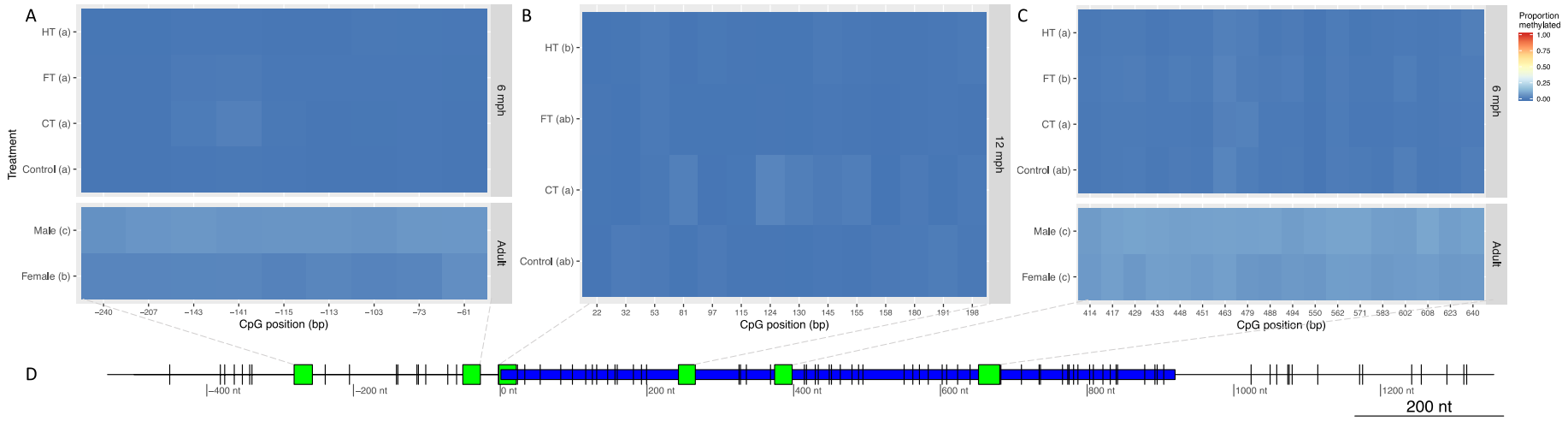

Figure 3.5 A. DNA methylation in the promoter and B and C. first exon of *foxl2* in *Lates calcarifer* at 6 and 12 months post hatch (mph) following treatment from 10-90 days post hatch obtained using bisulphite amplicon sequencing. Methylation levels for adult males and females are shown for reference. Bracketed letters denote pair-wise comparisons of whole gene averages using Tukey's Honest Significant Difference and Bonferroni correction following ANOVA of transformed data. D. Structure of *foxl2* gene showing exons (blue boxes), introns (black line), primer positions (green boxes) and CpG sites (black vertical lines). Control: 29 °C, CT: cold temperature; 24 °C, FT: fluctuating temperature 24 - 34 °C, HT: hot temperature; 34 °C.

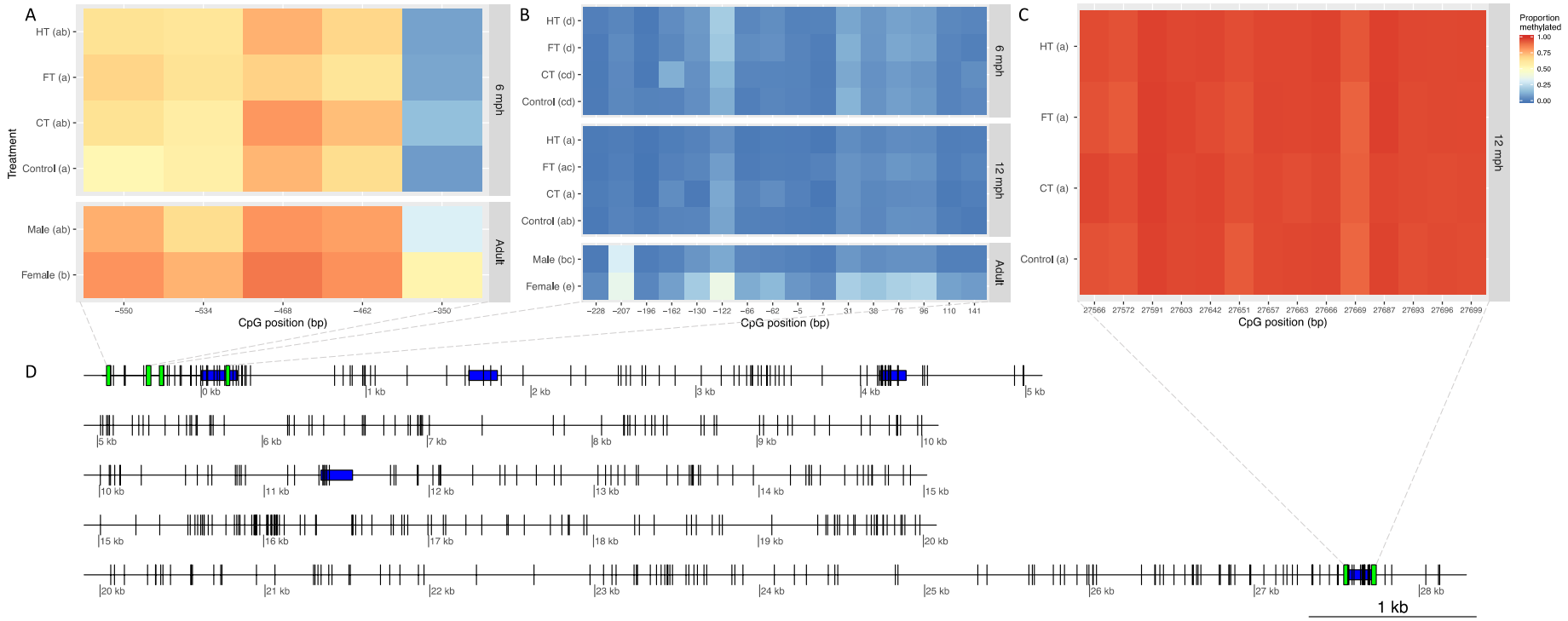

Figure 3.6 A. DNA methylation in the promoter of *dmrt1* in *Lates calcarifer* at 6 months post hatch (mph) following treatment from 10-90 days post hatch obtained using bisulphite amplicon sequencing. B. DNA methylation in the promoter and first exon of *dmrt1* in *L. calcarifer* at 6 and 12 mph following the same treatment and sequencing. Methylation levels for adult males and females are shown for reference. C. DNA methylation in the fifth (final) exon of *dmrt1* in *L. calcarifer* 12 mph following the same treatment. Bracketed letters denote pair-wise comparisons of whole gene averages using Tukey's Honest Significant Difference and Bonferroni correction following ANOVA of transformed data. C. Structure of *dmrt1* gene showing exons (blue boxes), introns (black line), primer positions (green boxes) and CpG sites (black vertical lines). Control: 29 °C, CT: cold temperature; 24 °C, FT: fluctuating temperature 24 - 34 °C, HT: hot temperature; 34 °C.

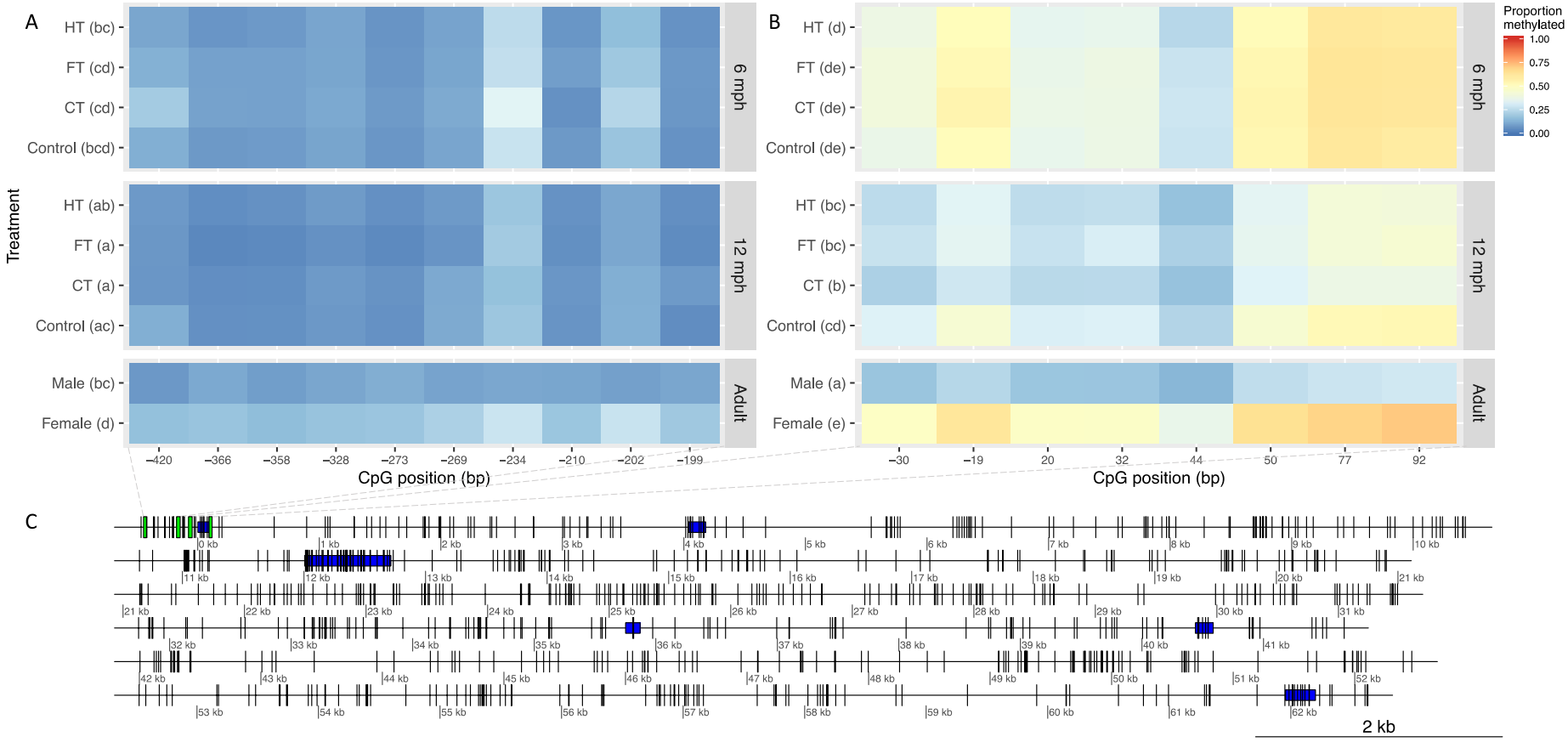

Figure 3.7 A. DNA methylation in the promoter of *nr5a2* in *Lates calcarifer* at 6 and 12 months post hatch (mph) following treatment from 10-90 days post hatch obtained using bisulphite amplicon sequencing. B. DNA methylation in the promoter and first exon of *nr5a2* in *L. calcarifer* at 6 and 12 mph following the same treatment and sequencing. Methylation levels for adult males and females are shown for reference. Bracketed letters denote pair-wise comparisons of whole gene averages using Tukey's Honest Significant Difference and Bonferroni correction following ANOVA of transformed data. C. Structure of *nr5a2* gene showing exons (blue boxes), introns (black line), primer positions (green boxes) and CpG sites (black vertical lines). Control: 29 °C, CT: cold temperature; 24 °C, FT: fluctuating temperature 24 - 34 °C, HT: hot temperature; 34 °C.

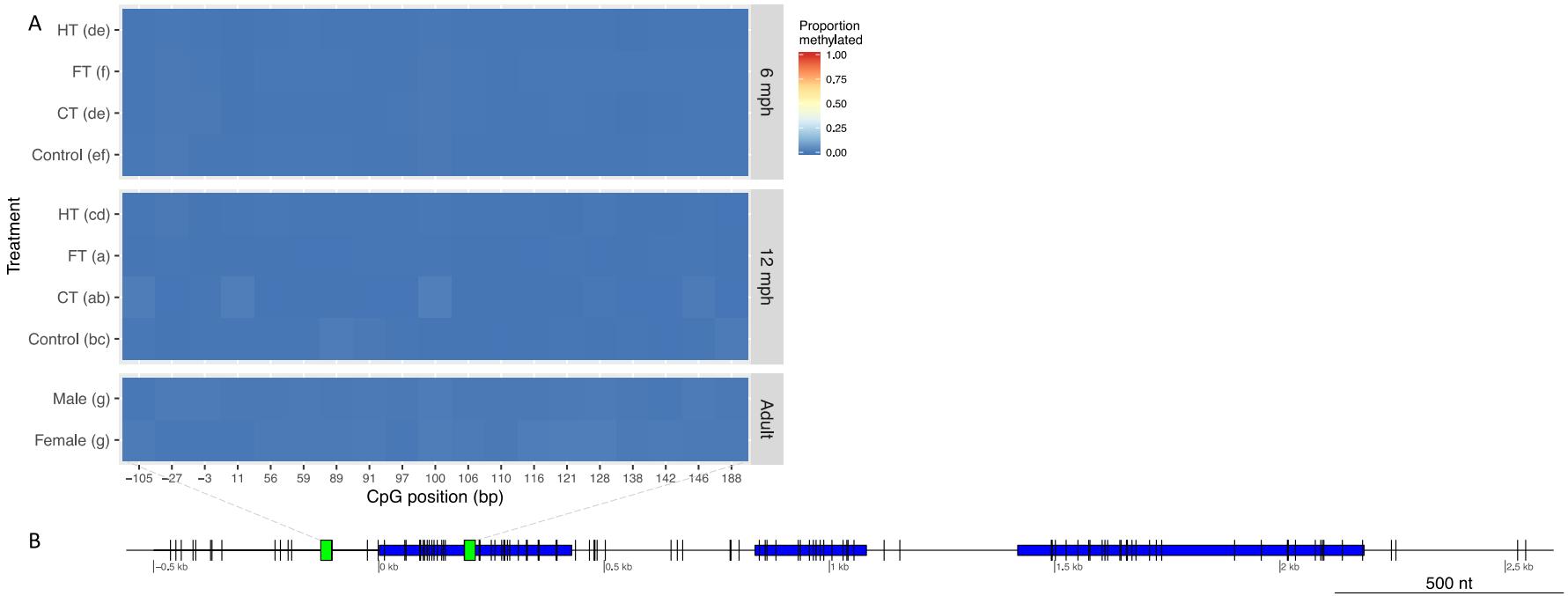

Figure 3.8 A. DNA methylation in the promoter and first exon of *sox9* in *Lates calcarifer* at 6 and 12 months post hatch (mph) following treatment from 10-90 days post hatch obtained using bisulphite amplicon sequencing. Methylation levels for adult males and females are shown for reference. Bracketed letters denote pair-wise comparisons of whole gene averages using Tukey's Honest Significant Difference and Bonferroni correction following ANOVA of transformed data. B. Structure of the *sox9* gene showing exons (blue boxes), introns (black line), primer positions (green boxes) and CpG sites (black vertical lines). Control: 29 °C, CT: cold temperature; 24 °C, FT: fluctuating temperature 24 - 34 °C, HT: hot temperature; 34 °C.

3.4.3 Dmrt1 *RT-qPCR*

RT-qPCR of *dmrt1* revealed no significant differences between expression of *dmrt1* mRNA between treatment groups, although expression in CT treated fish appeared higher than other treatments (Figure 3.9).

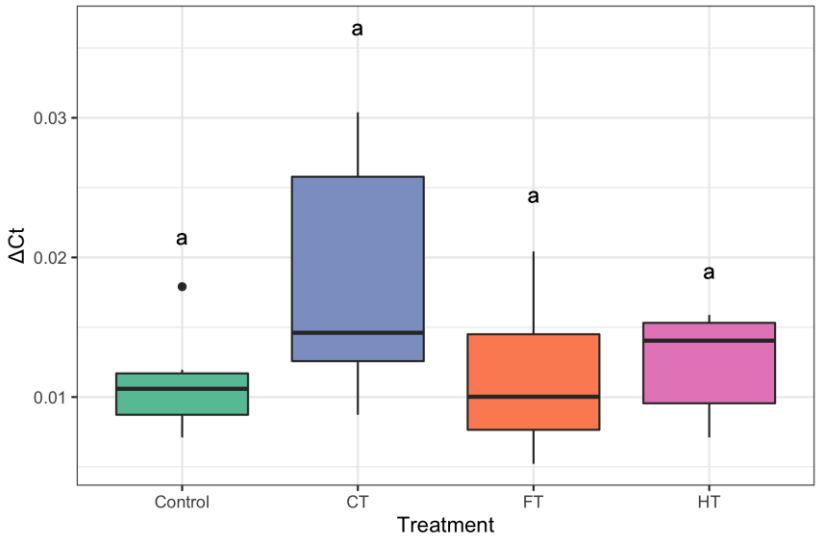

Figure 3.9 mRNA expression data for *Lates calcarifer dmrt1* at 12 months post hatch (mph) following temperature treatment from 10-90 days post hatch. N=8 fish per treatment. Letters denote pair-wise comparisons using Tukey's Honest Significant Difference and Bonferroni correction following ANOVA. Control: 29 °C, CT: cold temperature; 24 °C, FT: fluctuating temperature 24 - 34 °C, HT: hot temperature; 34 °C.

3.4.4 Length, weight and growth data

There was a significant effect of temperature treatment from 10-90 dph on fish length and weight as measured at 6 and 12 mph. At 6 mph, total lengths were significantly shorter in CT and FT treated fish compared to controls (Figure 3.10). Similarly, fish weight was significantly lower in CT treated fish compared to the control. By 12 mph, total length was significantly lower in the CT treated fish compared to the HT and FT treated fish, but not significantly different from the control. Conversely, fish weight was significantly lower in the CT treated compared to the control and HT treated, but not significantly different from the fluctuating treatment (Figure 3.10). Overall, the CT and FT temperature treatments had the lowest length and weight values at 6 mph but not 12 mph, with fewer and less consistent significant differences between the other treatments and time points (Figure 3.10). Von Bertalanffy growth curves modelled for data at 6, 9 and 12 mph indicated that growth was fastest in CT treated fish (K=0.298) and slowest in FT treated fish (K=0.172; Figure 3.11).

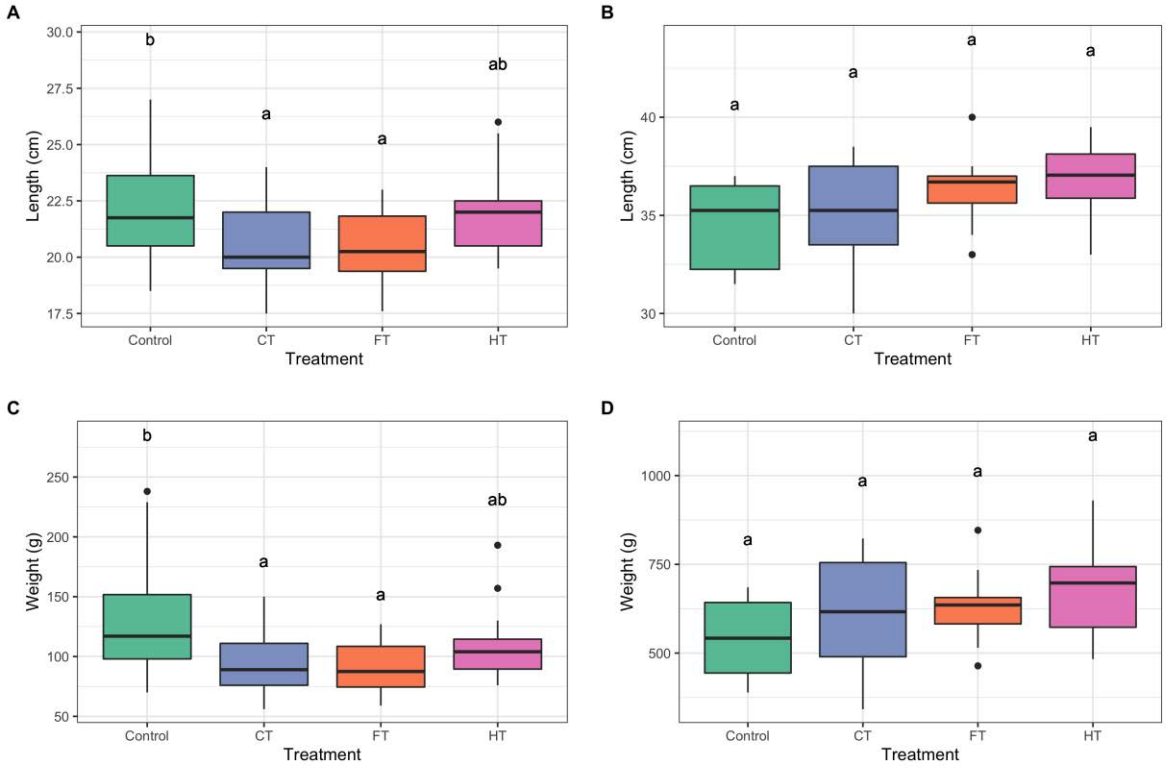

Figure 3.10 Length and weight comparison for *Lates calcarifer* at 6 (A and C) and 12 (B and D) months post hatch (mph) following temperature treatment from 10-90 days post hatch. N=10 fish per treatment. Letters denote pair-wise comparisons using Tukey's Honest Significant Difference and Bonferroni correction following ANOVA. Control: 29 °C, CT: cold temperature; 24 °C, FT: fluctuating temperature 24 - 34 °C, HT: hot temperature; 34 °C.

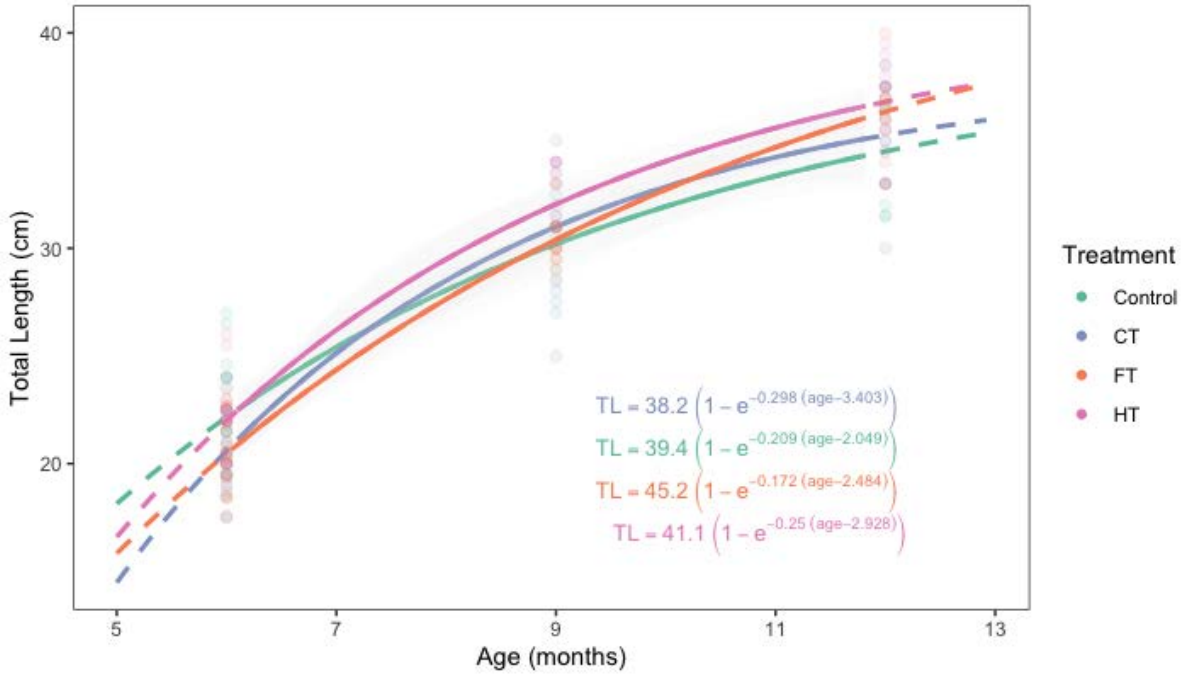

Figure 3.11 Fitted von Bertalanffy growth (solid lines) with raw data overlaid (dots) showing growth rate for *Lates calcarifer* following temperature treatment from 10-90 days post hatch. The equation for each treatment is written in the form of $TL = L_\infty(1\text{-}e^{-K(t-t0)})$ Where TL is the predicted length at age t years, L_∞ is the asymptotic mean length, K is the growth constant and t_0 the theoretical age at zero length. Control: 29 °C, CT: cold temperature; 24 °C, FT: fluctuating temperature 24 - 34 °C, HT: hot temperature; 34 °C.

3.4.5 Histology

Histological examination of the gonads revealed active spermatogenesis in the testes of all fish sampled at 12 mph and no germ cells differentiated into oocytes were identified (

Figure 3.12). Gonads from all treatments exhibited characteristics of M1 and M2 developmental stages, where M1 testis contain mostly spermatogonia and M2 testis, which are at a more advanced stage of development, are in active spermatogenesis containing mostly spermatocytes and spermatids (Guiguen *et al.*, 1994). Fish from the CT treatment typically exhibited less spermatogonia and more spermatocytes and spermatids compared to other treatments, signifying that fish from this treatment had progressed further towards the M2 stage of testis development (Figure 3.12).

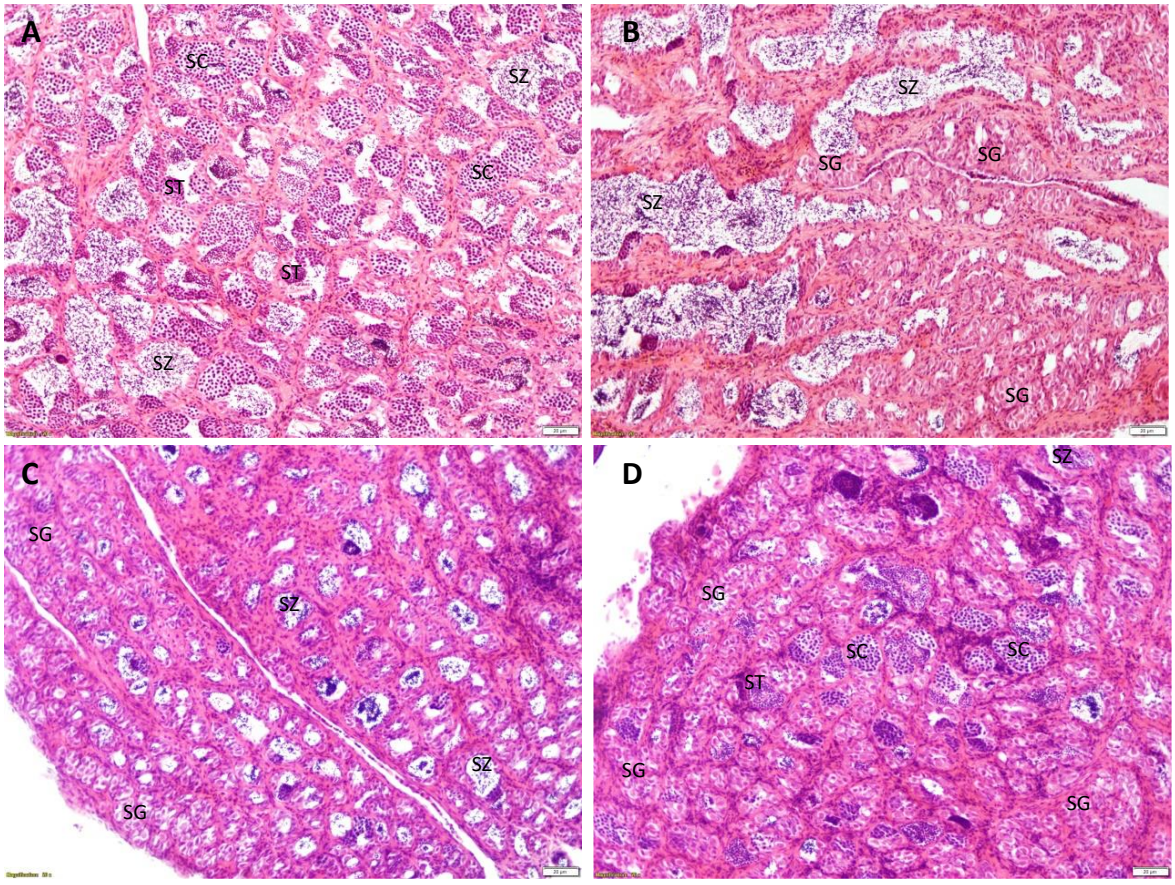

Figure 3.12 Haematoxylin and eosin staining on the gonads of *Lates calcarifer* at 12 months post hatch treated with A. CT; 24 °C, B. control; 29 °C, C. HT; 34 °C and D. FT; fluctuating 24 - 34 °C from 10 through 90 days post hatch. Labels indicate spermatogonia (SG), spermatocytes (SC), spermatids (ST), spermatozoa (SZ). Scale bar indicates 20 µm.

3.5 Discussion

The present study investigated the effect of exposure to differing temperatures during the development of juvenile barramundi on DNA methylation, mRNA expression and gonadal development revealing evidence for: 1) a significant but delayed effect of CT treatment during the predicted TSP (from 10-90 dph) on DNA methylation in amplicons of sex genes *amh, foxl2, sox9* and *esr1*, 2) a non-significant increase in mRNA expression of key male sex-determining gene *dmrt1* in CT treated fish, 2) a significant but not lasting effect of CT and FT treatment on fish length and weight and 3) histological differences between gonadal development stage (as males), but not phenotypic sex (no females). More specifically, at 6 mph, there were no significant differences in methylation levels for any of amplicons, however, by 12 mph CT treated fish were more similar to adult males in their DNA methylation patterns compared to other treatments. Both fish length and weight were significantly lower in CT and FT treated fish compared to the control at 6 mph, but no significant differences were present by 12 mph, which may be due to temperature related differences in growth rate. At 12 mph all fish were phenotypically male, with CT treated fish exhibiting a more advanced stage of male development. Although no phenotypic sex ratio shift occurred here, the results indicate that cold temperature treatment leads to advanced male development in juvenile *L. calcarifer*.

3.5.1 Low temperature treatment encourages male development

A finding of low temperatures contributing to advanced male development in hermaphroditic barramundi is in contrast to temperature effects in gonochoristic fish species, where high temperature treatment almost universally leads to the production of a greater proportion of males (Ospina-Alvarez and Piferrer, 2008). In mangrove killifish however, the only hermaphrodite species for which temperature treatment has a known effect on sexual differentiation, low temperature treatment also increases the proportion of male: hermaphrodite offspring, similar to what was observed here for barramundi (Ellison *et al.*, 2015). It may be that, in hermaphrodite fish, temperature exerts a different effect on gonadal development compared to gonochoristic species.

The common pattern of high temperature leading to male development is also disrupted in species where sexual differentiation is linked to size and/or growth. In such species, high temperature treatment typically leads to improved growth and subsequently encourages female development. For example, in the Southern Brook Lamprey, *Ichthyomyzon gagei*, lower

temperatures lead to development of a greater proportion of males, but only under favourable growth conditions (Beamish, 1993). Furthermore, in eels of the *Anguilla* genus, sexual differentiation is correlated with body size and dependent on temperature, whereby cooler temperatures result in more male and intersex individuals (see Geffroy and Bardonnet, 2016 and references therein). In eels, reduced growth rate is thought to delay development in both sexes, but encourage the *direct* development of males, which under typical conditions would involve an obligatory intersex stage (Geffroy *et al.*, 2013). In barramundi, sexual maturation, sex change and likely sexual differentiation are closely linked to size and growth (Moore, 1979; Davis, 1982; Guiguen *et al.*, 1994; Banh *et al.*, 2017). In this study, CT treated fish exhibited some evidence for compensatory growth, with significantly lower total length and weight than the control at 6 mph, but no significant differences by 12 mph as well as a faster growth rate throughout this period. It is well documented in fish that after a period of growth depression a phase of accelerated growth can occur once favourable conditions are restored (Ali *et al.*, 2003). For example, in both Atlantic salmon, *Salmo salar,* and cod, *Gadus morhua,* compensatory growth has been observed following periods of low temperature exposure (Mortensen and Damsgård, 1993; Purchase and Brown, 2001). Since sexual maturation and sex change is closely linked to size in barramundi, compensatory growth in CT fish may have led to an accelerated process of sexual differentiation and advanced gonadal development (as males) following low temperature treatment. Further investigation of the effects post 12 mph may reveal if the advanced gonadal development of CT-treated fish also leads to advanced male to female sex change later on in life (e.g. at 24 mph). Because sex change in sequential hermaphrodites is often linked to the attainment of a minimum body size and growth in fish is heavily influenced by temperature, it is likely that future research on the effect of temperature on sex in sequential hermaphrodites will be confounded with the effects of temperature on growth rate and differ from what is known in gonochoristic species (Warner, 1988a; Ospina-Alvarez and Piferrer, 2008).

3.5.2 A delayed response of temperature-induced DNA methylation

In this study, temperature treatment was applied during the first through third month post hatch (mph; specifically, at 10 - 90 days post hatch); however, an effect of temperature on sex-specific methylation was not observed until 12 mph. A delayed response of temperature on DNA methylation patterns has previously been observed in mice, where chronic exposure to radiation causes no immediate, but a pronounced delayed and sex-specific DNA methylation

changes in lung tissue (Pogribny *et al.*, 2004). It may be that chronic exposure to low temperature as incurred in this study caused the delayed effect on DNA methylation in barramundi.

3.5.3 The intensity, timing and type of treatment influences induction of phenotypic effects

In this study significant differences in DNA methylation of the gonads but no marked changes in phenotypic sex were observed. Such a result may indicate the intensity or timing of temperature treatment was insufficient to induce a greater effect. Here, a 10˚C total temperature differential (24 to 34 ˚C in CT compared to HT, respectively) was applied, with a 5 ˚C difference between the HT and CT temperature treatments compared to the control. This differential is slightly less in comparison to results published for the European seabass at 6˚C (15-21 ˚C; Navarro-Martin *et al.,* 2011) , the half-smooth tongue sole at 6 ˚C (22-28 ˚C; Shao *et al.,* 2014) and Nile tilapia at 8˚C (28-36 ˚C; Wang *et al.,* 2019) compared to their respective controls which may suggest that a more aggressive treatment may be necessary to induce a phenotypic effect. Furthermore, many species are most sensitive to treatments that occur outside the range of temperatures that they experience in their natural habitats (Baroiller *et al.*, 2009b). While the temperatures applied here were chosen due to optimal growth efficiency in culture conditions (see section 3.3.1) wild barramundi commonly experience minimum temperatures below the CT treatment (24 ˚C) and at and around HT treatment (34 ˚C; see Figure 2.7 from Chapter 2). Furthermore, the entire range of thermal tolerance for juvenile barramundi is 15 - 40 ˚C and, as such, more intense temperature treatments may be applicable without sacrificing survival (Katersky and Carter, 2007). Despite this broad thermal tolerance, at temperatures lower than 28 ˚C and higher than 35 ˚C, growth efficiency plateaus and survival decreases (Katersky and Carter, 2007). Therefore, if a more aggressive temperature treatment were to be applied it would be advantageous to reduce the treatment period in order not to sacrifice growth efficiency for aquaculture production.

The temporal window in which temperature treatments are most effectively applied in order to influence sexual differentiation processes, known as the TSP, also varies greatly between species. In fish, the TSP generally occurs just prior to the onset, or early in the process, of sexual differentiation (Baroiller and D'Cotta, 2001). Temperature treatments often become ineffective after the differentiation period, once the gonads become established and sex is stabilised (Valenzuela *et al.*, 2003; Ospina-Alvarez and Piferrer, 2008). In this study, an 80 day

(10-90 dpf) temperature treatment was applied, compared to research in the European seabass at 50 days (10-60 dpf; TSP at 0-60 dpf; Navarro-Martin *et al.,* 2011) , the half-smooth tongue sole at 75 days (25-100 dph; TSP at 30-80 dph; Chen *et al.,* 2014) and Nile tilapia at 10 days (12-22 dph; TSP at 12-14 dph; Wang *et al.,* 2019) . The specific TSP is yet to be identified in barramundi, however, sexual development is known to be complete by 140 dph under usual culture conditions with the first signs at 44 dph (Banh *et al.*, 2017). Because the TSP in fish occurs just prior to sexual differentiation, it is highly likely that the TSP occurred during the treatment period applied here. Despite this, it would be advantageous to apply multiple, short term temperature treatments just prior to and early on during the sexual differentiation period in barramundi in order to determine the precise TSP to best achieve sex manipulation in this species, and avoid any unnecessary adverse effects of prolonged temperature treatment on growth.

No binary change in phenotypic sex (i.e. male to female sex change) may also indicate that there are other factors other than temperature influencing sex in barramundi and/or that this species lacks sufficient responsiveness to external environmental triggers to induce sex reversal in juveniles and thus produce primary females. For example, while in eels sex change is influenced by temperature under rapid growth conditions, temperature has little effect when growth is slow (Beamish, 1993) and density is the main factor influencing sexual development (see Davey and Jellyman, 2005 and references therein). Furthermore, sexual development in protogynous (female to male) grouper is closely linked to total length, but also influenced by social factors such as density (Quinitio *et al.*, 1997), isolation (Liu and Sadovy de Mitcheson, 2011) and competitive interactions between dominant males (Mackie, 2000; Mackie, 2003). In *Apistogramma caetei* (a small tropical freshwater cichlid) changes in sex ratio occur due to changes in pH, but not temperature (Römer and Beisenherz, 1996). Finally, Atlantic silverside, *Menidia menidia*, are sensitive to temperature in low latitude but not at high latitude populations (Conover and Present, 1990). Previous research in adult barramundi suggests that sex change is influenced by temperature, but not salinity (Athauda and Anderson, 2014), however, further research is required to determine if sexual differentiation in juvenile barramundi is responsive to additional or alternative environmental cues.

3.5.4 Significant changes in DNA methylation occur between 6 and 12 mph

Marked temporal differences in DNA methylation levels in barramundi at 6 compared to 12 mph were observed, regardless of temperature treatment. At 12 mph, all fish exhibited methylation patterns more similar to adult males compared to 6 mph. Male-specific methylation patterns, as first identified by Domingos *et al.* (2018) are likely to occur as juveniles' approach or surpass sexual maturity. In Australia, wild barramundi reach sexual maturity at approximately 3-4 years of age and 50-70 cm (Davis, 1982; Grey, 1987) whereas in captivity, the testis are established as early as 160 dph at ~ 20 cm (Banh et al., 2017) and at least by 9 mph at ~ 28 cm (Szentes *et al.*, 2012). The average length of the barramundi sampled here was ~ 21 and 36 cm at 6 and 12 months, respectively. Histological examination revealed the gonads were fully differentiated at 12 mph, however, whether or not sexual maturity had occurred (identified by the presence of spermatozoa released into the ducts) was not determined. It is also interesting that at 6 mph, fish exhibited more female-specific methylation patterns than at 12 mph, and in some cases (e.g. *amh* and *esr1*) were not significantly different from adult females, indicating that methylation patterns in premature testis are more similar to ovarian tissue levels. While temporally induced changes in methylation were not the focus of this study, the results show that gonadal DNA methylation, which is known to differ in adult male and female barramundi, changes significantly during juvenile development.

3.5.5 Differences in DNA methylation vary greatly both within and between genes

DNA methylation levels between treatments and timepoints varied both within different amplicons within the same gene, and between different genes. For example, for *nr5a2* methylation in the amplicon positioned solely in the predicted promoter region (*nr5a2* P*;* -420 to -199 bp) was generally low, whereas in the amplicon spanning the TSS and surrounding region (*nr5a2* PE1*;*-30 to + 92 bp) had higher overall methylation and showed a greater differences among treatments at 12 mph, between the 6 and 12 mph time points and between the sexes. Similarly, heat-treated XX male and XY male tilapia exhibit low *dmrt1* methylation levels compared to XX females, but only in the region spanning intron three and four, with the remainder of the gene exhibiting the opposite pattern; high in all phenotypic males, low in females (Wang *et al.*, 2019). Together these results highlight the influence of specific gene region on DNA methylation levels observed, and in tilapia demonstrate that these differences do not always occur in the promoter and first exon as generally accepted. The results presented

here also show that methylation in 3' regions (final and second to last exons) was consistently higher than methylation in 5' regions in all three genes examined. Similar results in zebrafish, *Danio rerio*, revealed that final exons show a disproportionate amount of DNA methylation compared to the more 5' gene regions, with an overall dramatic positive increase in methylation with increased transcription (McGaughey *et al.*, 2014). As such, the high methylation in the 3' regions in *L. calcarifer* observed here may be a result of high gene transcription, as is the case in zebrafish, however additional data is necessary to confirm this in barramundi. In *foxl2* and *sox9,* CpG sites were highly dense yet demonstrated the lowest methylation levels, with no fish exhibiting DNA methylation higher than 20%. While many methylation studies target CpG islands (defined as regions where the observed-to-expected CpG ratio is greater than 60%), it has previously been observed that low CpG content promoter regions exhibit greater methylation-expression associations than high CpG content promoter regions (Hartung *et al.*, 2012). Indeed, Domingos *et al.* (2018) observed a negative correlation between DNA methylation and CpG density in *L. calcarifer* for many of the amplicons also examined here. Together, these results emphasize the importance of target gene and gene region in determining the ability to detect differences in DNA methylation in amplicon sequencing and caution the exclusive use of CpG rich regions such as CpG islands in global DNA methylation analyses.

3.6 Conclusion

The results provide evidence for a delayed effect of cold temperature treatment applied during development on DNA methylation in the gonads, a non-significant effect on gene expression, and an impermeant effect on fish weight and length, as well as differences between gonadal development stage but not phenotypic sex at 12 mph. Specifically, CT treated fish exhibited more male-specific methylation patterns, greater expression of the male sex determining gene *dmrt1*, evidence for compensatory growth and an advanced stage of testis development. The study also revealed significant changes in DNA methylation between 6 and 12 mph, with methylation patterns at 6 mph appearing more similar to adult females, and at 12 mph more similar to adult males. Additionally, the results highlighted large variability in DNA methylation levels between regions of the same gene and supported previous findings that gene regions with low CpG content may exhibit greater differences in methylation than regions with high CpG content, such as CpG islands. Further investigation of the effects of the current treatment post 12 mph may reveal if the advanced sexual maturation of CT-treated fish as males also leads to advanced male to female sex change and future research on the type and timing

of temperature (or other) treatment in juvenile barramundi will likely reveal if the production of primary females in this species, and thus sex reversal in sequential hermaphrodites, is possible. The results may assist in the development of sex control methods for aquaculture and are the first to examine the effects of temperature on juvenile sexual differentiation in a sequential hermaphrodite fish.

Chapter 4

Effect of year-long temperature treatment on methylation in the gonads of one-year-old, sexually differentiated barramundi, *Lates calcarifer*

4.1 Summary of results

In Chapter 4, previously reported effects of temperature treatment on sexually differentiated barramundi were reproduced by applying temperature treatments of 28 °C (Control), 24 °C (CT; cold temperature), 24 - 34 °C (FT; fluctuating temperature) and 34 °C (HT; hot temperature) to barramundi from 12 to 24 months post hatch. The effects on DNA methylation (WGBS), mRNA expression (RNA-Seq) and phenotypic sex (histology) were examined. The major results include:

1. Histological examination revealed 80% transitioning or female fish in the HT treatment compared to 0% in the control and CT treatments (all male; n=5 per treatment).
2. Differential expression analysis identified 8,310 differentially expressed genes between HT and control treatments ($p < 0.05$), with gene ontology analysis revealing strong representation of sequences involved in cellular signalling and ion binding.
3. Targeted analysis of eight sex-related genes revealed treatment-specific expression differences in seven of eight sex genes examined (*cyp19a1a, dmrt1, esr1, foxl2, nr5a2, sox8* and *sox9* but not *amh*; $p < 0.05$). Specifically, in the HT treatment, greater expression of *foxl2* ($p < 0.01$), *dmrt1* ($p < 0.001$), and *sox9* ($p < 0.01$) was observed.
4. No significant differences in average methylation of the eight sex-related genes over the predicted proximal promoter and gene body (-2000 bp to stop codon) were evident, but a significant difference in average methylation of *foxl2* [FT compared to control ($p < 0.05$)] and *nr5a2* [FT compared to control ($p < 0.05$); HT compared to control ($p < 0.001$); HT compared to CT($p < 0.05$)] over the -300 to 200bp region, was observed.
5. Single base-pair resolution visualisation of *cyp19a1a* and *dmrt1* revealed high variability in both methylation and expression along the length of each gene, including predicted proximal promoter, and demonstrated patterns of sex-specific alternative splicing consistent with previous reports (Domingos *et al.*, 2018).

This final data chapter provides the first examination of temperature treatment on gene expression, DNA methylation and gonadal phenotype in an adult sequentially sex changing fish, demonstrating a significant effect of high temperature treatment (34 °C) in barramundi. The results advance current knowledge of how temperature affects sexual phenotype in sequential hermaphrodites after the period of sexual differentiation and highlight the potential for an economically viable and environmentally friendly strategy for sex control in barramundi aquaculture.

4.2 Introduction

The gonads of sequential hermaphrodites are an extraordinary example of phenotypic plasticity (Gemmell *et al.*, 2019). While most fish species are gonochoristic, characterised by sexual phenotypes that are stable and binary, about 5% of fish species are sequentially hermaphroditic and undergo a drastic change in organ morphology and function during adult life (Pla Quirante *et al.*, 2018). In sequential hermaphrodites, sex change occurs as part of typical developmental processes, following primary sexual differentiation and sexual maturation and occurring in the form of either protogyny (female to male), protandry (male to female), or serial sex change (either direction, multiple times; Avise and Mank, 2009). While sex reversal can be achieved in some gonochoristic species, whereby genetically encoded gonadal phenotypes change in response to exogenous cues experienced during primary sexual differentiation, sex change in sequential hermaphrodites is cued by endogenous and/or exogenous environmental factors later in life. Among the vertebrates, sequential hermaphroditism is unique to teleost fish yielding powerful models with which to study molecular mechanisms underlying sexual development, particularly in response to environmental change.

It is well-established that in some gonochoristic fish species, the application of temperature during a specific period of development, known as the thermo-sensitive period, can lead to phenotypic reversal of genotypic sex, often via epigenetic changes (see Chapter 1, section 1.3.5 and 1.4.2 for details). For example, the application of high temperatures during development leads to changes in DNA methylation, gene expression and gonadal differentiation, such that phenotypic males are derived from genetic females in European seabass, *D. labrax,* half-smooth tongue sole, *C. semilaevis,* and Nile tilapia, *O. niloticus* (Navarro-Martín *et al.*, 2011; Shao *et al.*, 2014; Wang *et al.*, 2019). In the simultaneous hermaphrodite mangrove killifish, *Kryptolebias marmoratus*, it is low temperature treatment that achieves male development, such that individuals that would usually develop as hermaphrodites instead develop as male (Harrington Jr, 1967). This phenotypic change is accompanied by epigenetic change, specifically DNA methylation (Ellison *et al.*, 2015). In Chapter 3, it was shown that low temperature treatment of juvenile barramundi achieved advanced male development and was similarly accompanied by significant differences in DNA methylation. At least in gonochoristic species once the gonads have become established and sex is stabilised, temperature treatments typically become ineffective following the period of differentiation (Valenzuela *et al.*, 2003;

Ospina-Alvarez and Piferrer, 2008). In hermaphrodites, the effectiveness of temperature treatment following the period of primary sexual differentiation is unknown.

In contrast to gonochoristic species, the gonads of sequential or serial hermaphrodites often remain responsive to endogenous and/or exogenous environmental stimuli following the initial developmental period of primary sexual differentiation, and instead are influenced by changes such as social structure and/or the attainment of a minimum body size incurred later in life (Warner, 1984; Godwin, 2009; Kobayashi *et al.*, 2013). For example, in the protogynous half-moon grouper, *Epinephelus rivulatus,* sex change occurs at approximately 22-35 cm, but a threshold sex ratio is maintained by the suppressive dominance of large males over smaller females (Mackie, 2000; Mackie, 2003). More broadly in cultured grouper, *Epinephelus spp*., protogynous sex change and even male to female sex reversal can occur due to within-tank hierarchies, leading to unreliable seed production (Debas, 1989; Quinitio *et al.*, 1997; Mackie, 2003). Groupers are also very slow to reach sexual maturity and sex change, necessitating the use of testosterone implantation to reduce the number of years taken to gain access to viable eggs and sperm in culture environments (Sarter *et al.*, 2006). In captive protandrous seabream, *Sparus aurata*, the presence of old females decreases the frequency of sex change in young males and the introduction of young males increases the frequency of sex change among older males (Zohar *et al.*, 1984). In the protogynous ricefield eel, *Monopterus albus,* high stocking density accelerates female to male sex change and thus significantly decreases the proportion of female fish available for breeding (Yuan *et al.*, 2012). In this species sex change is known to be accompanied by substantial changes in DNA methylation and gene expression, but it is unknown if these changes can be induced by changes in stocking density, or other factors such as temperature (Zhang *et al.*, 2013a). In aquaculture, the ability to predict and control sex change in broodstock of sequentially hermaphroditic fish would enable reliable, economically viable and sustainable seed production. As such, if the gonad of a given species is sensitive to endogenous and/or exogenous stimuli, what the precise stimuli are and when are they most effectively applied are not only questions of biological interest, but in many cases are of economic importance as well.

Lates calcarifer, most broadly known as Asian seabass, but locally known as barramundi in Australia and Papua New Guinea, bhetki in India, ikan siakap in Malaysia and 75 other local names, supports a worldwide, multimillion-dollar aquaculture industry with a global production metric of over 100,000 tonnes annually (Mathew, 2009). The success in culture of

L. calcarifer has been attributed to the species' high fecundity, rapid growth rate and tolerance to a wide range of culture conditions (Reynolds and Moore, 1982; Davis, 1984a; Mackinnon, 1989; Garcia, 1990; Rimmer and Russell, 1998). *Lates calcarifer* are a protandrous hermaphrodite, typically maturing first as male at approximately 55-60 cm and sex changing at approximately 85-90 cm in most northern Australian populations (Davis, 1982). Thus, female barramundi broodstock are large and expensive to maintain, causing difficulties in supporting enough genetic diversity to initiate selective breeding in this species. Furthermore, because females mature several years later than males, same generation selection practices are inhibited (Robinson *et al.*, 2010). In captivity, barramundi can exhibit precocious (early) sexual maturation and change, maturing as males and sex changing to females earlier and at smaller size ranges (Guiguen *et al.*, 1994; Schipp, 1996). Whilst male to female sex change imposes significant expenses associated with maintaining large females, precocious sexual maturation leads to difficulties maintaining functional males, necessitating sex control methods to both encourage female development, or maintain functional males (Pankhurst, 1998; Allan and Stickney, 2000). While the aromatase inhibitor fadrozole holds some promise towards the maintenance of male barramundi broodstock, the use of the substance in farmed fish is currently cost prohibitive (Banh, 2019; J. Domingos *pers. comm.*). Furthermore, the use of hormones such as methyltestosterone to encourage male development and estrogen to encourage male to female sex change can be associated with adverse effects on farmers, consumers and the environment (Hoga *et al.*, 2018). Since hormone treatments incur high costs, require risk intensive practices and may lead to consumer resistance, thermal manipulations to control sex, where available, can offer more economically viable and environmentally sound methods for sex control in aquaculture.

It is well established that the effects of temperature on phenotypic sex typically become ineffective following the period of sexual differentiation in gonochoristic species (see section 1.3.5); but unknown if the same phenomenon occurs in sequential hermaphrodites. In barramundi, high temperature treatment in sexually differentiated (male) *L. calcarifer* has previously been shown to lead to the early induction of male to female sex change (Athauda *et al.*, 2012). This research demonstrates that temperature treatments in this sequential hermaphrodite are effective following the period of primary sexual differentiation. While the observed differences in phenotypic sex in barramundi corresponded to differences in the enzymatic activity of gonadal aromatase and levels of circulating estrogen (Athauda *et al.*, 2012), it is unknown whether these differences were accompanied by epigenetic changes. To

better understand both the reliability of temperature as a method of sex control in barramundi aquaculture, as well as the molecular mechanisms driving the previously observed changes in phenotypic sex, temperature treatments of 28 °C (control), 24 °C (cold temperature) and 34 °C (hot temperature) were applied to adult *L. calcarifer* from 12 - 24 months post hatch (mph). Given that sex change in barramundi occurs shortly after seasonal spawning (Davis, 1982), sex change may result from the cumulative effect of multiple seasonal fluctuations in temperature, rather than just the effect of a stable temperature exposure; thus a fluctuating temperature treatment of 24 - 34 °C was additionally applied. This research assessed the effect of temperature treatment on gonadal phenotype using histology, mRNA expression using RNA-Seq, and DNA methylation using WGBS in barramundi, focusing on several key sex determining genes and constitutes the first investigation of an epigenetic effect of temperature applied to sexually differentiated individuals in any sequential hermaphrodite.

4.3 Methods

4.3.1 Animals, rearing conditions and temperature treatments

Larvae from a single spawning event were produced at an on-site hatchery (James Cook University, Townsville, QLD, Australia). Spawning procedures, handling methods and rearing conditions followed standard Australian intensive barramundi hatchery practices (Schipp *et al.*, 2007), including a 12 h light and 12 h dark photoperiod and periodic grading after 18 dph to avoid cannibalism (Dhert *et al.*, 1992). From 2 dph to 12 mph *L. calcarifer* were reared at 29 ˚C and 30-35 ppt salinity. At 12 mph (34.5 ± 2.29 cm and 0.54 ± 0.11 kg), a total of 48 *L. calcarifer* were divided into eight 2000 L tanks (i.e. six fish per tank) held at 28 °C for a period of one week. Fish were acclimatized to their experimental treatments of 24 °C (cold temperature; CT), 28 °C (control), 34 °C (hot temperature; HT) or cycling though 24 - 34 °C (fluctuating temperature; FT). Each temperature treatment consisted of two tank replicates. Throughout the treatment period, the temperature of tanks subject to the FT treatment was increased by 1 °C per 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. The temperature of each tank was measured twice daily to ensure that the treatments were as close as practical to their target temperatures (Figure 4.1). Research was conducted under James Cook University Animal Ethics Permit Number A2014.

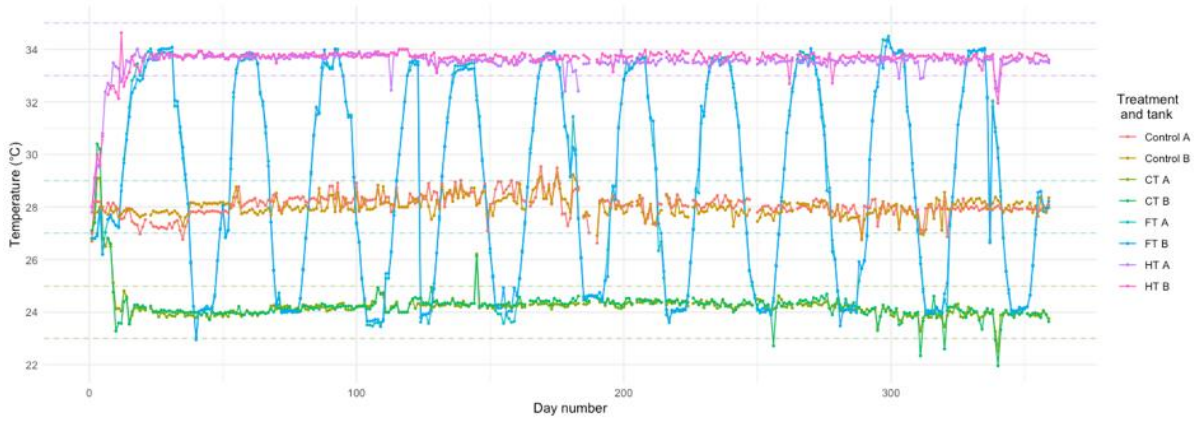

Figure 4.1 Recorded temperatures for each of four experimental treatments applied to *Lates calcarifer* from 12 – 24 months post hatch. Temperature readings were collected daily, and points represent raw data for each of the two tank replicates (A and B). Dashed lines represent target temperatures ±1 °C. Control: 28 °C, CT: cold temperature; 24 °C, FT: fluctuating temperature 24 - 34 °C, HT: hot temperature; 34 °C.

4.3.2 Sampling

At 24 mph, after 1 year of treatment, *L. calcarifer* were euthanized using AQUI-S aquatic anaesthetic (AQUI-S New Zealand), followed by immersion in an ice slurry and finally through severance of the head at the junction of the skull and the first vertebra. Tissue from the middle-most portion along the length of the right gonad was removed and immersed in RNAlater (Thermo Fisher Scientific). A portion of each gonad was fixed in 10 % neutral buffered formalin containing 4 % formalin for at least 24 h and used to determine the stage of gonadal development by histological examination as per Chapter 3 section 3.4.5. Total length and weight data to the nearest centimetre and gram, respectively, were also recorded.

4.3.3 DNA and RNA extraction

Gonadal 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 (Doyle and Doyle, 1987) for DNA extraction. Total RNA was extracted according to Chapter 3, section 3.3.3 and RNA integrity was analysed using a TapeStation 2200 (Aligent). Genomic DNA (gDNA) was extracted following the CTAB protocol (Doyle and Doyle, 1987), as described in Chapter 2, section 2.3.2. 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.

4.3.4 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). 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). All sequencing was performed at the Australian Genome Research Facility (AGRF).

4.3.5 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) specifying to remove Illumina-specific sequences (e.g. adapters), allowing for a maximum count of 2 mismatches, a minimum match score of 40 between aligned forward and reverse reads and a minimum match score of 15 for adapter sequence alignments (ILLUMINACLIP:<fastaWithAdaptersEtc>:2:40:15). Trimmomatic was also set to trim nucleotides from the start and end of each read if the read was below a Phred quality score of ≥2 (MacManes, 2014) and finally, 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 (Vij *et al.*, 2016) as reference. Because prebuilt indexes are not available for non-model organisms such as barramundi, a custom index was built to aid the mapping of RNA-Seq reads. For this, two python scripts included in the HISAT2 package (*extract_splice_sites.py* and *extract_exons.py*) were used along with the *L. calcarifer* genome and annotation file to create a set of known splice junctions and exons. The resulting files were

used to build a HISAT2 index using the *hisat2-build* function, specifying splice sites and exon options. Filtered reads were then aligned to the *L. calcarifer* genome using the indexed reference genome and 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). Resulting Sequence Alignment Map (SAM) files were then converted to Binary Alignment Map (BAM) files using the SAMtools version 1.5 *sort* function (Li *et al.*, 2009). For each sample, aligned transcripts were then assembled using StringTie version 2.0.4 (Pertea *et al.*, 2015) and the *L. calcarifer* genome annotation file for reference. Assembled transcripts from all samples were then merged using StringTie and again, the reference genome annotation, to create a final transcriptome assembly that included all genes and transcripts present in all samples. This assembly was assessed in comparison to the reference annotation using Cuffcompare from Cufflinks version 2.2.1 (Trapnell *et al.*, 2012) and overall summary statistics appear in Table 4.1. The final merged assembly was used for re-estimation of transcript abundances for each sample with StringTie, estimating only the transcripts matching those in the StringTie final merged assembly and outputting coverage data table files.

Table 4.1 Cuffcompare statistics related to the accuracy of transcripts in merged assembly compared to the reference genome annotation data. Sensitivity and specificity are as defined in Burset and Guigo (1996). Fuzzy variants of sensitivity and specificity allow for transcripts with small variation in exon boundaries to still be counted as matching.

Query mRNAs: 90297 in 30822 loci (87197 multi-exon transcripts)				
(17419 multi-transcript loci, ~ 2.9 transcripts per locus)				
Reference mRNAs: 19888 in 19573 loci (19288 multi-exon)				
Super-loci w/ reference transcripts: 17914				
Accuracy level	Sensitivity	Specificity	Fuzzy Sensitivity	Fuzzy Specificity
Base:	100	56.3	NA	NA
Exon:	99.8	58.6	100	59.9
Intron:	100	66.1	100	67.1
Intron chain	89.6	19.8	100	29.6
Transcript:	49.7	10.9	53.9	11.9
Locus:	89.6	54.2	99.3	58.5

Matching intron chains: 17282		
Matching loci: 17535		
	Number of transcripts	Percentage
Missed exons:	0/223970	0.00 %
Novel exons:	106510/381282	27.90 %
Missed introns:	0/203948	0.00 %
Novel introns:	65136/308329	21.10 %
Missed loci:	0/19573	0.00 %
Novel loci:	12330/30822	40.00 %

Total union super-loci across all input datasets: 30822

Expression analysis was performed in RStudio version 1.2.1335 (Allaire, 2012) using R version 2.15.2 (Ihaka and Gentleman, 1996). Tximport (Soneson *et al.*, 2015) was used to compute counts from the coverage information given by StringTie and then import the data into DESEq2 incorporation sequencing run, macroscopically determined sex (male or female) and treatment into the design (Love *et al.*, 2014). 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* (Tibshirani, 1988; Huber *et al.*, 2003; Anders and Huber, 2010). A heatmap of the top 10,000 most differentially expressed genes was created following the script provided by Huerlimann *et al.* (2018) using Pearson's correlation for the dendrogram. To test differences between treatment groups, 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. A Venn diagram of the number of differentially expressed genes from the top five of six comparisons was created using the *venn.plot* function from the VennDiagram package version 1.6.20 (Chen, 2018).

Functional analysis was performed for the comparison with the greatest number of differentially expressed genes; HT compared to control. A BED file for the genes differentially expressed (DE) between the HT and control treatment was exported from R, and the *bedtools getfasta* command was used to obtain a fasta file of the sequences for the DE genes. To create a teleost (Actinopterygii) specific database, the National Centre for Biotechnology Information (NCBI) non-redundant protein (nr) database was queried with the taxonomic identifier 7898 (txid7898[ORGN]) and the results were exported as a list of GenInfo Identifiers (i.e. a GI list; accessed 16/12/2019). The *blast* command *blastdb_aliastool* was then executed on the GI list to create an Actinopterygii-only alias blast database. The *blastx* command was then used to query the fasta file containing the DE gene sequences for the HT-control comparison, specifying *-outfmt* 16. The resulting .xml file was imported into OmicsBox version 1.2.0 (BioBam) for mapping and annotation following the suggested gene ontology (GO) annotation workflow including merging of Interpro scan and blast GO terms to produce the final

annotation. The OmicsBox Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways function was used to overlay annotated genes were overlaid onto their respective biological pathways.

To plot expression for individual genes, the DESeq2 function *plotCounts* count was used to normalise the data from the DESeq2 model by sequencing depth and add a pseudocount of 0.5 to allow for log-scale plotting. To identify which gene IDs from the StringTie annotation (e.g. MSTRG.####) corresponded to available full-length barramundi sex genes and plot their expression values, sex gene sequences from Domingos *et al.* (2015) were mapped to the *L. calcarifer* chromosome-level genome assembly version 3 (Vij *et al.*, 2016) using minimap2 (Li, 2018). The resultant SAM file was then converted and sorted into a BAM file and then converted again to a BED file containing the genomic coordinates for the eight queried genes. BEDTools (Quinlan, 2014) *intersect* was then used to identify overlapping genomic coordinates between the two files and thus retrieve the MSTRG StringTie ID's that corresponded to each of the mapped sex genes. Using the resultant StringTie sex gene IDs, it was then possible to use the DESeq2 *plotCounts* function 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). Pair-wise comparisons between treatments 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). Plots were drawn using ggplot2 v3.1.0 (Wickham, 2016). An overview of the RNA-Seq analysis pipeline is provided in Figure 4.2.

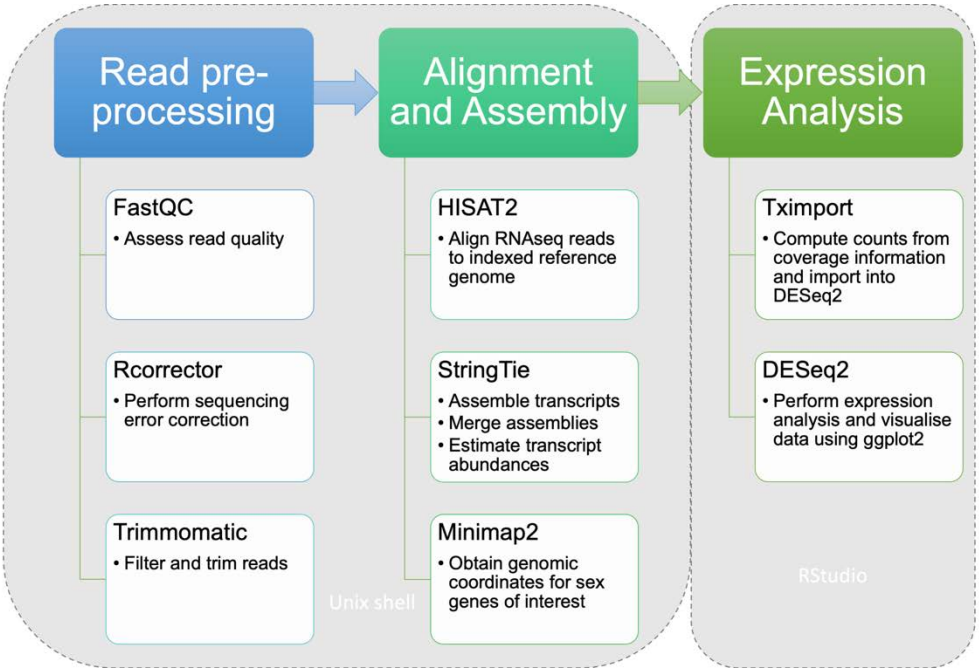

Figure 4.2 Overview of the computational pipeline used to analyse expression patterns of the eight *Lates calcarifer* sex genes targeted in this study

4.3.6 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 ('AGATCGGAAGAGC'), 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. Then, the trimmed reads were aligned to the reference genome using the default multi-seed lengths of 0 mismatches and 22 bp. A minimum alignment score of L's,0, -0.2 (roughly two mismatches) was applied. Options *reorder, ignore-quals, no-mixed, no-discordant* and *dovetail* were also selected, and all other specifications left to default settings. Reads were aligned twice by Bowtie2 within Bismark, once for the original top strand to a C-to-T converted genome and one for original bottom strand to a G-to-A converted genome as per the default for directional libraries. To analyse the CG methylation calls, the Bismark coverage files were imported into SeqMonk v1.46.0 (www.bioinformatics.babraham.ac.uk/projects/seqmonk/). Similar to the RNA-Seq analysis,

methylation analysis was targeted to eight available full-length barramundi sex genes. Specifically, the BED file containing genomic coordinates for the eight sex genes were converted to gene transfer format (GTF) using python script *bed2gtf.py* (github.com/pfurio/bed2gtf). The resultant GTF file, as well as the previously mentioned barramundi genome file and reference annotation were imported into 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. SeqMonk's methylation pipeline 'bisulphite methylation over features' was used to generate mean percent methylation values for each individual fish in each treatment. Methylation values were imported into RStudio version 1.2.1335 (Allaire, 2012) using R version 2.15.2 (Ihaka and Gentleman, 1996). Percentages were converted to proportions and logit transformed to allow subsequent ANOVA and pair-wise comparisons between treatments which were performed as per the RNA-Seq analysis. Box plots were drawn using ggplot2 v3.1.0 (Wickham, 2016). An overview of the WGBS pipeline is provided in Figure 4.3. To analyse methylation over the length of *cyp19a1a* and *dmrt1*, the methods of and scripts provided by Todd *et al.* (2019) were followed. Briefly, 2 bp running windows with a 2 bp step size were created over the gene and predicted proximal promoter and the 'bisulphite methylation over features' analysis was carried out again. Paired RNA-Seq values were created similarly, quantifying the 2 bp running window probes by total read count and normalising per million reads. Data were imported into RStudio using the version specified above and plots were created using the Gvis package (Hahne and Ivanek, 2016).

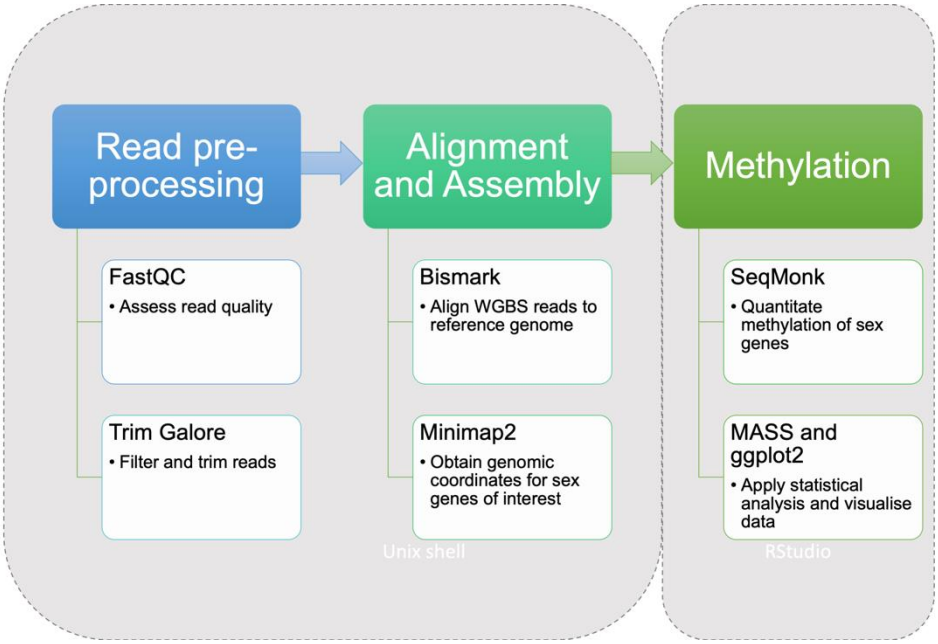

Figure 4.3 Overview of the computational pipeline used to analyse DNA methylation patterns in the eight *Lates calcarifer* sex genes targeted in this study

4.4 Results

4.4.1 Histology

Year-long temperature treatment resulted in substantial differences in gonadal development of *L. calcarifer* at 24 mph between treatments (Figure 4.4). Control fish were characterised by early male stages M1 and M2, exhibiting mostly spermatogonia, spermatocytes and spermatids, respectively (Figure 4.5B). Similarly, CT fish were mostly in the M2 stage, with one fish exhibiting mostly spermatozoa, indicating this individual was in the M3 stage of development (Figure 4.5A). The FT treatment also resulted in mostly male fish (stages M1 or M3), as well as one fish that was in an early transitional stage (T2), indicated by the presence of degenerating tissue and the appearance of ovarian tissue (Figure 4.5D). HT treated fish showed the greatest differences in gonadal development, with only one fish still in male phase (M2) and all remaining fish (80 %) either in transitional phases (T1 or T2) and one fish in the F2 female stage, exhibiting vitellogenic oocytes (Figure 4.5C).

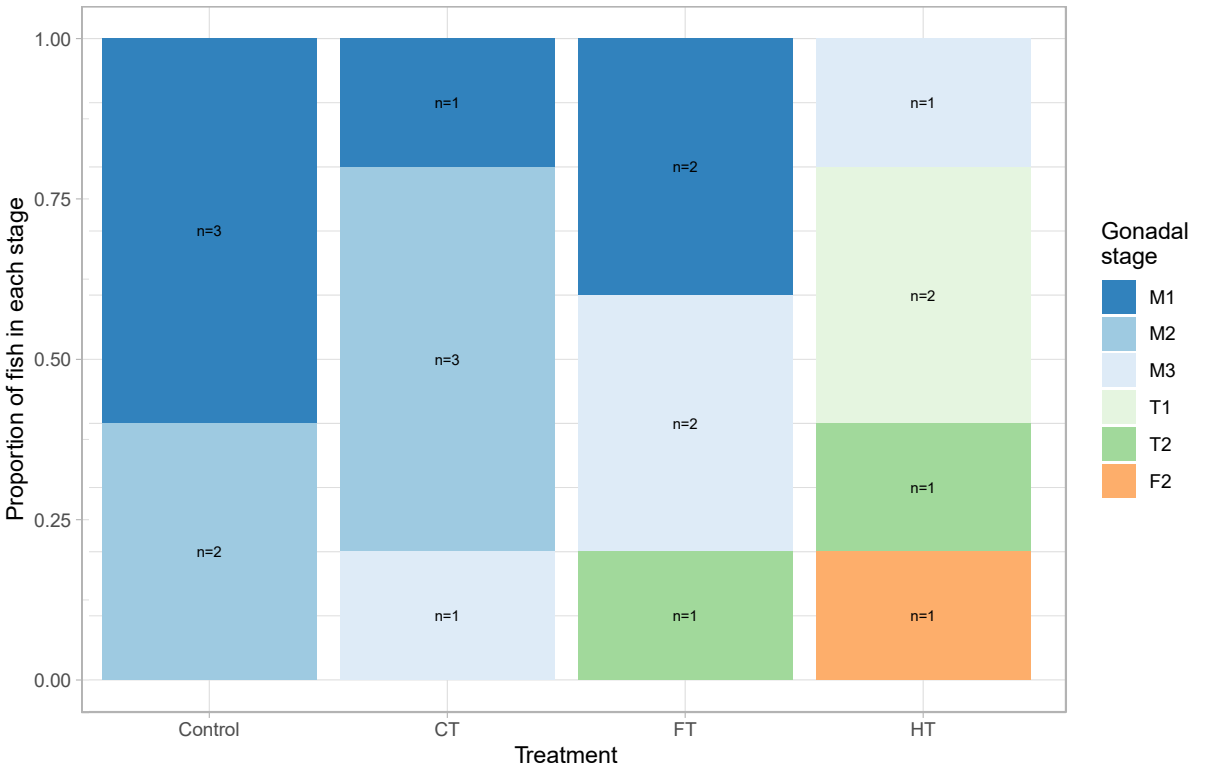

Figure 4.4 Gonadal development stage of *Lates calcarifer* gonads following temperature treatment from 12 to 24 months post hatch. N=5 fish per treatment. Control: 28 °C, CT: cold temperature; 24 °C, FT: fluctuating temperature 24 - 34 °C, HT: hot temperature; 34 °C. Gonadal stages are based on (Guiguen et al., 1994) where M1-M4 represent male stages,T1-T4 represent transitional stages and F1-F4 represent female stages. Not all stages where observed here.

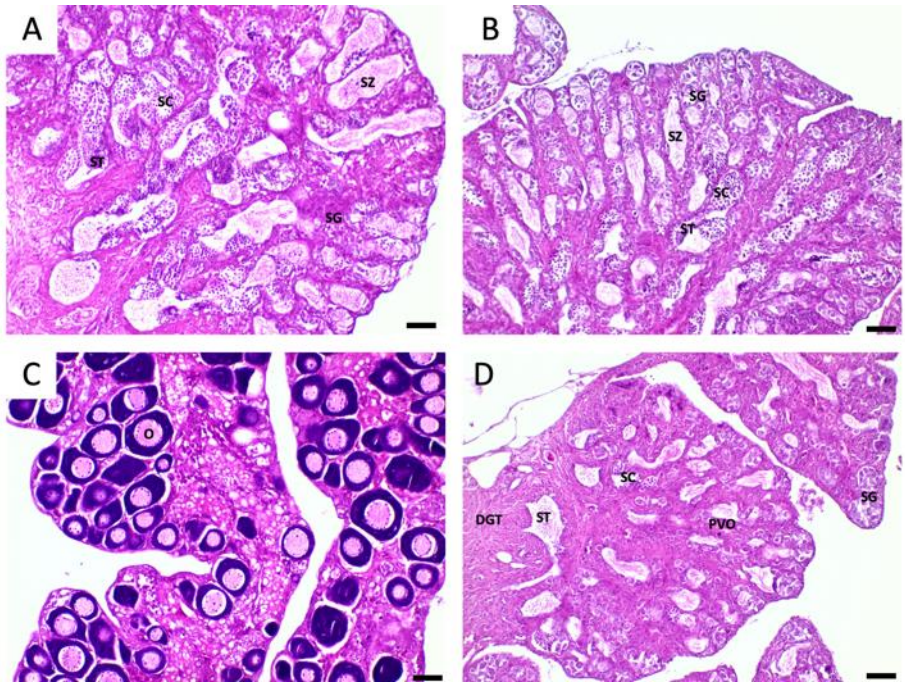

Figure 4.5 Haematoxylin and eosin staining on the gonads of *Lates calcarifer* at 24 months post hatch subject to A. CT: cold temperature; 24 °C, B. Control: 28 °C, C. HT: hot temperature; 34 °C and D. FT: fluctuating temperature 24 - 34 °C from 12 to 24 months post hatch. Labels indicate spermatogonia (SG), spermatocytes (SC), spermatids (ST), spermatozoa (SZ), oocyte (O), pre-vitellogenic oocyte (PVO) and degenerating tissue (DGT). Scale bars indicate 20 µm.

4.4.2 Length weight data

Length and weight data revealed that HT fish were the largest and CT fish were the smallest following 12 months of temperature exposure (Figure 4.6). HT-treated fish were significantly heavier and longer than CT-treated fish, but not significantly different from the controls. CT-treated fish were significantly smaller in length, but not weight compared to the controls. FT-treated fish were not significantly different from control-treated fish in either measurement (Figure 4.6).

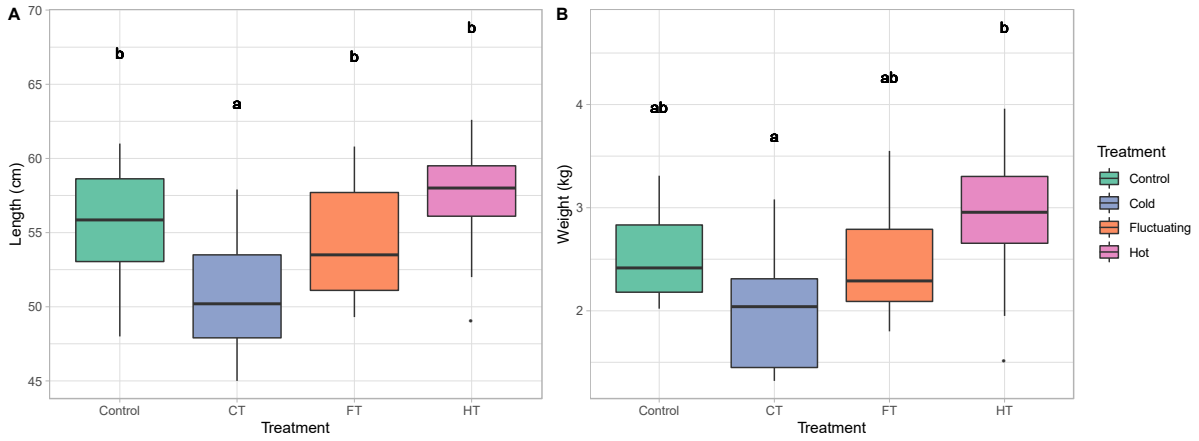

Figure 4.6 A. length and B. weight of *Lates calcarifer* following temperature treatment from 12 to 24 months post hatch. N=10-14 fish per treatment. Letters denote pair-wise comparisons using Tukey's Honest Significant Difference following ANOVA (P = 0.05). Control: 28 °C, CT: cold temperature; 24 °C, FT: fluctuating temperature 24 - 34 °C, HT: hot temperature; 34 °C.

4.4.3 Global expression patterns and functional analysis results

Visualisation of the top 10,000 differentially expressed genes highlights large within-treatment variation and clear distinction between the F2 stage fish and all other individuals (Figure 4.7). For among treatment comparisons, the highest number of differentially expressed genes between temperature treatments occurred between HT and control fish, with 8,310 differentially expressed genes at $p < 0.05$ (Figure 4.8).

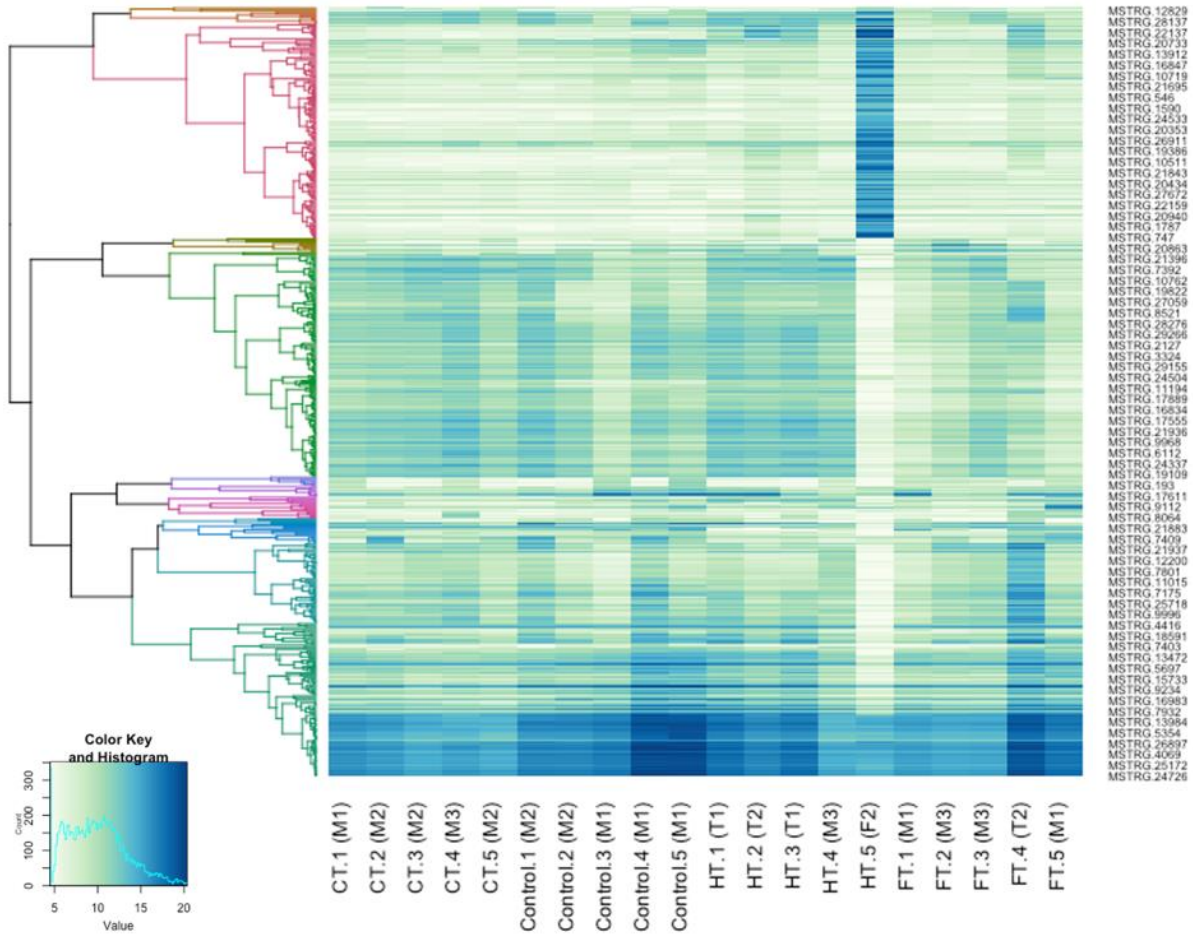

Figure 4.7 Heatmap of the 10,000 most differentially expressed genes from individual *Lates calcarifer* following temperature treatment from 12 to 24 months post hatch. Row dendrogram created using Pearson's correlation of variance stabilised DESeq2 count matrix, columns ordered by treatment. Control: 28 °C, CT: cold temperature; 24 °C, FT: fluctuating temperature 24 - 34 °C, HT: hot temperature; 34 °C.

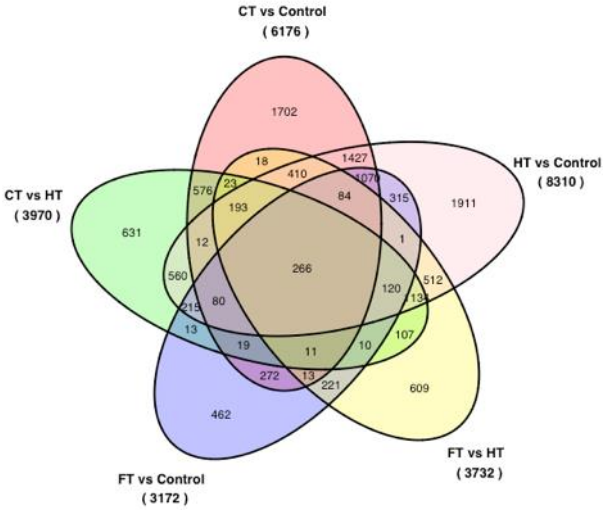

Figure 4.8 Venn diagram showing the distribution of differentially expressed genes among treatment by treatment comparisons of *Lates calcarifer* following temperature treatment from 12 to 24 months post hatch (n=5). Control: 28 °C, CT: cold temperature; 24 °C, FT: fluctuating temperature 24 - 34 °C, HT: hot temperature; 34 °C. The comparison with the least number of differentially expressed genes (CT vs FT) is not shown.

Gene ontology (GO) mapping and annotation revealed that the majority of these differentially expressed genes were involved in biological processes, including cellular and metabolic, followed by regulatory, and then responses to stimulus and signalling (Figure 4.9A). Specifically, the GO terms associated with signalling included signal transduction, G protein-coupled receptor signalling pathway, oxidation-reduction process, transmembrane transport, potassium ion transmembrane transport, regulation of ion transmembrane transport and neuropeptide signalling pathway (Figure 4.9B). The majority of the molecular functions of the differentially expressed genes were binding and catalytic activity, with most sequences associated with ATP, protein, DNA, nucleic acid, metal ion, zinc ion and calcium ion binding, but also methyltransferase activity, transmembrane transporter activity and transmembrane signalling receptor activity (Figure 4.9C). The cellular components associated with the differentially expressed genes were largely assigned to cellular anatomical entities, with approximately 950 sequences contributing to integral components of the cell membrane (Figure 4.9D). Finally, the enzyme code classes assigned to most of the sequences included Hydrolases, followed by transferases and reductases (Figure 4.10A). KEGG pathway analysis revealed that the top three biological pathways related to the differentially expressed genes were purine metabolism (126 sequences, 11 enzymes), thiamine metabolism (103 sequences, 4 enzymes) and the T cell receptor signalling pathway (36 sequences, 2 enzymes; Figure 4.10B and Figure 4.11).

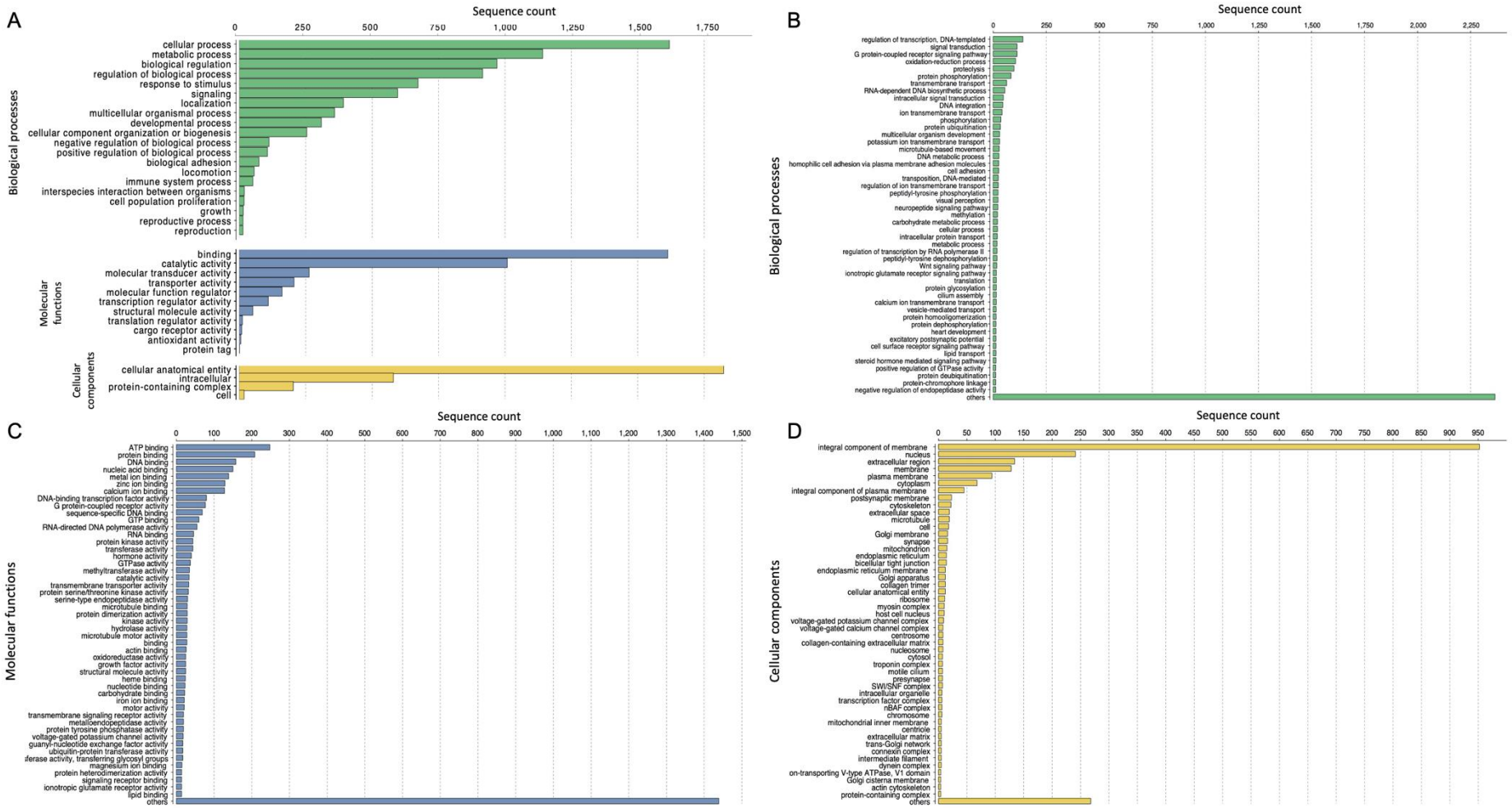

Figure 4.9 Gene Ontology (GO) distribution of gene sequences differentially expressed between HT (hot temperature; 34 °C) and control (28 °C) treated *Lates calcarifer* following temperature exposure from 12 to 24 months post hatch (n=5 per treatment). A. By category and those assigned specifically to B. biological processes C. molecular functioning or D. cellular components.

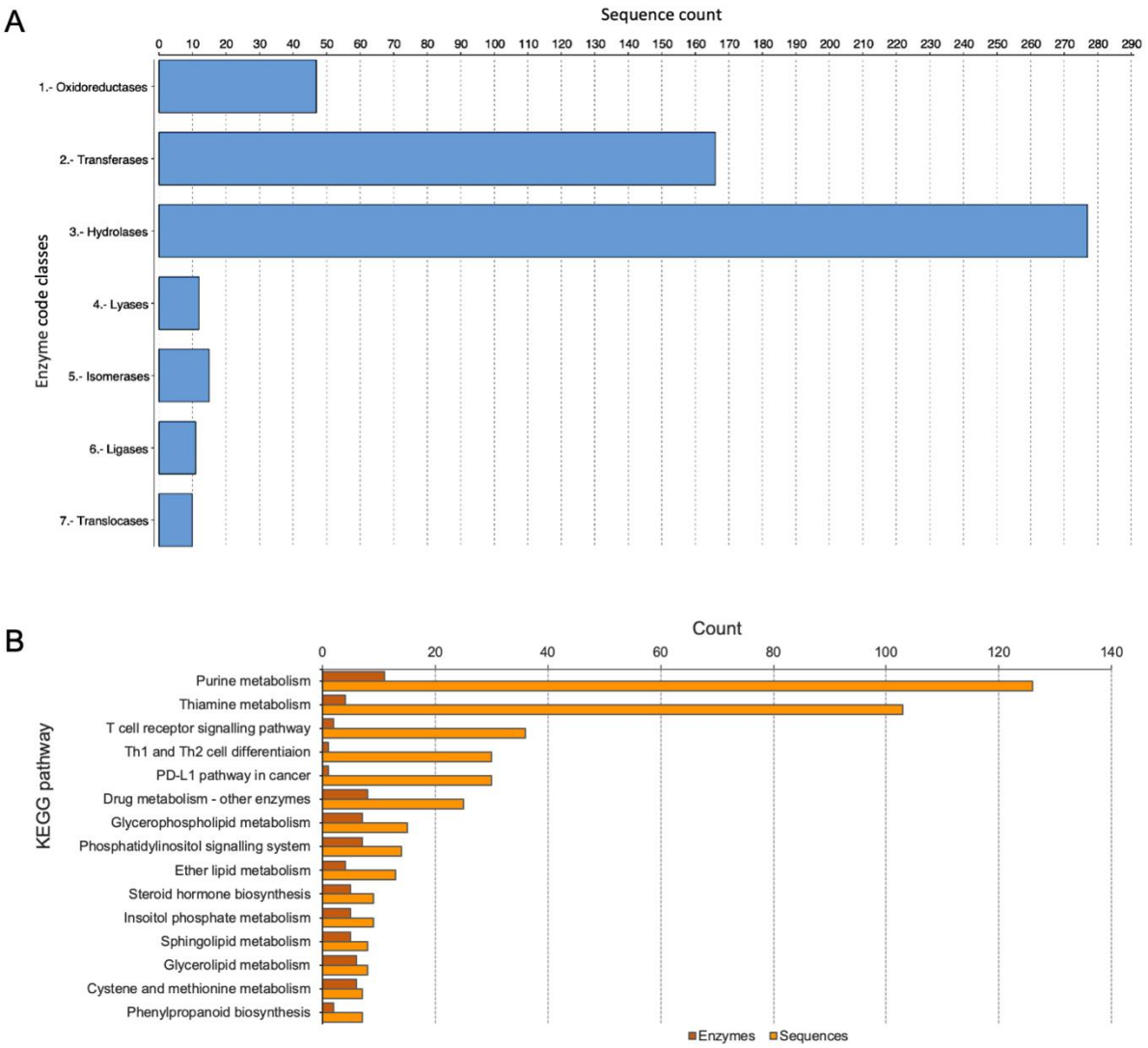

Figure 4.10 A. Enzyme code classes and B. Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways assigned to *Lates calcarifer* gene sequences differentially expressed between HT (hot temperature; 34 °C) and control (28 °C) treatments following temperature exposure from 12 to 24 months post hatch (n=5).

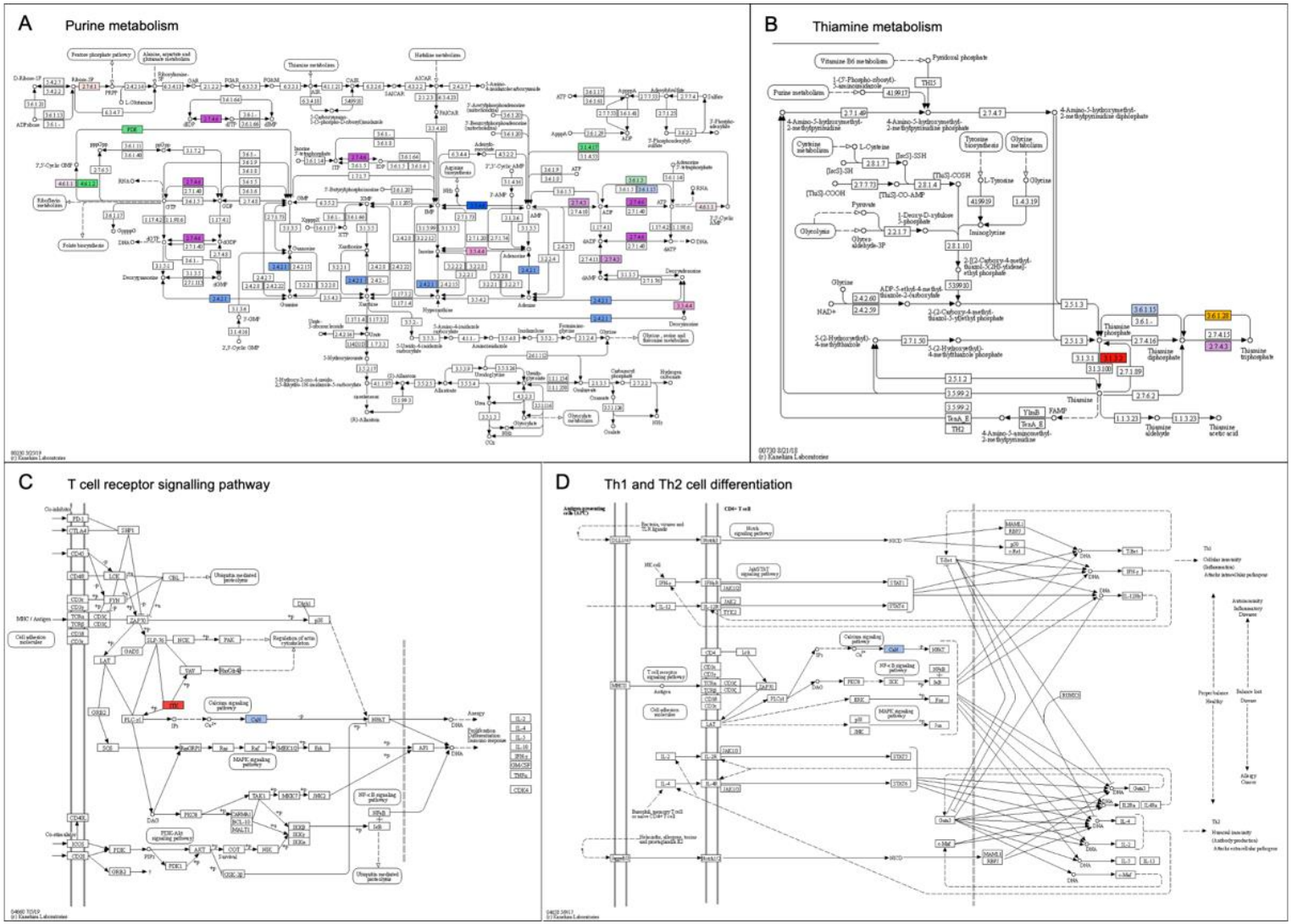

Figure 4.11 Top four Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways assigned to *Lates calcarifer* gene sequences differentially expressed between HT (hot temperature; 34 °C) and control (28 °C) treatments following temperature exposure from 12 to 24 months post hatch (n=5). Coloured boxes indicate enzymes to which sequences have been assigned, different colours indicate different enzymes as indicated by the enclosed text.

4.4.4 Methylation in the proximal predicted promoter and gene body

No significant differences in gonadal DNA methylation levels of the proximal predicted promoter and gene body (- 2000 bp to stop codon) between treatments were observed at 24 mph for the eight key sex-related genes investigated (Figure 4.12). Visualisation of the data suggests a general trend towards decreased methylation in HT-treated compared to control fish for *cyp19a1a, esr1, amh, nr5a2* and *dmrt1* (Figure 4.12A, C, D, E, F). The female fish (labelled F2) exhibited the lowest methylation value compared to all other individuals for *cyp19a1a, amh, nr5a2, dmrt1* and *sox9* (Figure 4.12A, D, E, F, G). Conversely, HT-treated fish tended to have the highest methylation values and control fish the lowest methylation in *foxl2*, although methylation of *foxl2* did not exceed 7.5 % in any fish (Figure 4.12). More generally, methylation was relatively high in *cyp19a1a, esr1, amh, nr5a2, dmrt1,* and *sox8* (> 50 % in all fish) and relatively low in *foxl2* and *sox9* (< 50 % in all fish; Figure 4.12). Targeted analysis of a smaller region of the promoter and first exon (- 300 to 200 bp) revealed significant differences in methylation of *nr5a*2 and *foxl2* between treatments (Figure 4.13). For *nr5a2*, methylation was significantly higher in HT fish than control and CT-treated fish (Figure 4.13E). In *foxl2*, methylation in the FT treatment was significantly different from control fish, but methylation did not exceed 5 % in any fish or treatment (Figure 4.13B).

4.4.5 Sex gene expression

Significant differences in gene expression among treatments occurred in all analysed genes excluding *amh* (Figure 4.14). Gene expression of *foxl2, dmrt1* and *sox9* was significantly increased in HT-treated fish compared to controls (Figure 4.14B, F, G). Gene expression in *cyp19a1a* and *foxl2* was significantly decreased in CT-treated fish compared to controls (Figure 4.14A and B). Similarly, expression in these genes (*cyp19a1a, foxl2, dmrt1* and *sox9*), as well as *nr5a2*, was significantly higher in HT-treated fish compared to CT-treated fish. Furthermore, gene expression was slightly, but not significantly higher in *esr1* and *sox8* in HT-treated fish compared to CT-treated fish (Figure 4.14C, H). Gene expression FT-treated fish was significantly higher than control fish in *dmrt1* and *sox9,* significantly higher than CT-treated fish in *cyp19a1a* and *sox9*, and significantly lower than HT fish in *esr1* and *sox8* (Figure 4.14). The largest changes in gene expression between treatments occurred in *esr1, amh, dmrt1 and sox9* (Figure 4.14C, D, F, G).

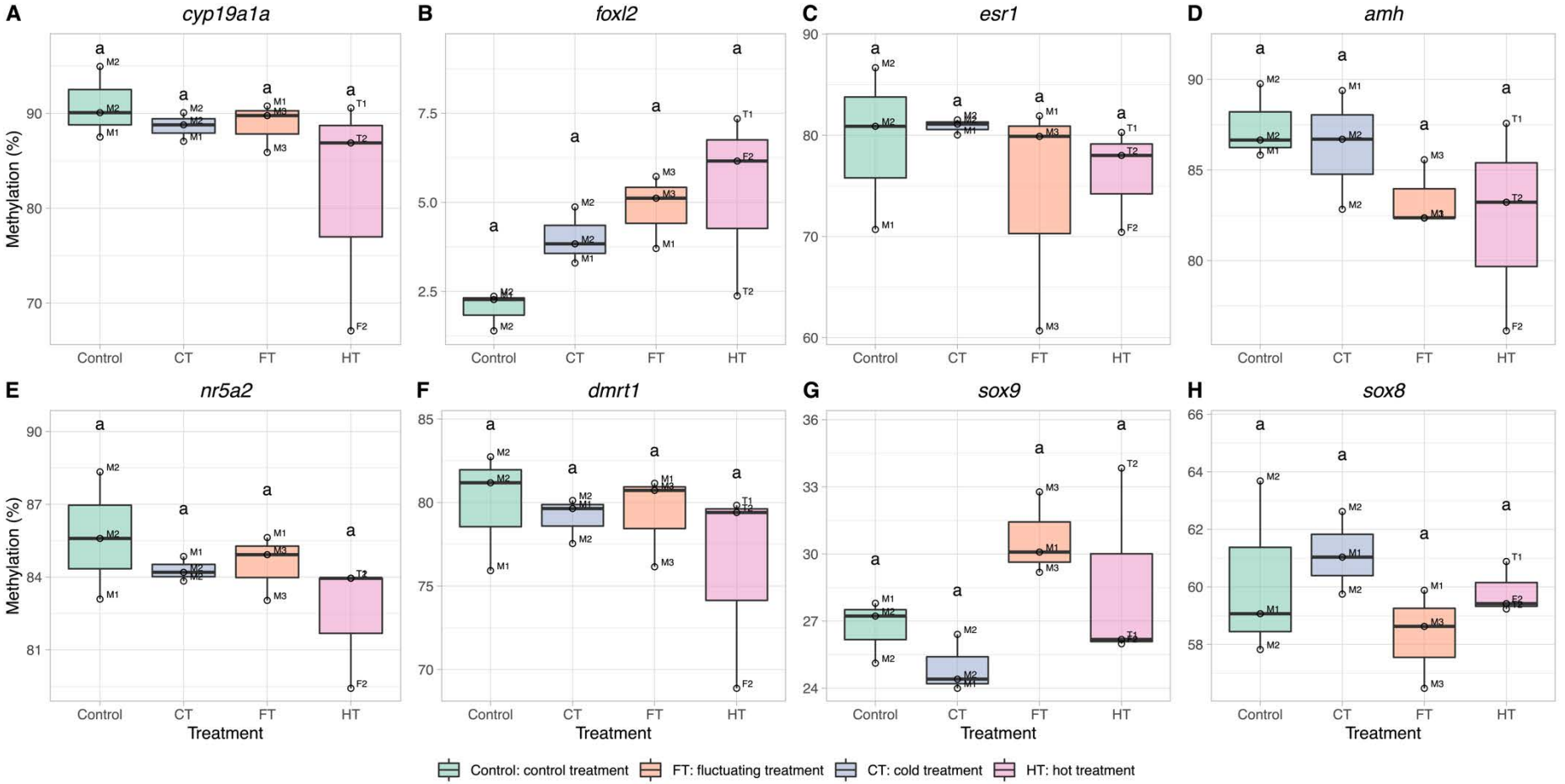

Figure 4.12 Gonadal DNA methylation of the predicted promoter and gene body (-2000 to stop codon) in *Lates calcarifer* sex-related genes following temperature treatment from 12 to 24 months post hatch. Data (n=3 fish per treatment) were obtained from whole genome bisulphite sequencing and represent average methylation. Letters denote pair-wise comparisons using Tukey's Honest Significant Difference corrected for false discovery rate following ANOVA on logit transformed data. Labels indicate gonadal status.

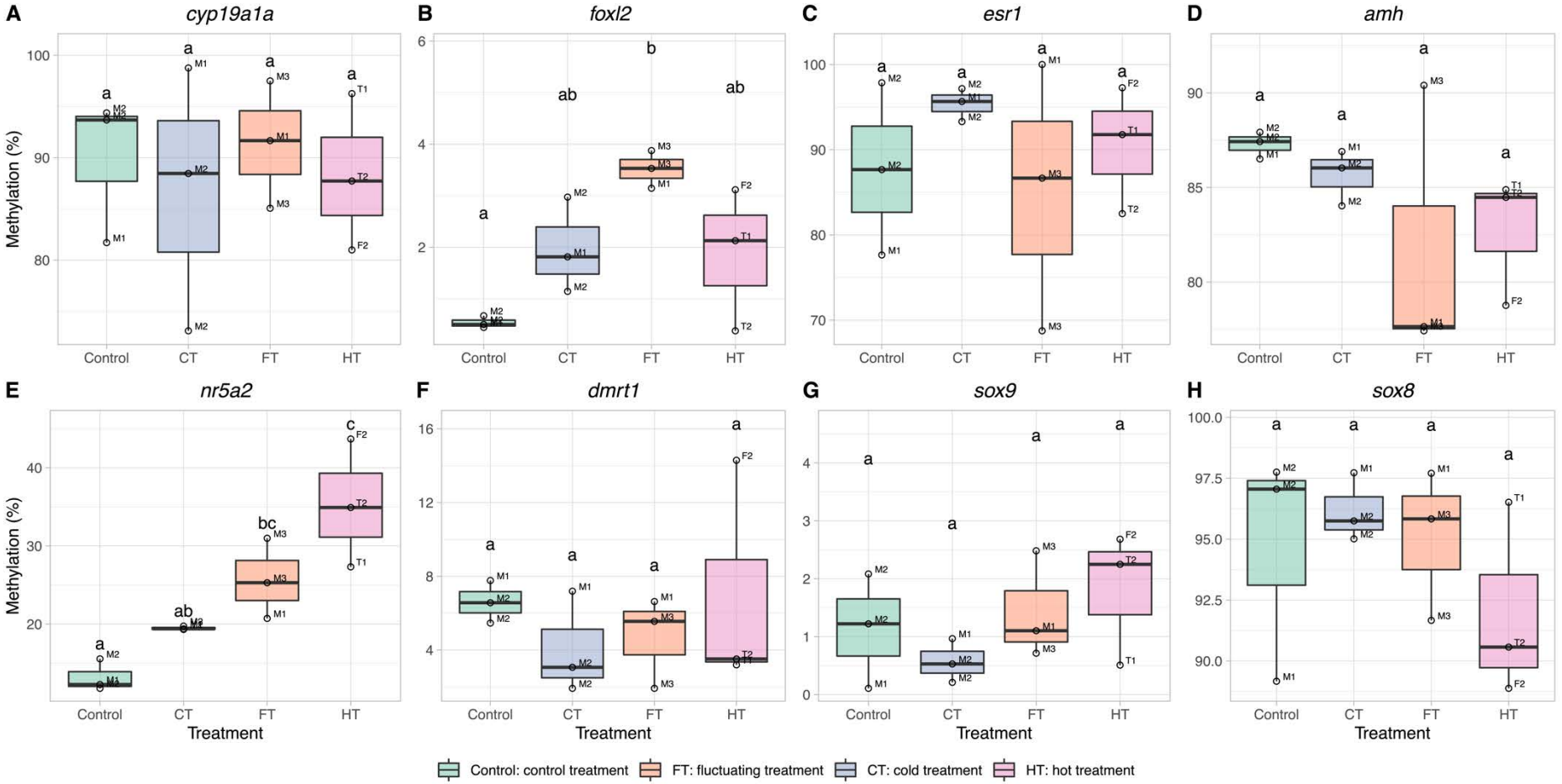

Figure 4.13 Gonadal DNA methylation of the region -300 to 200 bp in the proximal predicted promoter and gene body in *Lates calcarifer* sex-related genes following temperature treatment from 12 to 24 months post hatch. Data (n=3 fish per treatment) were obtained from whole genome bisulphite sequencing and represent average methylation. Letters denote pair-wise comparisons using Tukey's Honest Significant Difference corrected for false discovery rate following ANOVA on logit transformed data. Labels indicate gonadal status.

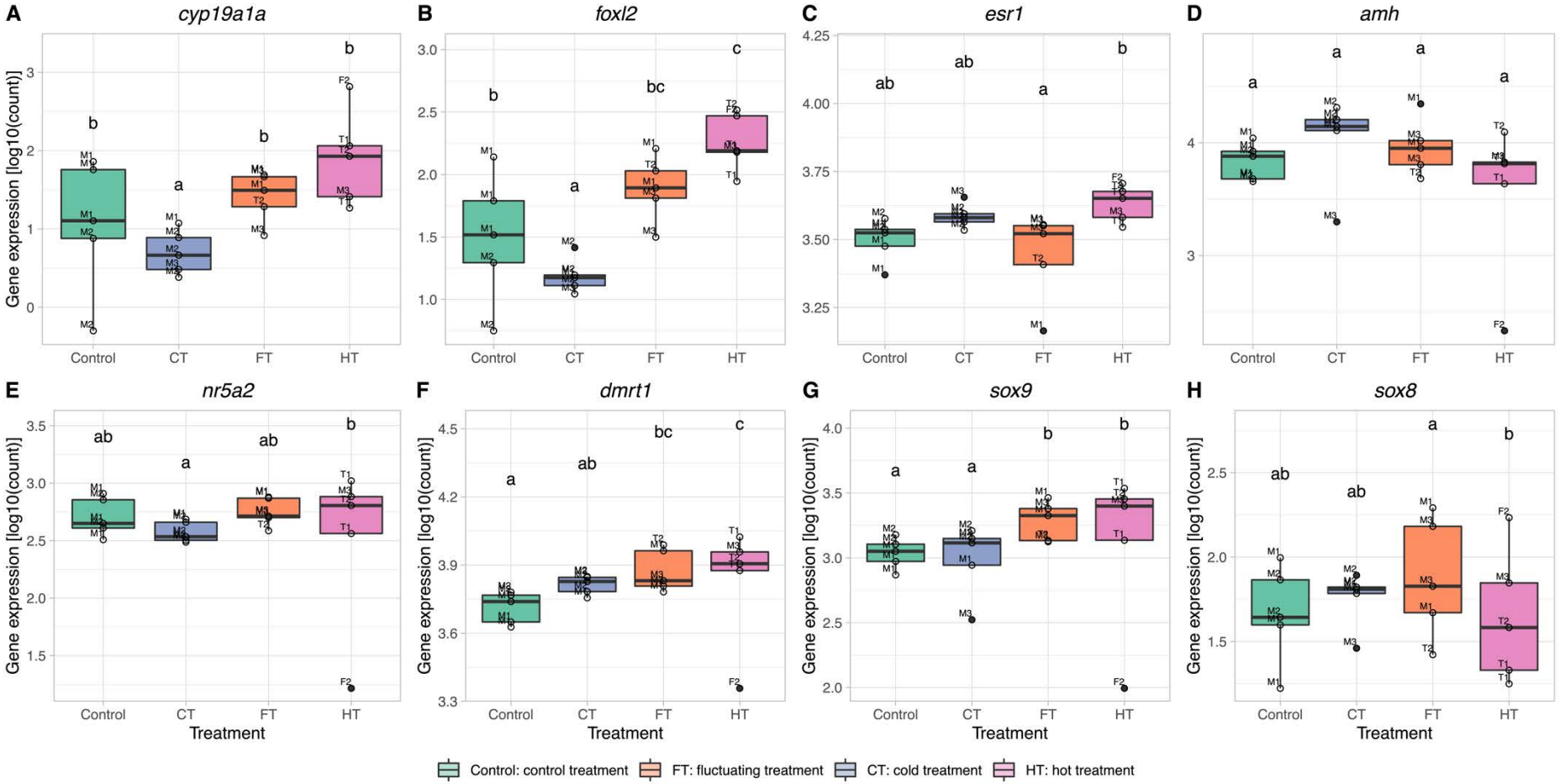

Figure 4.14 Gonadal gene expression in *Lates calcarifer* sex-related genes following temperature treatment from 12 to 24 months post hatch. Gene expression data (n=5 fish per treatment) were obtained from RNA-sequencing and are presented as normalised, log-transformed counts. Letters denote pair-wise comparisons using Tukey's Honest Significant Difference corrected for false discovery rate following negative binomial generalised linear modelling. Data point labels indicate gonadal status.

4.4.6 Coupled methylation and expression analysis

Due to large within-treatment variation and small sample size, methylation and expression values along the length of *cyp19a1a* and *dmrt1* in the three HT and three control individuals for which WGBS and RNA-Seq data were obtained were additionally assessed in greater detail (Figure 4.15). These data revealed that in HT fish, particularly in the phenotypic female (F2 stage), methylation of *cyp19a1a* was substantially lower in exons, as well as in a cluster of CpG sites in the upstream proximal promoter (Figure 4.15). RNA expression in the F2 individual was comparatively high in most exons, whereas expression levels in the transitional and male fish were over-represented in the first four exons, as well as the intron between exon three and four, with nearly absence in transcription of remaining exons five to nine (Figure 4.15). Furthermore, expression levels in exons one and two of *cyp19a1a* in male and transitional fish occurred in slightly truncated regions (as in GenBank MH784537.1: *Lates calcarifer cyp19a1a* short-form mRNA; alternatively spliced) compared to female fish and the *cyp19a1a* reference sequence (Figure 4.15). In comparison to *cyp19a1a*, methylation levels in *dmrt1* was high in both introns and exons (Figure 4.15 and Figure 4.16). Counterintuitively, *dmrt1* expression levels were comparably higher in the F2 individual compared to the male and transitional fish, with the exception of exon 1 which harbours the *dmrt1* DM domain (Figure 4.16). For exon 1 of *dmrt1*, expression was moderate compared to other exons in males and transitional fish, but the F2 individual in exon 1 exhibited almost no expression in this exon (as in GenBank MH784536.1: *Lates calcarifer dmrt1b* mRNA; alternatively spliced), which was also comparatively higher in methylation (Figure 4.16), particularly for CpGs -122 and -130 bp situated amidst putative transcription factor binding sites E2F and SP1 (data not shown).

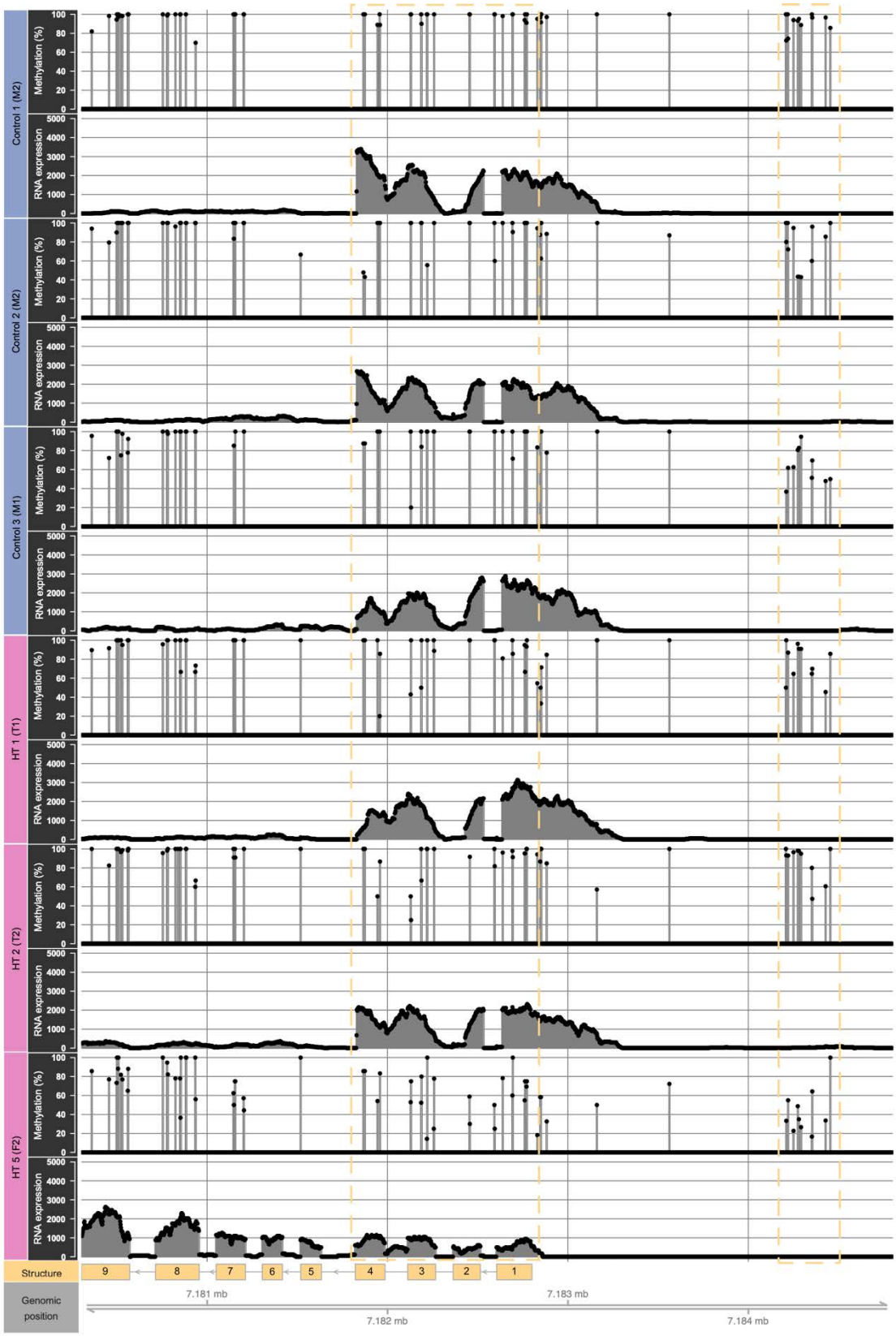

Figure 4.15 Relationship between gonadal DNA methylation and gene expression in *Lates calcarifer cyp19a1a* for individuals following hot temperature (HT; 34 °C) or control (28 °C) treatment from 12 to 24 months post hatch. Vertical grey bars represent CG methylation and expression values calculated at 2 bp running windows, where methylation is calculated as percentage and gene expression values are raw counts normalised per million reads. Yellow dashed boxes indicate regions of interest discussed in text. Gene structure is indicated by yellow numbered boxes (exons) and grey lines (introns); note the grey arrows indicating directionality; *L. calcarifer cyp19a1a* maps in reverse, therefore the promoter appears on the right of this figure.

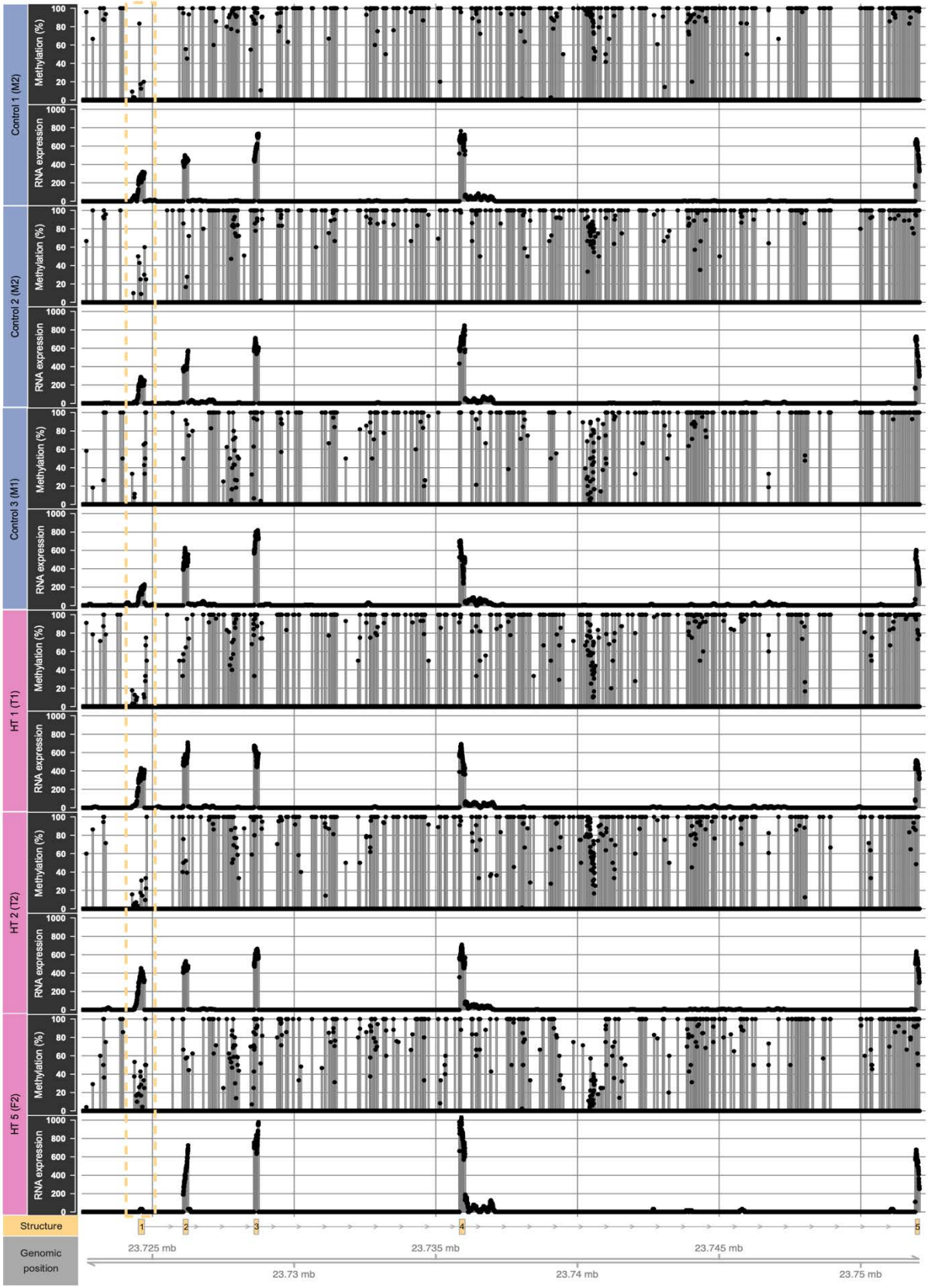

Figure 4.16 Relationship between gonadal DNA methylation and gene expression in *Lates calcarifer dmrt1* for individuals following hot temperature (HT; 34 °C) or control (28 °C) treatment from 12 to 24 months post hatch. Vertical grey bars represent CG methylation and expression values calculated at 2 bp running windows, where methylation is calculated as a percentage and gene expression values are raw counts normalised per million reads. Yellow dashed box indicates region of interest discussed in text. Gene structure is indicated by yellow numbered boxes (exons), and grey lines (introns).

4.5 Discussion

In this research, high temperature treatment of *L. calcarifer* at 34 °C in one-year-old fish for a period of one year led to advanced gonad development, skewing sex ratios towards transitional and female stage fish. GO term analysis between HT treated and control fish revealed an over-representation of sequences involved in cellular signalling and ion binding. High temperatures also led to an upregulation of female-associated genes *cyp19a1a* and *foxl2* compared to cold treated fish, and *esr1* compared to FT-treated fish. An overall upregulation in male-associated genes *nr5a2, dmrt1* and *sox9* was observed in HT-treated fish, with the lowest expression value occurring in the female individual and the higher expression most prevalent in transitional fish. Promoter and gene body DNA methylation showed a tendency towards low levels of methylation in heat treated fish in five of the eight genes examined, with the exception of *sox8*, *sox9* and *foxl2,* the latter of which showed the opposite effect. Further analysis of a more targeted region partially covering the predicted promoter and first exon (-300 to 200 bp) in each gene revealed increased methylation of *nr5a2* in heat treated fish, a known indicator for femaleness in this species (Domingos *et al.*, 2018). Fine scale analysis of methylation along the length of *cyp19a1a* and *dmrt1* revealed high variability in both methylation and expression along the length of each gene including predicted proximal promoter, and demonstrated patterns of sex-specific alternative splicing similar to a previous report whereby *L. calcarifer* males lacked the full-length aromatase coding *cyp19a1* mRNA due to partial or total exon splicing, and females lacked the *dmrt1* exon containing the DM-domain sequence (Domingos *et al.*, 2018). Additionally, it was observed that male and transitional fish lacked exons five through nine in *cyp19a1a*. These data are the first to show an experimentally induced effect of temperature on gonadal gene expression and DNA methylation in a sequentially sex changing fish and demonstrate the potential for the use of temperature treatment as a method of sex control in barramundi aquaculture.

4.5.1 GO term analysis reveals sequences involved in signalling but not methylation pathways

While DNA methylation has been identified as the mechanism linking changes in environmental temperature to differences in phenotypic sex in fish (Navarro-Martín *et al.*, 2011; Shao *et al.*, 2014; Ellison *et al.*, 2015; Wang *et al.*, 2019), 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); the enzymes responsible for the physical transfer of methyl groups onto the nucleic acids (Navarro-Martín *et al.*, 2011). For example, in *Xiphophorus* fishes, the activity of O^6-methylguanine-DNA-methyltransferase (O^6-MGMT) is optimal at 23 °C, but diminishes greatly, to less than 75 % of maximum efficiency, below 15 °C and above 40 °C (Walter *et al.*, 2001). Thus, in ectothermic species such as fish and reptiles, the activity of these enzymes is likely directly affected by the temperature of their external environment. Furthermore, O^6-MGMT activity in *Xiphophorus* fishes is tissue specific, with about six-fold higher levels in the brain compared to the liver, offering support for gonad-specific effects of temperature and thus DNMT activity in sex-reversed fish (Walter *et al.*, 2001). In this study, GO term analysis between HT and control treated fish did not reveal a marked overrepresentation in methylation or methyltransferase activity, but the results may have indicated otherwise if the comparison were to be made between male and female fish exclusively. Indeed, sex-biased expression of DNMT-related sequences was observed in the protogynous bluehead wrasse, but the functioning of their associated enzymes is yet to be investigated (Liu *et al.*, 2015).

Alternatively, it has also been proposed that cellular temperature sensors are responsible for the observed effect of temperature on epigenetic regulatory elements, gene expression and sex in TSD reptiles (Singh *et al.*, 2020). Specifically, temperature affects redox and calcium levels in the cell (Ahn and Thiele, 2003), which is proposed to serve as a sensor to environmental temperature and lead to the initiation of ubiquitous signal transduction pathways, influence epigenetic processes and drive the differential expression of sex genes (Castelli *et al.*, 2020). It has been shown in reptiles that an ortholog of a transient receptor potential cation channel, which mostly function as environmental sensors though calcium ion signalling, is activated by temperature and results in male-specific gene expression patterns (Yatsu *et al.*, 2015). There is also some support for this activity in temperature treated Nile tilapia, where transcriptome analysis revealed upregulation of genes involved in ion transport processes (Wang *et al.*, 2019). Specifically, these genes were upregulated in XX males, but not XX females or untreated XY males, suggesting that the upregulated genes are interacting with or responsive to the temperature cue itself, rather than induction of the male pathway more generally (Wang *et al.*, 2019). In the present study, GO term analysis of gene sequences differentially expressed between HT and control treated fish revealed that many differentially expressed genes were associated with biological pathways of signal transduction, signalling pathways, oxidation-reduction processes, transmembrane transport including ion transmembrane transport and

intracellular signal transduction, as well as molecular functions associated with binding, including calcium ion binding. Furthermore, enzyme code classes identified by KEGG pathway analysis revealed an over-representation of oxidoreductase-related sequences. Expression of calcium-dependent, calmodulin-stimulated protein phosphatase (represented by the CaN symbol) was also highlighted by KEGG pathway analysis, an enzyme which is known to play an essential role in the transduction of intracellular calcium ion mediated signals (Kilka *et al.*, 2009; Grigoriu *et al.*, 2013). Together, these findings provide support for a role of cellular calcium and redox signalling pathways as transducers of environmental temperature into sex-specific gene regulation in barramundi, as proposed more generally in Castelli *et al.* (2020).

4.5.2 Temperature treatment causes differential expression of key sex genes

High temperature treatment in adult barramundi led to an upregulation of five key sex genes when compared to cold temperature treatment. Specifically, an upregulation of female-associated genes *cyp19a1a* and *foxl2* in HT-treated fish was evident compared to CT-treated fish. *Cyp19a1a* plays a key role in sexual differentiation and sex change in fish, and its expression leads to ovarian development (Guiguen *et al.*, 2010). A strong upregulation of *cyp19a1a* has recently been shown to occur in the female stages of protandrous sex change in the clownfish *Amphiprion bicinctus,* similar to what was observed here (Casas *et al.*, 2016). *Foxl2* acts as a direct modulator of *cyp19a1a* expression, and as such is also known to play a determinant role in ovarian differentiation in fish (Wang *et al.*, 2007). Previous work in barramundi has shown that temperature-induced transitional stages and male to female sex change in barramundi were accompanied by increases in the enzymatic activity of aromatase in the gonad as well as levels or circulating estrogen in the blood, likely caused by the aromatisation of androgens into estrogens and resulting in an increase in transitional and female stage fish (Athauda *et al.*, 2012). Thus, the upregulation of female-associated *foxl2* and *cyp19a1a* observed here is likely to be associated with ovarian differentiation in HT-treated barramundi.

An upregulation in male-associated genes *nr5a2, dmrt1* and *sox9* was also observed in HT-treated fish compared to CT-treated fish; however, the expression values for these genes in the female F2 stage individual were the lowest. This indicates that while male-associated genes were expressed at low levels in the female individual, as expected, transitional stage individuals may be somewhat counterintuitively exhibiting an upregulation of male-associated genes.

While no sex biased expression of *nr5a2* has previously been observed in protandrous and clownfish or protogynous bluehead wrasse, *dmrt1* is upregulated in male stages of both species (Liu *et al.*, 2015; Casas *et al.*, 2016). *Dmrt1* is known to have multiple isoforms in many teleost species such as barramundi (Domingos *et al.*, 2018), grouper (Alam *et al.*, 2008) and eels (Huang *et al.*, 2005). Multiple isoforms in *dmrt1* may lead to diversified roles other than male development and therefore an upregulation in transitional fish as observed here. Similarly, *sox9* is also known to play diverse roles in sexual differentiation in fish, whereby *sox9* is predominantly observed in adult medaka ovary rather than the testis (Huang *et al.*, 2005). Further investigation into the roles of these genes in barramundi, specifically in transitional stages, would likely allow greater understanding of the upregulation of male-associated genes in HT-treated transitional individuals. While the precise roles of each of these genes in the sexual development of barramundi are unknown, it is clear that temperature treatment from 12 to 24 mph causes significant changes in their expression.

4.5.3 Targeted analysis of gene body and predicted proximal promoter methylation

The major biological consequence of DNA methylation is thought to be gene silencing (Colot and Rossignol, 1999), with a consistent inverse correlation between DNA methylation in the first intron and expression of a gene across species (Anastasiadi *et al.*, 2018). However, there are many exceptions to these general trends (Suzuki and Bird, 2008). In the present study, the majority of genes as examined here did not exhibit a correlation between methylation and expression. For example, analysis of total methylation levels over the predicted proximal promoter and gene body (-2000 bp to stop codon) of the eight sex-related genes examined here did not reveal any significant changes in methylation. However, restriction of the analysis to a more targeted region (-300 bp to 200 bp) revealed significant differences in methylation in *nr5a2*, which was most heavily methylated in HT treated fish. An *nr5a2* amplicon with overlapping coverage to that examined here was recently shown to exhibit the most significant sex-related differences in adult *L. calcarifer,* with similarly high levels occurring in female fish (Domingos *et al.*, 2018). This previous data, as well as that presented in Chapter 2 and Chapter 3, supports the current finding of high *nr5a2* methylation and the presence of transitional and female stages of development in HT-treated barramundi. The absence of an inverse correlation between methylation and expression is not uncommon in the existing body of literature. For example, sex-related genes *amh*, *cyp19a1a sox9a* and *sf1* showed either upregulated methylation *and* expression, or no change in methylation at all, in temperature sex-reversed

tongue sole (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). The absence of significant differences in promoter methylation and the absence of an inverse correlation between predicted promoter methylation and expression could indicate that the targeted region (- 2000 bp and into the first exon) may not coincide with the true promoter of the gene. It is also known, at least in mammals, that most genes have multiple promoters and each promoter has multiple transcription start sites which further complicates the identification of methylation responsive promoters in non-model species (Carninci *et al.*, 2006; Sandelin *et al.*, 2007; Frith *et al.*, 2008). The location of promoter regions in *L. calcarifer* sex genes is at present unknown, and until this is experimentally demonstrated the existence of a relationship between temperature treatments, promoter methylation and gene expression cannot be ruled out based on these analyses alone.

4.5.4 Base pair resolution data enables identification of differentially methylated gene regions

While the location of defined regulatory regions such as promoters is unknown in most species, single base pair resolution data provided by WGBS analysis can be used to identify differentially methylated regions between treatments and within genes of interest. For example, coupled analysis of methylation and expression along the length of *cyp19a1a* in bluehead wrasse revealed hypermethylation in males compared to females, most notably in a CpG island close to the transcription start site (Todd *et al.*, 2019). In tilapia, it was shown that methylation in *cyp19a1a* and the 2000 bp flanking regions was generally lower in temperature-induced males compared to females (Wang *et al.*, 2019). In the same study, it was shown that methylation levels in *dmrt1* were lower in XY males and XX males compared to XX females, but only in specific regions of the gene, rather than along its entire length (Wang *et al.*, 2019). Similar analysis performed here revealed that heat-treated fish, exhibited lower *cyp19a1a* methylation in exonic regions, but also in a cluster of CpG sites in the upstream promoter region. In male and transitional fish, transcription of *cyp19a1a* was heavily overrepresented in the first four exons (with shorter variants exons 1b and 2b) and introns, but absent for remaining coding sequence (exons 5 to 9). For *dmrt1*, while expression levels overall were higher in the female than in male and transitional individuals, the female lacked expression of the first exon (which contains the DM domain) almost entirely, which was also found to be heavily

methylated. While observed in the only heat-induced female here [notably a small (59.5 cm), young (2 year old) fish], this finding is supported by a recent study which showed that 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 gene splicing (Maor *et al.*, 2015) and may be leading to the sex specific differences observed in barramundi and other teleosts. For example, in tongue sole, DNA methylation appears to lead to alternative splicing of ovary-associated gene *figla* (factor in the germline alpha) and loss of functionally critical helix-loop-helix DNA binding domain in temperature-induced males (Shao *et al.*, 2014). Similarly, in barramundi, DNA methylation is associated with alternative splicing of testis-associated gene *dmrt1* leading to loss of the functionally important DM domain (Domingos *et al.*, 2018). Along with previous findings of alternatively spliced transcripts of *dmrt1* and *cyp19a1a* in other fish species (see He *et al.*, 2003; Yu *et al.*, 2003 for examples; Guo *et al.*, 2005; Zhang *et al.*, 2008) these results highlight the advantages of full-length sequence data in the identification of biologically relevant methylation and gene expression data in non-model organisms. While analysis of predicted proximal promoter methylation in target genes of interest was the major approach used here, the WGBS data generated provides genome-wide, single nucleotide level resolution data and offers an important resource to further investigate whether alternative changes in methylation contribute to temperature-induced sex change in barramundi.

4.5.5 The effect of temperature on phenotypic sex co-occurred with increased body size

While analysis of a greater number of replicates would likely allow better resolution of differences in DNA methylation between treatments, the lack of differentiation between treatments observed here may also indicate that an alternate mechanism is leading to the induction of sex change in HT-treated barramundi. Here it was observed that HT-treated fish were significantly larger than CT-treated fish in both length and weight, indicating that an increase in body size may have encouraged transitional and female stages in larger fish. It is well established that sex change in barramundi occurs in relation to body size, with larger fish generally changing sex from male to female (Moore, 1979; Davis, 1982; 1984b; Guiguen *et al.*, 1994). However, HT-treated fish were not significantly different in size compared to control treated fish. Furthermore, barramundi from the Queensland east coast of Australia typically change sex at between 85 and 100 cm (QFMA, 1991); however, the female individual attained only 59.5 cm and none of the fish in this study exceeded a body length of 65 cm. These

lines of evidence suggest that the induction of sex change in HT-treated barramundi was not due to the attainment of a larger body size alone. In other sex changing species, sex change has been linked more specifically to increases in growth rate (Munday, 2002). As such, it could be that increased growth rate may have led to early induction of sex change in heat-treated fish as observed here, but this metric was not measured. While methylation may be triggered by increased growth rate rather than directly by temperature, the current data on the heat-treated transitional and female individuals at relatively young age and small body size, suggests that this is an improbable cause. Moreover, temperature increase is a well-known epigenetic and phenotypic modifier of sex in teleost fish (see Chapter 1 section 1.4 for details) and functional analyses provided support for cellular calcium and redox signalling pathways as direct transducers of temperature on epigenetic regulation in barramundi, as proposed more generally in Castelli *et al.* (2020). Functional experiments on the cellular transduction of temperature and how these translate into changes in DNA methylation will likely best reveal precisely how temperature exerts an effect on gonadal phenotype in adult barramundi.

4.5.6 Temperature as a viable sex control strategy for barramundi aquaculture

Temperature exerts a substantial and reproducible effect on sexual development in barramundi. In this study, histological examination of the gonads revealed that all but one fish in the HT treatment were in transitional or in female stages of development and all fish in the CT and control treatments remained male. This confirms previous experimental evidence in barramundi whereby similar increases in temperature, specifically from 25 to 34 °C, induced transitional stages of female development (Athauda *et al.*, 2012). Given the frequency of females and transitional stages observed in HT-treated fish was 20 and 60 % respectively, a raw increase in the number of individuals alone may prove the technique viable for use in industry. Future work should investigate if temperature treated males (cold) and females (hot) produce viable gametes and can be spawned successfully. In addition to this, selection for temperature sensitive barramundi may lead to increases in the frequency of females resulting from temperature treatment. This is because in many other fish the responsiveness of sex ratio to temperature is family-specific. For example, in tilapia sex-related temperature sensitivity differs between different families (Baroiller *et al.*, 2009a; Baroiller and d'Cotta, 2016; Wessels *et al.*, 2017) and in European seabass, parental influence on sex ratios and genotype-temperature interactions suggest viability for selecting for specific temperature sensitivity in breeding programs (Saillant *et al.*, 2002). In addition to selection for temperature sensitivity,

transgenerational temperature treatments may lead to an additive effect on early sex change in barramundi. For example, breeding temperature-induced ZW male tongue sole with untreated ZW females lead to the development of ZW male offspring in the absence of temperature treatment, indicating that offspring retain the epigenetic signatures from their parents (Shao *et al.*, 2014). Breeding temperature-induced female barramundi with untreated males may therefore lead to retained early development in females, and transgenerational temperature treatments may result in a cumulative effect of temperature treatments on the early induction of sex change. As such, temperature treatment provides strong potential as a solution to sex control in barramundi aquaculture.

4.6 Conclusion

Data from this chapter provides the first examination of temperature treatment on gene expression, DNA methylation and gonadal phenotype an adult sequentially sex changing fish, revealing significant increases in *foxl2* and *cyp19a1a* expression, *nr5a2* DNA methylation and transitional and female stage fish in HT treated compared to and CT treated barramundi. The data revealed evidence to support for a recently proposed role of cellular calcium and redox signalling pathways as transducers of environmental temperature into sex-specific gene regulation and can help guide functional studies on the cellular transduction and epigenetic responses of external cues in sexual development. The results advance our knowledge of how temperature affects gene expression and sexual phenotype in sequential hermaphrodites beyond the completion of sexual differentiation and highlight the potential for an economically viable and environmentally friendly strategy for sex control in barramundi aquaculture.

Chapter 5

General Discussion

5.1 Summary

This chapter is intended to give an overview of the thesis, synthesise the major findings placing them into broader context and suggest some future directions with which to take the work. Section 5.2 outlines the significance of the research by recapitulating the state of knowledge at the beginning and throughout the duration of the project, and highlighting important research contributions from other authors. Section 5.3 provides an overview of the major outcomes provided by each chapter of this thesis and section 5.4 places these major outcomes in a broader context, specifically in relation to key fundamental questions of how and why fish change sex. Finally, the chapter and thus the thesis closes with some suggestions for future research, with the intent to build on the broad foundation that this exploratory work provides.

5.2 Significance

How do fish change sex? This question is at the core of some of the most interesting and challenging research in fish biology. Sex change in fish can occur as a typical part of sexual development, as in sequential or serial hermaphroditism, or as a result of exposure to exogenous environmental conditions experienced during primary sexual differentiation, as in sex reversal of many gonochoristic species (see Chapter 1 for details). While sex in fish is largely underpinned by a common network of genes, the specific gene pathways, regulatory mechanisms and environmental factors allowing for variation in sexual development within and between species are complex, rapidly evolving and not yet understood (Heule *et al.*, 2014). In sequential hermaphrodites, sex change is a pivotal variable in predicting fundamental population demographics used in fisheries modelling (Benvenuto *et al.*, 2017; Campbell *et al.*, 2017). Sex change also presents significant challenges associated with reproductive control and the management of breeding programs in aquaculture (Robinson *et al.*, 2010). In gonochoristic species, the sensitivity of sexual development to exogenous factors may lead to uneven sex ratios and thus compromised species reproductive capacity and viability amidst environmental change (Ospina-Alvarez and Piferrer, 2008). Conversely, the ability to skew sex ratios through administered changes in culture environment can offer opportunities for increased production of the economically more valuable sex in aquaculture (Budd *et al.*, 2015). Thus, understanding the exogenous environmental drivers and the underlying molecular mechanisms leading to sex reversal and sex change in fish will enable better fisheries management, allow identification and implementation of sex control in aquaculture and advance the current state of knowledge on how sex is established, maintained and transitioned in fish, and other vertebrates.

Over the last decade, and throughout the duration of this project, research into sex reversal and sex change in fish has expanded rapidly with many novel research contributions emerging in the literature (briefly summarised here, and in Figure 5.1). In 2011, Navarro-Martin *et al.* presented the first demonstration, in any vertebrate, that an epigenetic mechanism was mediating temperature effects on sex ratios. Using the European seabass as an experimental model, Navarro-Martín *et al.* (2011) were able to show that exposure of juvenile seabass to high temperatures during development increased DNA methylation levels in the promoter of *cyp19a1a* (gonadal aromatase; responsible for the conversion of androgens into estrogens), increasing the production of female fish (Navarro-Martín *et al.*, 2011). In 2013, Zhang *et al.*

revealed that DNA methylation of *cyp19a1a* also promotes male development in a sex changing species, the ricefield eel, observing a continual decrease in DNA methylation of *cyp19a1a* during protogynous sex change. The functional role of DNA methylation of *cyp19a1a* was further explored through abdominal implantation with a DNA methylation inhibitor, which resulted in reversal of the sex change process (Zhang *et al.*, 2013a). In 2014, Shao *et al*. found the reciprocal pattern, with methylation of key testis-promoting gene *dmrt1* inducing male development in genetically female fish as a result of high temperature treatment (Shao *et al.*, 2014). In 2015, Ellison *et al*. found similar effects in the simultaneously hermaphroditic mangrove killifish, with the proportion of male offspring increasing when egg incubation temperature was decreased. These differences were attributed to significant differential methylation of *cyp19a1a, dmrt1* and other sex-related genes (Ellison *et al.*, 2015). In 2016, Wu *et al*., investigated the relationship between DNA methylation and sex in the digonic black porgy, finding that methylation of *cyp19a1a* was higher in testis than in ovary, and that during sexual phase change from functional males to functional females, DNA methylation of *cyp19a1a* decreased (Wu *et al.*, 2016). In 2018, Domingos *et al*. similarly compared methylation levels in male and female protandrous barramundi, revealing that testis and ovaries were significantly differentially methylated in *cyp19a1a, dmrt1,* and other sex-related genes (Domingos *et al.*, 2018). In 2019, Wang *et al*. looked at global patterns of DNA methylation and gene expression, revealing that temperature-induced XX male tilapia are highly similar to XY males and distinct from XX females (Wang *et al.*, 2019). In 2019, Todd *et al*. showed that phenotypic changes during protogynous sex change in bluehead wrasse are accompanied by a progressive accumulation of DNA methylation in the gonads. Specifically, *cyp19a1a* becomes increasingly methylated and decreasingly expressed, and *dmrt1* became reciprocally methylated and expressed during female to male sex change (Todd *et al.*, 2019). Together, the current body of work on sex reversal and sequential sex change in fish suggests that DNA methylation is a common mechanism underlying temperature effects on sex in gonochoristic species and simultaneous hermaphrodites, as well as sex change in sequential hermaphrodites. Notably, high methylation in *cyp19a1a* and low methylation in *dmrt1* is broadly associated with male phenotypes, and the reciprocal pattern with female phenotypes; either ecologically, biologically or experimentally induced. What is yet to be revealed by work done elsewhere, and formed the focus of this thesis, is if temperature affects methylation in sequential hermaphrodites; a) when applied during development of the primary sex, or b) later in life, before development of the sequential, secondary sex.

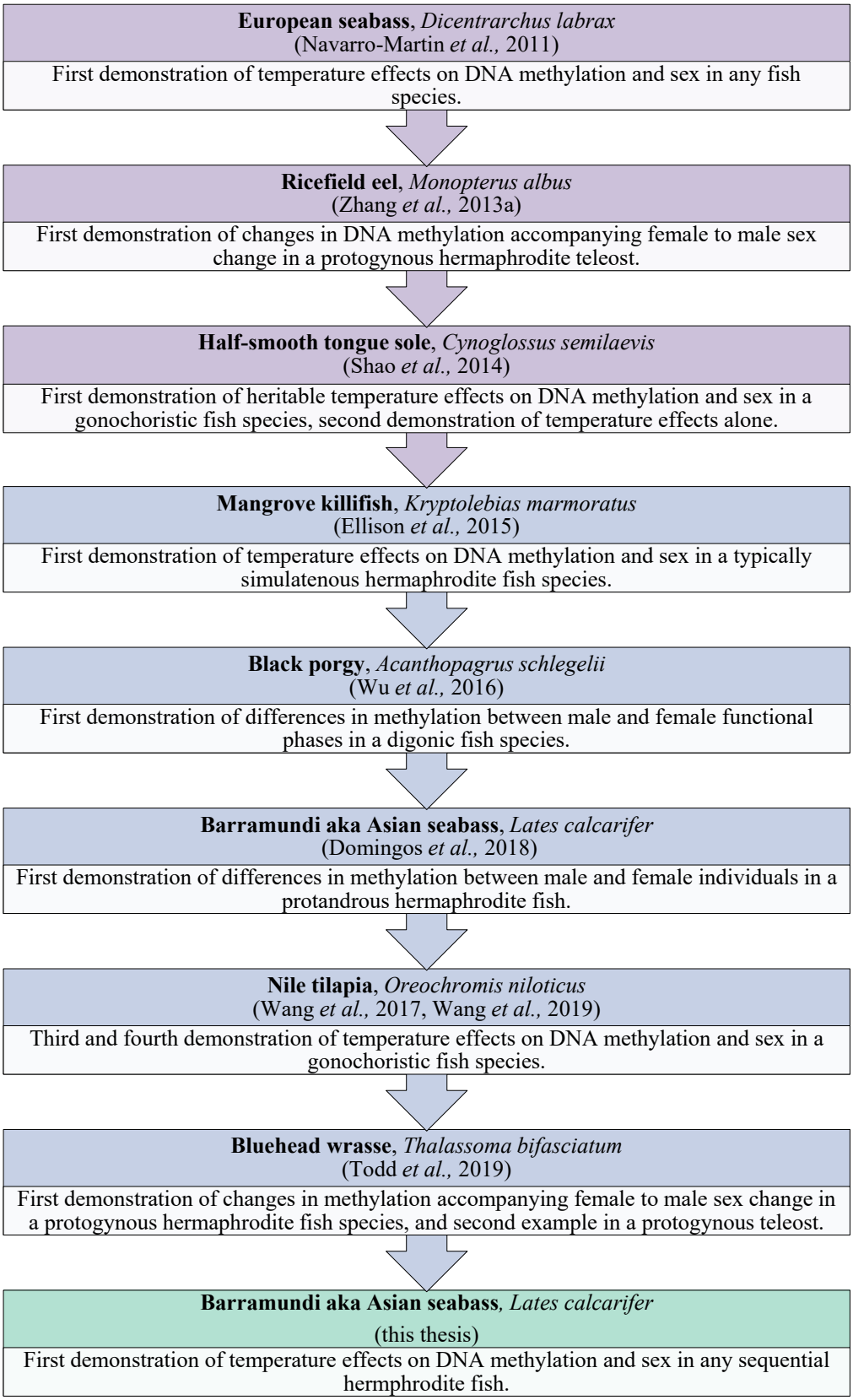

Figure 5.1 Summary of the position of research on DNA methylation and sex in teleost fish upon project commencement (purple), throughout the duration of the research (blue) and contribution of the work presented in this thesis (green).

5.3 Major outcomes

The overarching aim of this thesis was to investigate the effect of temperature on DNA methylation and sexual phenotype in the sequentially hermaphroditic, ecologically and economically important barramundi. The research first explored the relationship between DNA methylation and sex in wild-caught barramundi from different regions of Queensland, Australia. Following this initial exploration were two experimental components, the first of which investigated the effect of short-term temperature treatment on DNA methylation and sex in juvenile barramundi preceding and during the period of male sexual differentiation, and the second examines the effect of prolonged temperature exposure on DNA methylation, gene expression and sex in one-year-old barramundi, where male sex had been established.

In Chapter 2, the research explores a potential association between DNA methylation and length-at-sex change in wild-caught barramundi from Queensland, Australia and in doing so, investigated the hypothesis that gradual changes in DNA methylation lead to male-female sex change in this species. Using a total of 95 barramundi from three geographic regions, it was shown that male and female barramundi are significantly differentially methylated in male-associated genes *dmrt1* and *nr5a2*, as well as female-associated genes *esr1* and *cyp19a1a.* This confirms the findings of Domingos *et al.* (2018) but with a larger sample size, covering a broader geographic range and additionally contributes information on the relationship between DNA methylation and fish total length. Using a beta regression to model this relationship, it was found that despite the protandrous nature of this species and a known relationship between increasing size and the frequency of females, male barramundi become more male-specific in their methylation patterns with increasing total length. Highly male specific DNA methylation patterns in large barramundi supports previous observations that suggest not all barramundi undergo male to female sex change (Moore 1979). Furthermore, because these large male barramundi exhibit overlapping size classes with female barramundi within the same region, this emphasises that factors in addition to total length are leading to sex change in this species (Guiguen *et al.*, 1994). It was also observed that there are differences in total length-at-sex change between regions within the Gulf of Carpentaria, and that these differences in length-at-sex change are reflected in differences in DNA methylation patterns. Specifically, barramundi from the mid-northern Gulf of Carpentaria exhibited a significantly smaller total length-at-sex change and significantly different DNA methylation patterns in all four genes examined (*cyp19a1a, esr1, drmt1* and *nr5a2*) compared to the Southern Gulf of Carpentaria and North

Queensland east coast, which were not significantly different from each other. The observed differences in length-at-sex change and DNA methylation occurred in the absence of substantial genetic variation. While differences in length-at-sex change and DNA methylation could not be correlated with differences in average ambient temperatures, there was some evidence for slowed growth in the mid-northern Gulf of Carpentaria population. While similar findings within the Gulf of Carpentaria were observed by Davis (1984b), this research shows, for the first time, that sex-related phenotypic differences are accompanied by differences in DNA methylation. This constitutes one of the few published examples of population-level epigenetic variation concurrent with phenotypic variation in wild fish. A similar study in Atlantic salmon revealed variation in DNA methylation in the absence of genetic variation, which was proposed to allow for differences in the timing of sexual maturation but the two were not directly linked (Morán and Pérez-Figueroa, 2011). In *C. eosneogaeus*, concurrent variation in pH and epigenetic signatures was suggested to indicate alternative responses of identical genotypes to differing environments (Massicotte and Angers, 2012). Here, the work not only showed epigenetic variation consistent with phenotypic differences in total length-at-sex change, but that this epigenetic variation occurs in the precise genes that have been implicated in sex in this and other sequential hermaphrodites (Zhang *et al.*, 2013a; Liu *et al.*, 2015; Casas *et al.*, 2016; Domingos *et al.*, 2018). While the environmental correlates merit further investigation, this chapter provides a unique example of population level, epigenetically driven phenotypic plasticity in a commercially important, sex changing species and is the first to show that differential DNA methylation of trait-of-interest related genes may be associated with phenotypic plasticity.

In Chapter 3, research in this thesis examined the effect of temperature treatment on DNA methylation and phenotypic sex during development in cultured barramundi (Figure 5.2). Juvenile barramundi were treated with hot (34 °C), cold (24 °C) and fluctuating temperatures (24 - 34 °C) during the predicted temperature sensitive period (10-90 days post hatch) and their DNA methylation and gonadal development was analysed at 6 and 12 months post hatch. It was found that at 12 months post hatch, cold temperature treated fish were more male-specific in their DNA methylation patterns, and in some cases not significantly different from adult males. Cold temperature-treated fish also exhibited more advanced gonadal development, with greater proportions of cells in the later stages of spermatogenesis compared to hot, fluctuating and control temperature treatments. While no differences in DNA methylation levels among treatments were found at 6 months post hatch, it was observed more generally, that fish at this

earlier timepoint had either more similar DNA methylation patterns to adult females, or were not significantly differentially methylated from adult females. Thus, it appears that gonadal DNA methylation levels in barramundi sex genes at 6 months post hatch are similar to adult females, but at 12 months post hatch are more similar to adult males. Following this, developmental methylation and/or demethylation processes in barramundi may occur, such that a) methylation patterns originate as more female-specific (high in male-associated genes, low in female-associated genes), b) become more male-specific as fish approach sexual maturity (low in male-associated genes high in female-associated genes) as observed here at 12 months post hatch, and c) methylation patterns likely return to female-specific levels upon male to female sex change (as observed in Chapter 2). It was also noted that there was high variability in methylation levels between amplicons both within the same and between different genes, with DNA methylation in amplicons positioned in final exons notably exhibiting much higher methylation compared to their associated promoter and first exon amplicons. While there was some evidence for compensatory growth in juveniles following the completion of temperature treatment, this compensatory growth was not specifically associated with the cold treatment, where advanced gonadal maturation and differences in methylation were observed. Quantitative PCR of gene expression in *dmrt1* revealed no significant differences in expression, likely due to the assays inability to detect highly sex-specific, alternatively spliced transcripts of this gene, as first identified by Domingos *et al.* (2018) and additionally seen in Chapter 4. In summary, cold temperatures led to advanced gonad development and a delayed effect on DNA methylation in barramundi. While the effect of temperature on phenotypic sex was not as marked as the complete reversal of genetic sex observed in gonochoristic European seabass (Navarro-Martín *et al.*, 2011), tongue sole (Shao *et al.*, 2014) or tilapia (Wang *et al.*, 2019), the significant effects of temperature on DNA methylation in many of the same genes indicates that greater responses may be achieved with more intense temperature treatments. This chapter is the first demonstration of an effect of temperature treatment on development of the primary sex in a sequential hermaphrodite. It revealed not only an effect on sexual development, but also significant changes in DNA methylation of known male- and female-specific markers. While reversal of the primary sex was not achieved, this work provides a foundation on which future work can build towards this goal.

Throughout Chapter 4, the final data chapter, the thesis examined the effect of year-long temperature treatment on DNA methylation, gene expression and sexual development in one-year-old cultured barramundi (Figure 5.2). In this chapter, it was found that hot temperature

treatment (34 °C) leads to advanced gonad maturation, skewing sex ratios towards transitional and female stage fish (80 %) compared to the cold (24 °C; 0 %), fluctuating (24 - 34 °C; 20 %) and control (29 °C; 0 %) treatments. Importantly, the work demonstrates that the effect of temperature on gonadal development in adult barramundi previously reported by Athauda *et al.* (2012) is reproducible. This research further enquired about the mechanism by which temperature can affect sex in fish that have already undergone primary sexual differentiation. Functional analysis of the 8,310 genes differentially expressed between hot and control temperature treatments revealed that many of these genes were involved in biological responses to stimulus, cell signalling and regulatory processes, including methylation, providing some support for a role of calcium and redox signalling in sex specific epigenetic responses to temperature (Castelli *et al.*, 2020). Targeted analysis of sex-related gene expression revealed significant differences in seven of the eight genes examined (*cyp19a1a, foxl2, esr1, nr5a2, dmrt1, sox8* and *sox9,* but not *amh*). However, quantification of DNA methylation of the whole gene body and promotor revealed no significant differences, whereas analysis of more targeted regions revealed significant differences in the male-associated gene *nr5a2* and female-associated gene *foxl2* and single base pair resolution analysis suggests also methylation differences in specific regions along the length of *cyp19a1a* and *dmrt1*. Specifically, these results in experimental barramundi demonstrated patterns of sex-specific alternative splicing similar to previous reports in both farmed and wild-caught individuals (Domingos *et al.*, 2018), as well as revealing additional sex-specific differences in transcription of *cyp19a1a*. These results emphasise that methylation in specific gene and/or regulatory regions is likely more indicative of changes in expression than whole gene averages. It is also important to note that this study investigated 5mC DNA methylation in isolation, and that methylation of other bases, as well as two other classes of epigenetic modification, histone modification and the activity of micro RNAs, often work in combination to lead to changes in gene expression and future research would benefit from the investigation of all three. In this experiment, hot temperature treated barramundi were also significantly larger than cold-treated fish, however, all individuals were substantially smaller than wild-caught females suggesting that size was not the sole or primary contributor to the sexual development outcomes observed here. Overall the data demonstrate that temperature treatment of one-year-old barramundi for a period of one year causes significant alterations in gene expression, changes in patterns of DNA methylation and leads to marked differences in phenotypic sex, similar to those observed by Athauda *et al.* (2012). While further investigation is required to better understand the underlying biological and molecular mechanisms, which may be guided by the results obtained here, temperature

treatment offers an economically viable and environmentally friendly solution to sex control in *L. calcarifer* aquaculture.

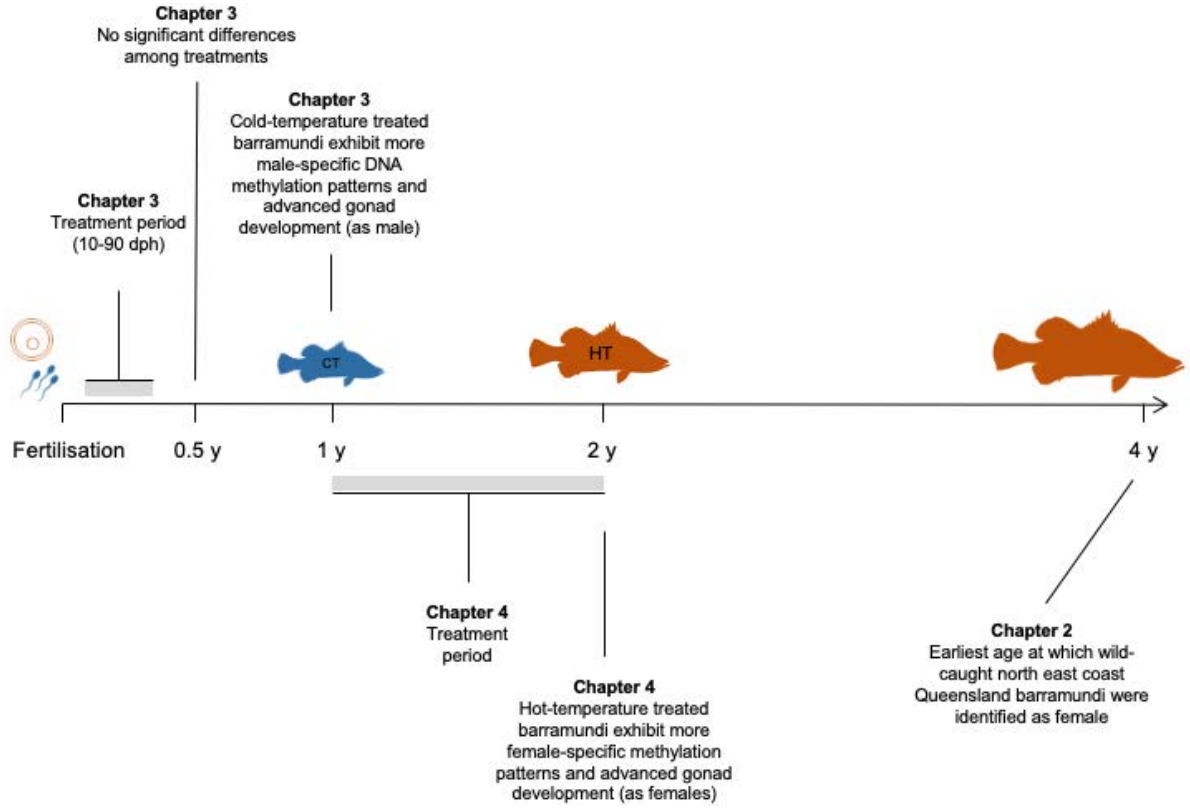

Figure 5.2 Diagram of temperature treatments carried out in Chapter 3 and Chapter 4 in relation to each other including brief description of major results. Time is in years (y), grey boxes indicate temperature treatment periods, colours indicate maleness (blue) and femaleness (orange). Capitalised labels indicate the type of temperature treatment associated with the observed effect, where CT indicates cold temperature treatment (24°C) and HT indicates hot temperature treatment (34°C). Four-year-old wild-caught female from Chapter 2 is included for reference.

5.4 Contributions to a broader understanding

5.4.1 Why do fish change sex?

In the context of evolutionary biology, the most broadly accepted theory on why sex change occurs is the size advantage model (Ghiselin, 1969; Warner, 1975). The model predicts that sex change will evolve when the reproductive success of an individual is size-dependent, and unequal between sexes (Ghiselin, 1969; Warner, 1975). Thus, sex change will occur when an individual attains a body size that confers better reproductive success. Protandry (male-first) often evolves where female functioning is more costly than male functioning and mating is monogamous or near-random (Warner, 1975; 1988b). In the case of barramundi, females are

large and highly fecund, and there is a larger energetic investment into egg production compared to sperm production (Moore, 1982). Furthermore, mating in barramundi occurs through broadcast spawning with little known evidence for social hierarchies, and as such, is thought to be near random (Benvenuto *et al.*, 2017). The research in Chapter 2 found significant differences in total length-at-sex change between barramundi from different regions within the Gulf of Carpentaria, Australia. While environmental correlates of these differences where not uncovered, it was found that barramundi from the mid-northern Gulf of Carpentaria sex change at smaller total lengths and exhibit a slower growth rate compared to other regions. It has previously been shown that barramundi from this region also sexually mature at a faster rate (Davis 1984). Thus, the size advantage model may predict that for barramundi from the northern Gulf of Carpentaria, a smaller size at sexual maturity and sex change confers better reproductive success. In salmonids, it has been established that the relationship between growth and maturity has evolved to maximise the trade-off between reproductive success versus increased mortality, with growth thresholds for maturity exhibiting long term decline due to increased mortality from anthropogenic and/or environmental factors (Siegel *et al.*, 2018). Taking both the theoretical framework of the size advantage model and the applied example from salmon, it follows that in barramundi, fish from the mid-northern Gulf of Carpentaria may be incurring increased mortality risk causing selection pressure on faster maturing and faster sex changing individuals. While this region of the Gulf is remote, and as such is not subject to high fishing pressure, increased mortality may result from predation by saltwater crocodiles, *Crocodylus porosus*, which are the major predator of barramundi and are present in markedly high numbers in the northern Gulf region (Read *et al.*, 2005). The size advantage model may therefore help to explain why barramundi change sex at different lengths between regions, because the reproductive success associated with a given rate of sexual maturation and sex change differs with variation in mortality risk.

The findings here may also contribute to the underlying assumptions of the size-advantage model. Traditionally, the model assumes that size at sex change is genetically determined (Warner *et al.*, 1975; Leigh *et al.*, 1976; Charnov, 1982; Goodman, 1982) and in barramundi, differences in length-at-sex change have been theorised to have a genetic basis as well (Davis, 1984b). However, many species are known to change sex in response to local conditions, leading to criticisms of the model due to its inability to explain changes in sexual phenotypes in the absence or low prevalence of genetic variation (Shapiro, 1987). In the case of barramundi, in Chapter 2 it was found that differences in total length-at-sex change occur in

the absence of substantial population genetic variation (Loughnan *et al.*, 2019). Instead, Chapter 2 revealed that phenotypic variation in length-at-sex change in barramundi was associated with epigenetic variation, specifically with differences in DNA methylation patterns. This research provides the first applied evidence that epigenetic modifications accompany region-specific differences in sex change and, as such, offer additional insight into the biological mechanisms underpinning the theoretical size advantage model.

5.4.2 How do fish change sex?

The research in this thesis builds on our fundamental understanding of mechanisms of sexual development, and how fish change sex. The data presented constitutes the first evidence for an effect of temperature on DNA methylation and sexual phenotype in a sequentially hermaphroditic fish, demonstrating that temperature treatment leads to changes in DNA methylation and advanced sexual development in barramundi. Manipulation of sex by experimental temperature treatment was achieved in both juvenile and one-year-old fish. Specifically, it was observed that cold treatment in juveniles led to advanced gonadal development (as male), and heat treatment in one-year-old fish led to advanced gonadal development (transitional or female; Figure 5.2). While temperature has a known effect on growth performance in barramundi (Katersky and Carter, 2005) and sex change is closely related to body size (Moore, 1979; Davis, 1982; Guiguen *et al.*, 1994), the observed differences in sexual phenotype in both experimental manipulations were unable to be attributed to differences in growth or size alone. In both experimental chapters, differences in phenotypic sex were associated with differences in DNA methylation, suggesting that temperature has a direct effect on DNA methylation and sexual phenotype in barramundi. The research took a focus on two key genes associated with ovary and testis development, *cyp19a1a* and *dmrt1*, respectively, which are heavily studied elsewhere and therefore enable direct comparisons to other studies and broader interpretation and conclusions about the conserved role of epigenetic regulation in sexual development (see Piferrer *et al.*, 2019 for example). However, while *cyp19a1a* and *dmrt1* show a consistent and inverse correlation between methylation and expression in a broad range of species, whether these and other genes implicated in temperature-induced changes in sex respond to temperature directly, or via upstream cellular responses remains unknown (Li and Gui, 2018; Piferrer *et al.*, 2019). Very recently, it has been proposed that redox and calcium levels serve as a direct cellular sensor to environmental temperature and leads to the initiation of ubiquitous signal transduction pathways, influence

epigenetic processes (such as DNA methylation) and regulate sexual phenotype (Castelli *et al.*, 2020). In Chapter 4, analysis of gene sequences differentially expressed between hot temperature treated and control barramundi revealed that many were associated with signal transduction, oxidation-reduction processes and calcium ion binding; including the identification of an enzyme (calcium-dependent, calmodulin-stimulated protein phosphatase) known to play an essential role in the transduction of intracellular calcium ion mediated signals (Kilka *et al.*, 2009; Grigoriu *et al.*, 2013). As such, the findings of Chapter 4 provide support for the newly proposed theory of a role of cellular calcium and redox signalling pathways as transducers of environmental temperature into sex-specific gene regulation, and may assist in the design of future functional studies that further explore this phenomenon. To conclude, the research presented in this thesis reveals, for the first time, that barramundi and likely other sequential hermaphrodites are responsive to temperature treatment during and preceding primary sexual development, exhibiting differential methylation and expression of sex-related genes and altered sexual phenotypes. The work contributes to our rapidly building understanding of environmentally inducible changes in the sexual development of fish, and other vertebrates.

5.5 Future directions

This exploratory work forms a broad foundation on which future research on the relationships between fish length or growth, geographic origin and/or the effects of temperature treatment on DNA methylation, gene expression and phenotypic sex in sequentially hermaphroditic fish can build. Firstly, tracking gene expression and DNA methylation changes in individual barramundi through the process of sex change would enable a greater understanding of the sequence of molecular events that directly precede, accompany and proceed the sex change process, and enable greater understanding to the static data collected from wild-caught fish in Chapter 2. Whilst barramundi do not show externally visible signs of sex change, these samples could be obtained through the collection of routine gonadal cannulation practices in cultured broodstock individuals. The process would benefit even further by using methods such as single-cell RNA-Seq and WGBS to allow discrimination between cellular processes occurring in gametic versus somatic cells, and could be complemented with long-read sequencing such as Oxford Nanopore to uncover not only 5-methyl-cytosine DNA methylation, as detected using the bisulphite sequencing methods employed here, but also N6-methyladenine and 4-methylcytosine. The barramundi gonadal samples could also be subject chromatin remodelling

assessment via chromatin immunoprecipitation and related techniques (see Granada *et al.*, 2018) to allow genome wide mapping of chemical chromatin modifications that occur during sex change. Similar methods have successfully been used to gain insight into the structural organisation of the inactive X chromosome in mammals (Giorgetti *et al.*, 2016). For temperature treatments of barramundi performed in Chapter 3 and Chapter 4, further development should first focus on inducing greater phenotypic effects, directly improving applied outcomes for industry but also offering increased statistical power and greater resolution of the underlying mechanisms leading to the observed and marked phenotypic effects. For example, given that the thermal tolerance of juvenile barramundi ranges from 15 - 40 ˚C (Katersky and Carter, 2007), treatment with a more extreme range of temperatures has potential to yield greater differences in phenotypic sex. Ideally, temperature treatments would approach the thermal minima and maxima for barramundi, and be applied over multiple periods during development to enable identification of the precise thermal sensitive period for this species. This would best enable conclusions on whether to accept or preclude the practicality of producing primary females by temperature treatment in the culture of *L. calcarifer*. Following this, analysis of gene expression and DNA methylation though global methods of RNA-Seq and WGBS, as implemented in Chapter 4 or through single-cell sequencing, would allow identification of any and all of the differentially methylated and expressed genes contributing to the observed changes in sexual phenotype and enable analysis of functional pathways that are upregulated in temperature treated juveniles. Such analyses will likely provide a basis for functional experiments to test the cellular and biochemical reactions that enable temperature to be sensed by cells and tissues and be transduced into biological signals in fish and other ectotherms. The dataset originating from the temperature treatment of one-year-old barramundi could be further exploited to investigate differential methylation patterns across the genome as suggested above. In addition to DNA methylation, the relationship between histone methylation, *dmrt1* expression and sex could also be explored, similar to recent studies in TSD reptiles (Ge *et al.*, 2018). To further develop the technique as a method of sex control for aquaculture, selection for temperature sensitive barramundi and/or transgenerational treatment would likely yield to a greater skew in sex ratios, and smaller, younger females. For example, changes in sex ratio in response to temperature treatment are family-specific in Tilapia (Baroiller *et al.*, 2009a; Baroiller and d'Cotta, 2016; Wessels *et al.*, 2017), and temperature-induced epigenetic modifications and changes in phenotypic sex are known to be passed on from parent to offspring in half-smooth tongue sole (Shao *et al.*, 2014). Beyond barramundi, the data presented in this thesis suggest that temperature may be an

effective method for sex control in other commercially important sequential hermaphrodites, and suggest that the epigenetic mechanisms underlying the plasticity of sex determination and differentiation in fish exhibit similarity, even where systems of sexual development are disparate.

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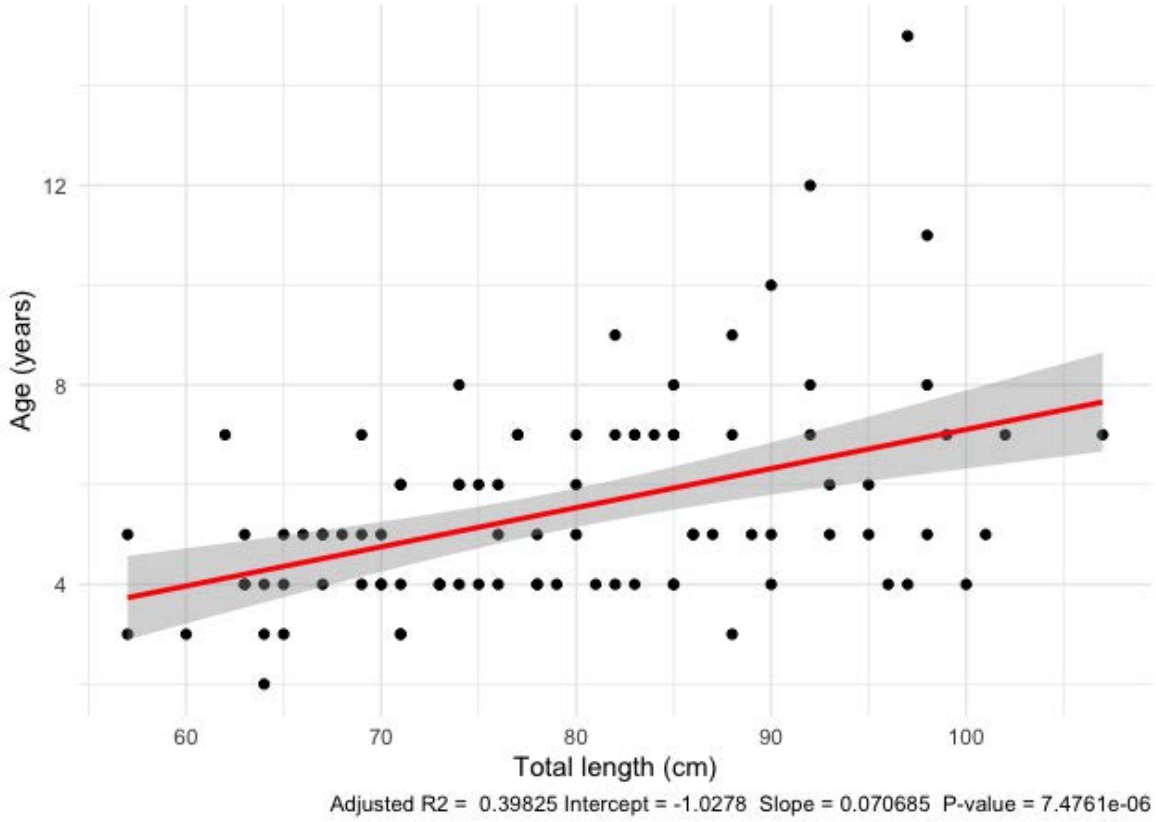
APPENDICES

Appendix A Generalized variance-inflation factors (GVIF) calculated for beta regression model of proportion methylation in gonadal DNA from wild-caught *Lates calcarifer* from Queensland, Australia is modelled for amplicons of four sex-related genes using CpG site, geographic region, age and implementing the logit link function. Larger GVIF values indicate greater levels of multicollinearity. GVIF^(1/(2*Df)) is calculated to allow comparability by reducing the GVIF to a linear measure. Table corresponds to methods reported in Chapter 2.

Amplicon	Covariate	Generalised VIF (GVIF)	Degrees of freedom (Df)	GVIF^(1/(2*Df))
nr5a2	CpG site	1.00595358	1	1.00297237
	Region	1.75316219	2	1.15068254
	Sex	1.35747873	1	1.16510889
	Total length	1.47220899	1	1.2133462
	Age	1.69586818	1	1.30225504
esr1	CpG site	1.00122668	1	1.00061315
	Region	1.73401266	2	1.1475274
	Sex	1.31478924	1	1.14664259
	Total length	1.44123471	1	1.20051435
	Age	1.68235364	1	1.29705576
dmrt1	CpG site	1.000003	1	1.0000015
	Region	1.82879974	2	1.16289777
	Sex	1.35920914	1	1.16585125
	Total length	1.46918985	1	1.21210142
	Age	1.7125676	1	1.30865106
cyp19a1a	CpG site	1.03618795	1	1.01793317
	Region	1.7651271	2	1.15264082
	Sex	1.33384353	1	1.15492144
	Total length	1.44832027	1	1.20346179
	Age	1.6992645	1	1.3035584

Appendix B Model selection table based on the Bayesian information criterion (BIC) where proportion methylation in gonadal DNA from wild-caught *Lates calcarifer* from Queensland, Australia is modelled for amplicons of four sex-related genes using beta regression with CpG site, geographic region and the covariate(s) listed in the table with all possible interaction terms and implementing the logit link function. K is the number of estimated parameters for each model. Lower BIC values indicate a better fit. Table corresponds to methods reported in Chapter 2.

Amplicon	Model covariate(s)	K	Log-likelihood	BIC	Delta BIC	Relative likelihood	BIC weight
nr5a2	Age	23	548.104232	-944.63154	18.275613	0.00010752	9.23E-05
	Total length	23	557.242039	-962.90715	0	1	0.85833473
	Total length and age	38	604.867118	-959.3028	3.60435755	0.16493913	0.14157298
esr1	Age	23	568.262792	-984.94866	176.589863	4.51E-39	4.51E-39
	Total length	23	617.955366	-1084.3338	77.2047156	1.72E-17	1.72E-17
	Total length and age	38	705.984982	-1161.5385	0	1	1
dmrt1	Age	23	1414.28117	-2676.9854	0	1	0.99930036
	Total length	23	1407.01692	-2662.4569	14.5284919	0.00070013	0.00069964
	Total length and age	38	1434.79409	-2619.1567	57.8286651	2.77E-13	2.77E-13
cyp19a1a	Age	23	871.669112	-1591.7613	27.5239397	1.05E-06	1.05E-06
	Total length	23	877.166631	-1602.7563	16.5289024	0.00025751	0.00025744
	Total length and age	38	934.85834	-1619.2852	0	1	0.9997415



Appendix C Linear model of age in *Lates calcarifer* from Queensland, Australia as predicted by total length and geographic region indicates a significant relationship between age and total length. Figure corresponds to methods reported in Chapter 2.

Appendix D Comparison of DNA methylation in amplicons covering partial sex gene and promoter regions in barramundi, *Lates calcarifer*, between three geographic regions in Queensland, Australia. Results are derived from linear hypothesis testing to compare regression coefficients for each region specified in the comparison column based on the beta regression model specified in text and *p* values are corrected based on false discovery rate (FDR) methods of Benjamini & Hochberg (1995). Results show that regression coefficients and therefore methylation levels are significantly different in the mid-northern gulf compared to both other study regions region in male barramundi for all four amplicons investigated and female barramundi for *nr5a2*. Table corresponds to data presented in Chapter 2.

Gene/Amplicon	Sex	Factor	Df	Chi-squared	FDR adjusted *p* value	
cyp19a1a	Both	CpG site	7	1382.784	0.000	***
		Region	2	67.599	0.000	***
		Sex	1	1765.567	0.000	***
		Total length	1	18.754	0.000	***
		CpG site:Sex	7	48.442	0.000	***
		Region:Sex	2	24.817	0.000	***
		Region:Total length	2	3.832	0.147	
		Sex:Total length	1	120.142	0.000	***
		CpG site:Sex:Total length	14	35.452	0.001	**
		Region:Sex:Total length	2	22.802	0.000	***
	Female	CpG site	7	477.027	0.000	***
		Region	2	44.098	0.000	***
		Total length	1	21.395	0.000	***
		Region:Total length	2	9.062	0.011	*
	Male	CpG site	7	958.279	0.000	***
		Region	2	51.193	0.000	***
		Total length	1	191.789	0.000	***
		Region:Total length	2	16.194	0.000	***
esr1	Both	CpG site	7	32.006	0.000	***
		Region	2	37.079	0.000	***
		Sex	1	474.481	0.000	***
		Total length	1	41.435	0.000	***
		CpG site:Sex	7	0.120	1.000	
		Region:Sex	2	29.547	0.000	***
		Region:Total length	2	5.625	0.077	.
		Sex:Total length	1	207.238	0.000	***
		CpG site:Sex:Total length	14	0.290	1.000	
		Region:Sex:Total length	2	8.210	0.023	*
	Female	CpG site	7	10.302	0.194	
		Region	2	36.761	0.000	***
		Total length	1	37.317	0.000	***
		Region:Total length	2	3.758	0.183	
	Male	CpG site	7	24.407	0.002	***
		Region	2	44.241	0.000	***

		Total length	1	364.975	0.000	***
		Region:Total length	2	11.583	0.005	**
nr5a2	Both	CpG site	7	527.011	0.000	***
		Region	2	39.733	0.000	***
		Sex	1	1279.933	0.000	***
		Total length	1	218.559	0.000	***
		CpG site:Sex	7	17.786	0.016	*
		Region:Sex	2	60.546	0.000	***
		Region:Total length	2	44.252	0.000	***
		Sex:Total length	1	81.116	0.000	***
		CpG site:Sex:Total length	14	5.580	0.976	
		Region:Sex:Total length	2	3.879	0.152	
	Female	CpG site	7	1034.809	0.000	***
		Region	2	24.707	0.000	***
		Total length	1	3.853	0.056	*
		Region:Total length	2	21.173	0.000	***
	Male	CpG site	7	134.091	0.000	***
		Region	2	56.186	0.000	***
		Total length	1	262.177	0.000	***
		Region:Total length	2	28.047	0.000	***
dmrt1	Both	CpG site	7	1178.499	0.000	***
		Region	2	41.243	0.000	***
		Sex	1	1306.356	0.000	***
		Total length	1	127.496	0.000	***
		CpG site:Sex	7	13.016	0.081	.
		Region:Sex	2	105.312	0.000	***
		Region:Total length	2	27.070	0.000	***
		Sex:Total length	1	49.981	0.000	***
		CpG site:Sex:Total length	14	5.511	0.977	
		Region:Sex:Total length	2	0.714	0.741	
	Female	CpG site	7	987.945	0.000	***
		Region	2	98.161	0.000	***
		Total length	1	5.935	0.018	*
		Region:Total length	2	17.530	0.000	***
	Male	CpG site	7	349.991	0.000	***
		Region	2	61.046	0.000	***
		Total length	1	203.301	0.000	***
		Region:Total length	2	11.730	0.004	**

Appendix E Beta-regression models for methylation levels in Queensland barramundi, *Lates calcarifer*. Final models were subset be gene and sex, using the formula: Methylation ~ CpG site + region + total length + region :total length and logit link. Table corresponds to data presented in Chapter 2.

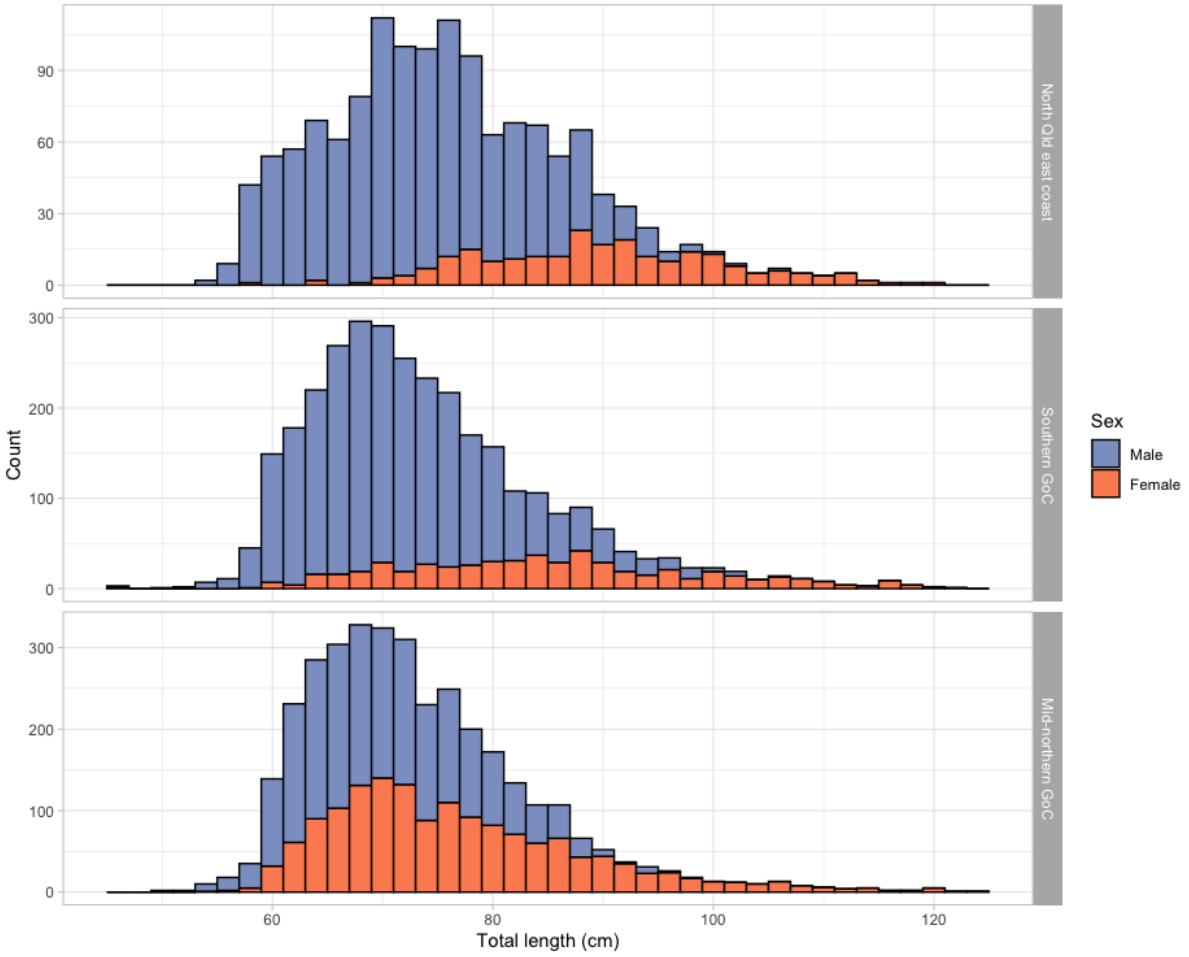
Gene	Sex	Beta	Level	Coefficient		Z value	FDR Adjusted *p* value
cyp19a1a	Female	(Intercept)	-83	3.442	3.569	0.001	***
		CpG site	-52	0.860	8.839	0.000	***
			-48	0.573	5.903	0.000	***
			-31	0.446	4.589	0.000	***
			28	1.340	13.454	0.000	***
			38	0.992	10.161	0.000	***
			92	1.701	16.491	0.000	***
			106	1.613	15.792	0.000	***
		Region	Mid-northern Gulf of Carpentaria	-3.409	-3.403	0.001	**
			Southern Gulf of Carpentaria	-3.414	-3.407	0.001	**
		Total length	Variable	-0.041	-3.980	0.000	***
		Region:Total length	Mid-northern Gulf of Carpentaria: variable	0.031	2.860	0.006	**
			Southern Gulf of Carpentaria: variable	0.032	2.983	0.004	**
		Total length + Region:Total length	Variable + Mid-northern Gulf of Carpentaria: variable	-0.010			
		Total length + Region:Total length	Variable + Southern Gulf of Carpentaria: variable	-0.009			
Pseudo R2: 0.66							
	Male	(Intercept)	-83	-1.797	-7.094	0.000	***
		CpG site	-52	0.946	11.669	0.000	***
			-48	0.384	5.197	0.000	***
			-31	0.221	3.063	0.003	**
			28	1.911	18.891	0.000	***
			38	1.324	15.079	0.000	***
			92	2.049	19.566	0.000	***
			106	1.899	18.826	0.000	***
		Region	Mid-northern Gulf of Carpentaria	1.697	3.964	0.000	***
			Southern Gulf of Carpentaria	-0.052	-0.133	0.908	
		Total length	Variable	0.037	11.320	0.000	***
		Region:Total length	Mid-northern Gulf of Carpentaria: variable	-0.022	-3.952	0.000	***
			Southern Gulf of Carpentaria: variable	-0.004	-0.745	0.585	

		Total length + Region:Total length	Variable + Mid-northern Gulf of Carpentaria: variable	0.015			
		Total length + Region:Total length	Variable + Southern Gulf of Carpentaria: variable	0.033			
Pseudo R2: 0.76							
Esr1	Female	(Intercept)	-71	4.841	3.319	0.005	**
		CpG site	-41	0.259	1.776	0.156	
			-36	0.254	1.739	0.164	
			-29	0.299	2.048	0.096	.
			-20	0.360	2.454	0.040	*
			-18	0.337	2.300	0.057	.
			8	0.400	2.722	0.019	*
			18	0.359	2.447	0.040	*
		Region	Mid-northern Gulf of Carpentaria	-3.006	-1.986	0.103	
			Southern Gulf of Carpentaria	-3.388	-2.239	0.061	.
		Total length	Variable	-0.048	-3.074	0.009	**
		Region:Total length	Mid-northern Gulf of Carpentaria: variable	0.024	1.465	0.277	
			Southern Gulf of Carpentaria: variable	0.030	1.870	0.131	
		Total length + Region:Total length	Variable + Mid-northern Gulf of Carpentaria: variable	-0.024			
		Total length + Region:Total length	Variable + Southern Gulf of Carpentaria: variable	-0.017			
Pseudo R2: 0.18							
	Male	(Intercept)	-71	-3.181	-10.470	0.000	***
		CpG site	-41	0.311	2.961	0.011	*
			-36	0.317	3.017	0.009	**
			-29	0.347	3.296	0.005	**
			-20	0.405	3.820	0.001	**
			-18	0.374	3.542	0.002	**
			8	0.435	4.084	0.000	***
			18	0.381	3.605	0.002	**
		Region	Mid-northern Gulf of Carpentaria	1.854	3.591	0.002	**
			Southern Gulf of Carpentaria	-0.231	-0.484	0.999	
		Total length	Variable	0.058	14.626	0.000	***
		Region:Total length	Mid-northern Gulf of Carpentaria: variable	-0.023	-3.240	0.005	**
			Southern Gulf of Carpentaria: variable	-0.001	-0.157	0.999	

		Total length + Region:Total length	Variable + Mid-northern Gulf of Carpentaria: variable	0.035			
		Total length + Region:Total length	Variable + Southern Gulf of Carpentaria: variable	0.057			
Pseudo R2: 0.52							
nr5a2	Female	(Intercept)	-30	1.401	2.360	0.055	.
		CpG site	-19	0.519	8.445	0.000	***
			20	0.027	0.440	0.987	
			32	0.010	0.161	0.987	
			44	-0.440	-6.957	0.000	***
			50	0.760	12.244	0.000	***
			77	0.984	15.592	0.000	***
			92	1.112	17.418	0.000	***
		Region	Mid-northern Gulf of Carpentaria	-2.109	-3.412	0.003	**
			Southern Gulf of Carpentaria	-1.060	-1.714	0.204	
		Total length	Variable	-0.017	-2.603	0.029	*
		Region:Total length	Mid-northern Gulf of Carpentaria: variable	0.022	3.190	0.005	**
			Southern Gulf of Carpentaria: variable	0.009	1.347	0.379	
		Total length + Region:Total length	Variable + Mid-northern Gulf of Carpentaria: variable	0.005			
		Total length + Region:Total length	Variable + Southern Gulf of Carpentaria: variable	-0.008			
Pseudo R2: 0.82							
	Male	(Intercept)	-30	2.940	9.176	0.000	***
		CpG site	-19	0.318	2.749	0.020	*
			20	0.034	0.287	0.987	
			32	0.028	0.236	0.987	
			44	-0.272	-2.222	0.072	.
			50	0.515	4.522	0.000	***
			77	0.650	5.749	0.000	***
			92	0.719	6.386	0.000	***
		Region	Mid-northern Gulf of Carpentaria	-3.092	-5.707	0.000	***
			Southern Gulf of Carpentaria	-0.556	-1.121	0.494	
		Total length	Variable	-0.058	-14.069	0.000	***
		Region:Total length	Mid-northern Gulf of Carpentaria: variable	0.038	5.296	0.000	***
			Southern Gulf of Carpentaria: variable	0.012	1.857	0.155	

		Total length + Region:Total length	Variable + Mid-northern Gulf of Carpentaria: variable	-0.020			
		Total length + Region:Total length	Variable + Southern Gulf of Carpentaria: variable	-0.046			
Pseudo R2: 0.48							
dmrt1	Female	(Intercept)	-5	-0.210	-0.395	0.880	
		CpG site	8	-0.383	-4.569	0.000	***
			32	1.087	16.488	0.000	***
			39	0.745	10.896	0.000	***
			77	0.918	13.700	0.000	***
			97	1.003	15.098	0.000	***
			111	-0.051	-0.653	0.771	
			142	-0.044	-0.571	0.798	
		Region	Mid-northern Gulf of Carpentaria	-2.557	-4.570	0.000	***
			Southern Gulf of Carpentaria	-1.904	-3.398	0.002	**
		Total length	Variable	-0.022	-3.850	0.000	***
		Region:Total length	Mid-northern Gulf of Carpentaria: variable	0.024	3.919	0.000	***
			Southern Gulf of Carpentaria: variable	0.016	2.621	0.028	*
		Total length + Region:Total length	Variable + Mid-northern Gulf of Carpentaria: variable	0.002			
		Total length + Region:Total length	Variable + Southern Gulf of Carpentaria: variable	-0.006			
Pseudo R2: 0.81							
	Male	(Intercept)	-5	-0.510	-2.042	0.109	
		CpG site	7	-0.331	-3.014	0.008	**
			31	0.886	9.899	0.000	***
			38	0.562	6.012	0.000	***
			76	0.658	7.130	0.000	***
			96	0.696	7.585	0.000	***
			110	-0.129	-1.223	0.443	
			141	-0.183	-1.712	0.198	
		Region	Mid-northern Gulf of Carpentaria	-1.757	-3.956	0.000	***
			Southern Gulf of Carpentaria	-0.402	-1.054	0.551	
		Total length	Variable	-0.038	-11.996	0.000	***
		Region:Total length	Mid-northern Gulf of Carpentaria: variable	0.020	3.369	0.003	**
			Southern Gulf of Carpentaria: variable	0.009	1.731	0.197	

	Total length + Region:Total length		Variable + Mid-northern Gulf of Carpentaria: variable	-0.018
	Total length + Region:Total length		Variable + Southern Gulf of Carpentaria: variable	-0.030
Pseudo R2: 0.60				



Appendix F Length frequency distribution of male and female *Lates calcarifer* collected from three regions in Queensland (Qld), Australia from 2000-2015 showing that female barramundi of shorter length classes are more frequent in the Mid-northern Gulf of Carpentaria (GoC) compared to the north Qld east coast and southern GoC. Figure corresponds to data presented in Chapter 2.

Appendix G Mean (±SD) proportion of DNA methylation values by amplicon for *Lates calcarifer* at six and twelve months post hatch (mph) following treatment from 10-90 days post hatch obtained using bisulphite amplicon sequencing, as depicted by coloured heatmaps in figures two through eight. Control: 29 °C, CT: cold temperature; 24 °C, FT: fluctuating temperature 24 - 34 °C, HT: hot temperature; 34 °C. Adult male and female methylation values from Chapter 2 and Domingos *et al.* (2018) are included for reference. Table corresponds to data presented in Chapter 3.

Fish age	**Treatment**	***amh E7***	***amh PE1***	***cyp19a1a E8***	***cyp19a1a PE1***	***dmrt1 E5***	***dmrt1 P***	***dmrt1 PE1***	***esr1 PE1***	***foxl2 E1.1***	***foxl2 E1.2***	***foxl2 P***	***nr5a2 P***	***nr5a2 PE1***	***sox9 PE1***
12 mph	Control	0.9165 (0.0571)	0.6829 (0.1114)	0.9511 (0.041)	0.8695 (0.1076)	0.9566 (0.0286)	NA	0.0385 (0.0351)	0.6932 (0.1025)	0.0089 (0.0137)	NA	NA	0.0981 (0.0718)	0.3964 (0.1304)	0.0055 (0.0095)
12 mph	CT	0.9339 (0.0486)	0.747 (0.1206)	0.9546 (0.043)	0.9092 (0.0802)	0.9609 (0.0163)	NA	0.0387 (0.0396)	0.8184 (0.0784)	0.0126 (0.0308)	NA	NA	0.0937 (0.0776)	0.2889 (0.1474)	0.0064 (0.0193)
12 mph	FT	0.9357 (0.0358)	0.6489 (0.113)	0.9503 (0.0503)	0.8876 (0.1003)	0.9594 (0.0287)	NA	0.0406 (0.0375)	0.6272 (0.1898)	0.0054 (0.0055)	NA	NA	0.0789 (0.0549)	0.3353 (0.1526)	0.0033 (0.0033)
12 mph	HT	0.927 (0.0378)	0.685 (0.1334)	0.9559 (0.0344)	0.8816 (0.1087)	0.9643 (0.0138)	NA	0.0301 (0.0225)	0.7213 (0.1301)	0.0067 (0.0061)	NA	NA	0.0872 (0.0565)	0.3128 (0.1545)	0.0048 (0.0037)
6 mph	Control	NA	0.551 (0.1418)	NA	0.808 (0.1211)	NA	0.5274 (0.3259)	0.0565 (0.0452)	0.5319 (0.1599)	NA	0.0148 (0.0098)	0.0064 (0.0024)	0.1212 (0.069)	0.4641 (0.1297)	0.0064 (0.0042)
6 mph	CT	NA	0.5112 (0.0633)	NA	0.8075 (0.1226)	NA	0.594 (0.3027)	0.0621 (0.0819)	0.5498 (0.0872)	NA	0.013 (0.0112)	0.0095 (0.0182)	0.148 (0.1044)	0.4831 (0.1263)	0.006 (0.0047)
6 mph	FT	NA	0.5655 (0.0826)	NA	0.8186 (0.1144)	NA	0.5571 (0.3177)	0.068 (0.0512)	0.5204 (0.0875)	NA	0.0155 (0.0075)	0.0081 (0.0044)	0.1259 (0.0669)	0.4772 (0.1291)	0.0069 (0.0031)
6 mph	HT	NA	0.472 (0.0639)	NA	0.8164 (0.1243)	NA	0.5673 (0.3494)	0.0673 (0.0577)	0.46 (0.0689)	NA	0.0129 (0.0074)	0.0069 (0.0048)	0.1126 (0.0617)	0.4551 (0.1321)	0.005 (0.003)
Adult	Female	NA	0.4987 (0.0567)	NA	0.625 (0.1836)	NA	0.7678 (0.1582)	0.1537 (0.094)	0.6492 (0.1956)	NA	0.087 (0.025)	0.0391 (0.0172)	0.2063 (0.0505)	0.5602 (0.1321)	0.0128 (0.0071)
Adult	Male	NA	0.7564 (0.1556)	NA	0.8723 (0.1155)	NA	0.6905 (0.2401)	0.0516 (0.0576)	0.8167 (0.1209)	NA	0.1005 (0.0327)	0.074 (0.0554)	0.1071 (0.0363)	0.2254 (0.1562)	0.0109 (0.0064)

Appendix H Pair-wise comparisons of mean DNA methylation using Tukey's Honest Significant Difference and Bonferroni correction following ANOVA of logit transformed data for *Lates calcarifer* at six and twelve months post hatch (mph) following treatment from 10-90 days post hatch obtained using bisulphite amplicon sequencing, as depicted by compact letter display in heatmaps in figures two through eight. Control: 29 °C, CT: cold temperature; 24 °C, FT: fluctuating temperature 24 - 34 °C, HT: hot temperature; 34 °C. Adult male and female methylation values from Chapter 2 and Domingos *et al.* (2018) are included for reference. Table corresponds to data presented in Chapter 3.

Amplicon	Comparison	Estimate	Std. error	T-value	*p* value	
amh E7	12 mph control - 12 mph CT	-0.3297	0.0893	-3.691	0.001459146	**
	12 mph control - 12 mph FT	-0.253	0.0893	-2.8324	0.024568077	*
	12 mph FT - 12 mph CT	-0.0767	0.0893	-0.8586	0.826054219	
	12 mph HT - 12 mph control	0.0688	0.0865	0.7956	0.856387108	
	12 mph HT - 12 mph CT	-0.2609	0.0865	-3.0165	0.014089948	*
	12 mph HT - 12 mph FT	-0.1842	0.0865	-2.1297	0.144796479	
amh PE1	12 mph control - 12 mph CT	-0.4052	0.1225	-3.3075	0.033354076	*
	12 mph control - 12 mph FT	0.1584	0.1225	1.2931	0.954149691	
	12 mph FT - 12 mph CT	-0.5637	0.1225	-4.6006	0.000228745	***
	12 mph HT - 12 mph control	0.0503	0.1225	0.4103	0.999994497	
	12 mph HT - 12 mph CT	-0.355	0.1225	-2.8972	0.108637557	
	12 mph HT - 12 mph FT	0.2087	0.1225	1.7034	0.791083256	
	6 mph control - 12 mph control	-0.5818	0.1225	-4.7484	0.000119178	***
	6 mph control - 12 mph CT	-0.987	0.1225	-8.0559	7.05E-14	***
	6 mph control - 12 mph FT	-0.4234	0.1225	-3.4553	0.020225732	*
	6 mph control - 12 mph HT	-0.6321	0.1225	-5.1587	1.79E-05	***
	6 mph control - 6 mph CT	0.1844	0.1225	1.5048	0.888226496	
	6 mph control - 6 mph FT	-0.038	0.1225	-0.3102	0.999999526	
	6 mph CT - 12 mph control	-0.7662	0.1225	-6.2532	1.49E-07	***
	6 mph CT - 12 mph CT	-1.1714	0.1225	-9.5607	0	***
	6 mph CT - 12 mph FT	-0.6077	0.1225	-4.9601	3.96E-05	***
	6 mph CT - 12 mph HT	-0.8164	0.1225	-6.6635	1.31E-09	***
	6 mph FT - 12 mph control	-0.5438	0.1225	-4.4382	0.000468883	***
	6 mph FT - 12 mph CT	-0.949	0.1225	-7.7457	4.37E-13	***
	6 mph FT - 12 mph FT	-0.3853	0.1225	-3.1451	0.054405468	.
	6 mph FT - 12 mph HT	-0.5941	0.1225	-4.8485	8.41E-05	***
	6 mph FT - 6 mph CT	0.2224	0.1225	1.815	0.722447168	
	6 mph HT - 12 mph control	-0.9264	0.1225	-7.5613	3.13E-12	***
	6 mph HT - 12 mph CT	-1.3317	0.1225	-10.8688	0	***
	6 mph HT - 12 mph FT	-0.768	0.1225	-6.2682	5.28E-08	***
	6 mph HT - 12 mph HT	-0.9767	0.1225	-7.9716	1.12E-13	***
	6 mph HT - 6 mph control	-0.3446	0.1225	-2.8129	0.132984912	
	6 mph HT - 6 mph CT	-0.1603	0.1225	-1.3081	0.950660963	
	6 mph HT - 6 mph FT	-0.3827	0.1225	-3.1231	0.058447171	.

amh PE1	adult female - 12 mph control	-0.8181	0.1522	-5.3743	3.60E-06	***
	adult female - 12 mph CT	-1.2233	0.1522	-8.0366	1.21E-13	***
	adult female - 12 mph FT	-0.6596	0.1522	-4.3335	0.000675697	***
	adult female - 12 mph HT	-0.8683	0.1522	-5.7046	5.99E-07	***
	adult female - 6 mph control	-0.2363	0.1522	-1.5523	0.868234189	
	adult female - 6 mph CT	-0.0519	0.1522	-0.3411	0.999998908	
	adult female - 6 mph FT	-0.2743	0.1522	-1.802	0.730831244	
	adult female - 6 mph HT	0.1084	0.1522	0.7118	0.999423172	
	adult male - 12 mph control	0.665	0.1357	4.899	5.42E-05	***
	adult male - 12 mph CT	0.2597	0.1357	1.9134	0.656537712	
	adult male - 12 mph FT	0.8234	0.1357	6.0662	5.77E-08	***
	adult male - 12 mph HT	0.6147	0.1357	4.5286	0.000346913	***
	adult male - 6 mph control	1.2467	0.1357	9.1852	0	***
	adult male - 6 mph CT	1.4311	0.1357	10.5435	0	***
	adult male - 6 mph FT	1.2087	0.1357	8.9052	1.11E-16	***
	adult male - 6 mph HT	1.5914	0.1357	11.7243	0	***
	adult male - adult female	1.483	0.163	9.0961	0	***
cyp19a1a E8	12 mph control - 12 mph CT	-0.1464	0.1632	-0.8974	0.806207492	
	12 mph control - 12 mph FT	-0.0704	0.1632	-0.4317	0.972989015	
	12 mph FT - 12 mph CT	-0.076	0.1632	-0.4657	0.966459825	
	12 mph HT - 12 mph control	-0.0308	0.1632	-0.1889	0.99760259	
	12 mph HT - 12 mph CT	-0.1773	0.1632	-1.0863	0.698213575	
	12 mph HT - 12 mph FT	-0.1013	0.1632	-0.6206	0.925422002	
cyp19a1a PE1	12 mph control - 12 mph CT	-0.4546	0.1708	-2.6624	0.185903054	
	12 mph control - 12 mph FT	-0.2342	0.1708	-1.3718	0.932622236	
	12 mph FT - 12 mph CT	-0.2204	0.1708	-1.2906	0.953571242	
	12 mph HT - 12 mph control	0.1533	0.1708	0.8977	0.996317822	
	12 mph HT - 12 mph CT	-0.3013	0.1708	-1.7647	0.74997157	
	12 mph HT - 12 mph FT	-0.0809	0.1708	-0.474	0.999980196	
	6 mph control - 12 mph control	-0.6303	0.1708	-3.6915	0.009050608	**
	6 mph control - 12 mph CT	-1.085	0.1708	-6.3539	5.34E-09	***
	6 mph control - 12 mph FT	-0.8646	0.1708	-5.0633	2.08E-05	***
	6 mph control - 12 mph HT	-0.7836	0.1708	-4.5893	0.000241686	***
	6 mph control - 6 mph CT	-0.0073	0.1708	-0.043	1	
	6 mph control - 6 mph FT	-0.067	0.1708	-0.3924	0.999996148	
	6 mph CT - 12 mph control	-0.623	0.1708	-3.6485	0.010137585	*
	6 mph CT - 12 mph CT	-1.0776	0.1708	-6.3109	4.75E-08	***
	6 mph CT - 12 mph FT	-0.8572	0.1708	-5.0203	2.76E-05	***
	6 mph CT - 12 mph HT	-0.7763	0.1708	-4.5462	0.00028497	***
	6 mph FT - 12 mph control	-0.5633	0.1708	-3.2992	0.032961592	*
	6 mph FT - 12 mph CT	-1.018	0.1708	-5.9616	6.71E-08	***
	6 mph FT - 12 mph FT	-0.7976	0.1708	-4.6709	0.000162692	***

	6 mph FT - 12 mph HT	-0.7166	0.1708	-4.1969	0.001281773	**
	6 mph FT - 6 mph CT	0.0597	0.1708	0.3493	0.999998599	
	6 mph HT - 12 mph control	-0.5402	0.1708	-3.1636	0.049586454	*
	6 mph HT - 12 mph CT	-0.9948	0.1708	-5.826	1.62E-07	***
	6 mph HT - 12 mph FT	-0.7744	0.1708	-4.5354	0.000234735	***
	6 mph HT - 12 mph HT	-0.6935	0.1708	-4.0613	0.002152995	**
	6 mph HT - 6 mph control	0.0901	0.1708	0.5279	0.999950256	
	6 mph HT - 6 mph CT	0.0828	0.1708	0.4849	0.999975898	
	6 mph HT - 6 mph FT	0.0231	0.1708	0.1355	1	
	adult female - 12 mph control	-1.6683	0.1767	-9.4388	0	***
	adult female - 12 mph CT	-2.1229	0.1767	-12.0109	0	***
	adult female - 12 mph FT	-1.9025	0.1767	-10.764	0	***
	adult female - 12 mph HT	-1.8215	0.1767	-10.3061	0	***
	adult female - 6 mph control	-1.0379	0.1767	-5.8724	2.97E-07	***
	adult female - 6 mph CT	-1.0453	0.1767	-5.914	1.58E-07	***
	adult female - 6 mph FT	-1.1049	0.1767	-6.2515	2.30E-08	***
	adult female - 6 mph HT	-1.1281	0.1767	-6.3824	4.67E-09	***
	adult male - 12 mph control	0.0169	0.1369	0.1231	1	
	adult male - 12 mph CT	-0.4378	0.1369	-3.1975	0.044929897	*
	adult male - 12 mph FT	-0.2174	0.1369	-1.5878	0.848804205	
	adult male - 12 mph HT	-0.1364	0.1369	-0.9965	0.991997209	
	adult male - 6 mph control	0.6472	0.1369	4.7273	0.000151442	***
	adult male - 6 mph CT	0.6398	0.1369	4.6736	0.000153747	***
	adult male - 6 mph FT	0.5802	0.1369	4.2379	0.001091646	**
	adult male - 6 mph HT	0.5571	0.1369	4.0689	0.002047015	**
	adult male - adult female	1.6851	0.1443	11.6769	0	***
dmrt1 E5	12 mph control - 12 mph CT	-0.0701	0.0571	-1.2276	0.608667815	
	12 mph control - 12 mph FT	-0.1101	0.062	-1.7774	0.284563307	
	12 mph FT - 12 mph CT	0.0401	0.0607	0.6601	0.911665185	
	12 mph HT - 12 mph control	0.1406	0.0644	2.1836	0.128776579	
	12 mph HT - 12 mph CT	0.0705	0.0632	1.1163	0.678840539	
	12 mph HT - 12 mph FT	0.0304	0.0676	0.4502	0.969461617	
dmrt1 P	6 mph control - 6 mph CT	-0.4148	0.3738	-1.1098	0.876643466	
	6 mph control - 6 mph FT	-0.1973	0.3738	-0.5278	0.994997725	
	6 mph FT - 6 mph CT	-0.2176	0.3738	-0.582	0.992109813	
	6 mph HT - 6 mph control	0.2353	0.3738	0.6295	0.988687128	
	6 mph HT - 6 mph CT	-0.1795	0.3738	-0.4803	0.996792981	
	6 mph HT - 6 mph FT	0.038	0.3738	0.1017	0.999998454	
	adult female - 6 mph control	1.3786	0.4	3.4467	0.00873707	**
	adult female - 6 mph CT	0.9638	0.4	2.4096	0.156781105	
	adult female - 6 mph FT	1.1814	0.4	2.9535	0.039975466	*
	adult female - 6 mph HT	1.1433	0.4	2.8585	0.052060365	.

	adult male - 6 mph control	1.0018	0.3579	2.7995	0.060788902	.
	adult male - 6 mph CT	0.587	0.3579	1.6403	0.571948165	
	adult male - 6 mph FT	0.8045	0.3579	2.2482	0.219460722	
	adult male - 6 mph HT	0.7665	0.3579	2.1419	0.26899714	
	adult male - adult female	-0.3768	0.3852	-0.9784	0.924211253	
dmrt1 PE1	12 mph control - 12 mph CT	0.1428	0.1423	1.0038	0.991605688	
	12 mph control - 12 mph FT	-0.0794	0.1429	-0.5556	0.999924154	
	12 mph FT - 12 mph CT	0.2222	0.1403	1.5844	0.850157763	
	12 mph HT - 12 mph control	-0.0847	0.1463	-0.579	0.999892922	
	12 mph HT - 12 mph CT	0.0581	0.1437	0.4041	0.999995057	
	12 mph HT - 12 mph FT	-0.1641	0.1444	-1.1369	0.979812941	
	6 mph control - 12 mph control	0.4632	0.1301	3.561	0.013164018	*
	6 mph control - 12 mph CT	0.606	0.1272	4.7657	0.000115937	***
	6 mph control - 12 mph FT	0.3838	0.1278	3.0018	0.077603334	.
	6 mph control - 12 mph HT	0.5479	0.1317	4.1611	0.001273856	**
	6 mph control - 6 mph CT	0.0087	0.121	0.0718	1	
	6 mph control - 6 mph FT	-0.2854	0.1102	-2.5907	0.216159348	
	6 mph CT - 12 mph control	0.4545	0.1368	3.3232	0.029844086	*
	6 mph CT - 12 mph CT	0.5973	0.134	4.4577	0.000348575	***
	6 mph CT - 12 mph FT	0.3751	0.1347	2.7856	0.137345117	
	6 mph CT - 12 mph HT	0.5392	0.1383	3.8992	0.004099899	**
	6 mph FT - 12 mph control	0.7486	0.1273	5.8792	8.63E-08	***
	6 mph FT - 12 mph CT	0.8914	0.1243	7.1686	7.36E-12	***
	6 mph FT - 12 mph FT	0.6692	0.1251	5.351	2.21E-06	***
	6 mph FT - 12 mph HT	0.8333	0.129	6.4615	1.48E-09	***
	6 mph FT - 6 mph CT	0.2941	0.118	2.4923	0.26616188	
	6 mph HT - 12 mph control	0.6938	0.1282	5.413	2.16E-06	***
	6 mph HT - 12 mph CT	0.8366	0.1252	6.6815	3.14E-10	***
	6 mph HT - 12 mph FT	0.6144	0.1259	4.8794	4.99E-05	***
	6 mph HT - 12 mph HT	0.7786	0.1298	5.9979	4.94E-08	***
	6 mph HT - 6 mph control	0.2307	0.1111	2.0752	0.53546633	
	6 mph HT - 6 mph CT	0.2393	0.1189	2.0127	0.581015423	
	6 mph HT - 6 mph FT	-0.0547	0.1079	-0.5073	0.999964957	
	adult female - 12 mph control	1.7821	0.1402	12.7064	0	***
	adult female - 12 mph CT	1.9249	0.1376	13.994	0	***
	adult female - 12 mph FT	1.7027	0.1382	12.3209	0	***
	adult female - 12 mph HT	1.8668	0.1417	13.1707	0	***
	adult female - 6 mph control	1.3189	0.1249	10.5614	0	***
	adult female - 6 mph CT	1.3276	0.1318	10.0698	0	***
	adult female - 6 mph FT	1.0335	0.122	8.4699	4.44E-16	***
	adult female - 6 mph HT	1.0882	0.1229	8.8541	0	***
	adult male - 12 mph control	0.3238	0.1156	2.8014	0.131588829	

dmrt1 PE1	adult male - 12 mph CT	0.4666	0.1123	4.1551	0.00140406	**
	adult male - 12 mph FT	0.2444	0.1131	2.1613	0.474641505	
	adult male - 12 mph HT	0.4085	0.1174	3.4801	0.017120072	*
	adult male - 6 mph control	-0.1394	0.0964	-1.4461	0.90884412	
	adult male - 6 mph CT	-0.1307	0.1052	-1.2418	0.963747085	
	adult male - 6 mph FT	-0.4248	0.0926	-4.5855	0.00020925	***
	adult male - 6 mph HT	-0.37	0.0938	-3.9448	0.003166865	**
	adult male - adult female	-1.4582	0.1097	-13.2906	0	***
esr1 PE1	12 mph control - 12 mph CT	-0.7388	0.1367	-5.4035	2.50E-06	***
	12 mph control - 12 mph FT	0.2862	0.1367	2.0933	0.52235066	
	12 mph FT - 12 mph CT	-1.0251	0.1367	-7.4968	1.31E-12	***
	12 mph HT - 12 mph control	0.2293	0.1367	1.6767	0.800929399	
	12 mph HT - 12 mph CT	-0.5096	0.1367	-3.7267	0.007796445	**
	12 mph HT - 12 mph FT	0.5155	0.1367	3.7701	0.006558256	**
	6 mph control - 12 mph control	-0.7097	0.1415	-5.0145	2.76E-05	***
	6 mph control - 12 mph CT	-1.4485	0.1415	-10.2348	0	***
	6 mph control - 12 mph FT	-0.4235	0.1415	-2.9921	0.081548632	.
	6 mph control - 12 mph HT	-0.939	0.1415	-6.6344	4.26E-09	***
	6 mph control - 6 mph CT	-0.0483	0.1521	-0.3175	0.999999388	
	6 mph control - 6 mph FT	0.0734	0.1462	0.5023	0.999966934	
	6 mph CT - 12 mph control	-0.6614	0.1477	-4.4783	0.000321191	***
	6 mph CT - 12 mph CT	-1.4002	0.1477	-9.481	0	***
	6 mph CT - 12 mph FT	-0.3752	0.1477	-2.5403	0.241336961	
	6 mph CT - 12 mph HT	-0.8907	0.1477	-6.0307	1.01E-07	***
	6 mph FT - 12 mph control	-0.7831	0.1415	-5.5332	1.55E-06	***
	6 mph FT - 12 mph CT	-1.522	0.1415	-10.7535	0	***
	6 mph FT - 12 mph FT	-0.4969	0.1415	-3.5109	0.01653522	*
	6 mph FT - 12 mph HT	-1.0124	0.1415	-7.1531	1.75E-11	***
	6 mph FT - 6 mph CT	-0.1217	0.1521	-0.8001	0.998468295	
	6 mph HT - 12 mph control	-1.0304	0.1477	-6.9769	6.37E-11	***
	6 mph HT - 12 mph CT	-1.7692	0.1477	-11.9795	0	***
	6 mph HT - 12 mph FT	-0.7442	0.1477	-5.0388	1.30E-05	***
	6 mph HT - 12 mph HT	-1.2597	0.1477	-8.5292	3.66E-15	***
	6 mph HT - 6 mph control	-0.3207	0.1521	-2.1078	0.511922307	
	6 mph HT - 6 mph CT	-0.369	0.1579	-2.3372	0.356956397	
	6 mph HT - 6 mph FT	-0.2473	0.1521	-1.6253	0.828702531	
	adult female - 12 mph control	-0.103	0.1415	-0.7279	0.999277877	
	adult female - 12 mph CT	-0.8419	0.1415	-5.9482	6.11E-08	***
	adult female - 12 mph FT	0.1832	0.1415	1.2944	0.952285324	
	adult female - 12 mph HT	-0.3323	0.1415	-2.3478	0.349781172	
	adult female - 6 mph control	0.6067	0.1462	4.1505	0.001358936	**
	adult female - 6 mph CT	0.5584	0.1521	3.6701	0.00911321	**

esr1 PE1	adult female - 6 mph FT	0.6801	0.1462	4.6527	0.000163403	***
	adult female - 6 mph HT	0.9274	0.1521	6.0955	1.83E-07	***
	adult male - 12 mph control	0.8941	0.1096	8.1555	1.73E-14	***
	adult male - 12 mph CT	0.1553	0.1096	1.4162	0.918166612	
	adult male - 12 mph FT	1.1803	0.1096	10.7664	0	***
	adult male - 12 mph HT	0.6648	0.1096	6.0643	3.04E-08	***
	adult male - 6 mph control	1.6038	0.1156	13.8785	0	***
	adult male - 6 mph CT	1.5555	0.123	12.6439	0	***
	adult male - 6 mph FT	1.6772	0.1156	14.5138	0	***
	adult male - 6 mph HT	1.9245	0.123	15.6433	0	***
	adult male - adult female	0.9971	0.1156	8.6285	9.99E-16	***
foxl2 E1.2	12 mph control - 12 mph CT	0.3334	0.1598	2.086	0.159251781	
	12 mph control - 12 mph FT	0.1706	0.1605	1.0623	0.712654482	
	12 mph FT - 12 mph CT	0.1628	0.1605	1.0142	0.741193983	
	12 mph HT - 12 mph control	0.1596	0.1598	0.9988	0.750136472	
	12 mph HT - 12 mph CT	0.493	0.1598	3.0848	0.011762086	*
	12 mph HT - 12 mph FT	0.3302	0.1605	2.0566	0.169107571	
	6 mph control - 6 mph CT	0.152	0.0616	2.4687	0.133184233	
	6 mph control - 6 mph FT	-0.0855	0.0616	-1.3882	0.731554091	
	6 mph FT - 6 mph CT	0.2375	0.057	4.1659	0.000490015	***
	6 mph HT - 6 mph control	-0.1272	0.0634	-2.0041	0.33780278	
	6 mph HT - 6 mph CT	0.0249	0.059	0.4218	0.998269364	
	6 mph HT - 6 mph FT	-0.2127	0.059	-3.6029	0.004467343	**
	adult female - 6 mph control	1.9518	0.0739	26.4029	0	***
	adult female - 6 mph CT	2.1039	0.0702	29.9852	0	***
	adult female - 6 mph FT	1.8663	0.0702	26.5995	0	***
	adult female - 6 mph HT	2.079	0.0718	28.9552	0	***
	adult male - 6 mph control	2.0918	0.0711	29.4128	0	***
	adult male - 6 mph CT	2.2438	0.0672	33.3902	0	***
	adult male - 6 mph FT	2.0063	0.0672	29.8552	0	***
	adult male - 6 mph HT	2.2189	0.0689	32.2021	0	***
	adult male - adult female	0.14	0.0787	1.7793	0.476538774	
foxl2 P	6 mph control - 6 mph CT	-0.0864	0.0954	-0.9058	0.944058489	
	6 mph control - 6 mph FT	-0.1943	0.0922	-2.1081	0.282298234	
	6 mph FT - 6 mph CT	0.1079	0.0954	1.1308	0.86661089	
	6 mph HT - 6 mph control	7.00E-04	0.0922	0.0079	1	
	6 mph HT - 6 mph CT	-0.0857	0.0954	-0.8982	0.945977274	
	6 mph HT - 6 mph FT	-0.1936	0.0922	-2.1002	0.28638746	
	adult female - 6 mph control	1.8079	0.114	15.8622	0	***
	adult female - 6 mph CT	1.7215	0.1166	14.7628	0	***
	adult female - 6 mph FT	1.6136	0.114	14.1571	0	***
	adult female - 6 mph HT	1.8072	0.114	15.8558	0	***

foxl2 P	adult male - 6 mph control	2.2148	0.1058	20.9259	0	***
	adult male - 6 mph CT	2.1284	0.1087	19.5856	0	***
	adult male - 6 mph FT	2.0205	0.1058	19.0898	0	***
	adult male - 6 mph HT	2.2141	0.1058	20.919	0	***
	adult male - adult female	0.4069	0.1253	3.2482	0.015718641	*
nr5a2 P	12 mph control - 12 mph CT	0.3481	0.1419	2.4531	0.287442567	
	12 mph control - 12 mph FT	0.349	0.1419	2.4594	0.283966642	
	12 mph FT - 12 mph CT	-9.00E-04	0.1419	-0.0063	1	
	12 mph HT - 12 mph control	-0.0727	0.1419	-0.5123	0.999961636	
	12 mph HT - 12 mph CT	0.2754	0.1419	1.9407	0.631623735	
	12 mph HT - 12 mph FT	0.2763	0.1419	1.947	0.627311275	
	6 mph control - 12 mph control	0.3791	0.1533	2.4733	0.276606746	
	6 mph control - 12 mph CT	0.7272	0.1533	4.7444	8.68E-05	***
	6 mph control - 12 mph FT	0.7281	0.1533	4.7502	0.000110334	***
	6 mph control - 12 mph HT	0.4518	0.1533	2.9476	0.09125102	.
	6 mph control - 6 mph CT	-0.1543	0.1793	-0.8602	0.997328342	
	6 mph control - 6 mph FT	-0.0454	0.1607	-0.2828	0.999999783	
	6 mph CT - 12 mph control	0.5334	0.1697	3.1428	0.052599976	.
	6 mph CT - 12 mph CT	0.8815	0.1697	5.1939	1.44E-05	***
	6 mph CT - 12 mph FT	0.8824	0.1697	5.1992	7.41E-06	***
	6 mph CT - 12 mph HT	0.6061	0.1697	3.5712	0.013382323	*
	6 mph FT - 12 mph control	0.4245	0.1499	2.8327	0.123409222	
	6 mph FT - 12 mph CT	0.7726	0.1499	5.1554	1.03E-05	***
	6 mph FT - 12 mph FT	0.7735	0.1499	5.1614	1.06E-05	***
	6 mph FT - 12 mph HT	0.4972	0.1499	3.3178	0.030535477	*
	6 mph FT - 6 mph CT	-0.1088	0.1764	-0.6168	0.999815698	
	6 mph HT - 12 mph control	0.3001	0.1419	2.1147	0.50772762	
	6 mph HT - 12 mph CT	0.6482	0.1419	4.5678	0.000234139	***
	6 mph HT - 12 mph FT	0.6491	0.1419	4.5741	0.000204944	***
	6 mph HT - 12 mph HT	0.3728	0.1419	2.6271	0.20107912	
	6 mph HT - 6 mph control	-0.079	0.1533	-0.5154	0.99995941	
	6 mph HT - 6 mph CT	-0.2333	0.1697	-1.3745	0.93162537	
	6 mph HT - 6 mph FT	-0.1244	0.1499	-0.8304	0.99797197	
	adult female - 12 mph control	1.1102	0.2244	4.9478	4.02E-05	***
	adult female - 12 mph CT	1.4583	0.2244	6.4992	3.06E-09	***
	adult female - 12 mph FT	1.4592	0.2244	6.5032	1.60E-09	***
	adult female - 12 mph HT	1.1829	0.2244	5.2718	6.45E-06	***
	adult female - 6 mph control	0.7311	0.2317	3.1548	0.050682046	.
	adult female - 6 mph CT	0.5768	0.2429	2.3745	0.333925171	
	adult female - 6 mph FT	0.6856	0.2295	2.9875	0.081203434	.
	adult female - 6 mph HT	0.8101	0.2244	3.6103	0.011418812	*
	adult male - 12 mph control	0.3115	0.1661	1.8755	0.676466921	

nr5a2 P	adult male - 12 mph CT	0.6596	0.1661	3.9717	0.002958702	**
	adult male - 12 mph FT	0.6605	0.1661	3.977	0.003008835	**
	adult male - 12 mph HT	0.3842	0.1661	2.3133	0.37265393	
	adult male - 6 mph control	-0.0676	0.1759	-0.3845	0.999996762	
	adult male - 6 mph CT	-0.2219	0.1904	-1.1655	0.97602234	
	adult male - 6 mph FT	-0.1131	0.1729	-0.6538	0.999702603	
	adult male - 6 mph HT	0.0114	0.1661	0.0685	1	
	adult male - adult female	-0.7987	0.2404	-3.3225	0.029677171	*
nr5a2 PE1	12 mph control - 12 mph CT	0.5632	0.1505	3.7423	0.007168797	**
	12 mph control - 12 mph FT	0.3149	0.1505	2.0921	0.522363128	
	12 mph FT - 12 mph CT	0.2484	0.1454	1.7082	0.78245451	
	12 mph HT - 12 mph control	-0.4732	0.1505	-3.1445	0.052457072	.
	12 mph HT - 12 mph CT	0.09	0.1454	0.6189	0.999809054	
	12 mph HT - 12 mph FT	-0.1584	0.1454	-1.0893	0.984728862	
	6 mph control - 12 mph control	0.2935	0.1505	1.9503	0.623836473	
	6 mph control - 12 mph CT	0.8567	0.1454	5.8924	3.61E-07	***
	6 mph control - 12 mph FT	0.6084	0.1454	4.1842	0.001305495	**
	6 mph control - 12 mph HT	0.7667	0.1454	5.2735	6.90E-06	***
	6 mph control - 6 mph CT	-0.0809	0.1454	-0.5563	0.999921316	
	6 mph control - 6 mph FT	-0.057	0.1454	-0.3922	0.999996088	
	6 mph CT - 12 mph control	0.3744	0.1505	2.4877	0.268327474	
	6 mph CT - 12 mph CT	0.9376	0.1454	6.4487	2.00E-09	***
	6 mph CT - 12 mph FT	0.6892	0.1454	4.7405	0.000116943	***
	6 mph CT - 12 mph HT	0.8476	0.1454	5.8298	1.56E-06	***
	6 mph FT - 12 mph control	0.3505	0.1505	2.3291	0.361177857	
	6 mph FT - 12 mph CT	0.9137	0.1454	6.2846	1.01E-08	***
	6 mph FT - 12 mph FT	0.6654	0.1454	4.5764	0.000187141	***
	6 mph FT - 12 mph HT	0.8238	0.1454	5.6657	8.24E-07	***
	6 mph FT - 6 mph CT	-0.0239	0.1454	-0.1641	0.999999998	
	6 mph HT - 12 mph control	0.2543	0.1505	1.6895	0.793303778	
	6 mph HT - 12 mph CT	0.8175	0.1454	5.6225	1.01E-06	***
	6 mph HT - 12 mph FT	0.5691	0.1454	3.9144	0.003784577	**
	6 mph HT - 12 mph HT	0.7275	0.1454	5.0037	2.97E-05	***
	6 mph HT - 6 mph control	-0.0392	0.1454	-0.2699	0.999999855	
	6 mph HT - 6 mph CT	-0.1201	0.1454	-0.8261	0.998024466	
	6 mph HT - 6 mph FT	-0.0963	0.1454	-0.662	0.999665695	
	adult female - 12 mph control	0.7091	0.1441	4.9226	4.14E-05	***
	adult female - 12 mph CT	1.2724	0.1387	9.1722	0	***
	adult female - 12 mph FT	1.024	0.1387	7.3819	5.35E-12	***
	adult female - 12 mph HT	1.1824	0.1387	8.5236	9.45E-14	***
	adult female - 6 mph control	0.4156	0.1387	2.9962	0.079566349	.
	adult female - 6 mph CT	0.3347	0.1387	2.4131	0.309971002	

nr5a2 PE1	adult female - 6 mph FT	0.3586	0.1387	2.5851	0.219223679	
	adult female - 6 mph HT	0.4549	0.1387	3.279	0.034733239	*
	adult male - 12 mph control	-1.0752	0.121	-8.8852	3.33E-16	***
	adult male - 12 mph CT	-0.5119	0.1146	-4.4674	0.0003912	***
	adult male - 12 mph FT	-0.7603	0.1146	-6.6346	8.85E-10	***
	adult male - 12 mph HT	-0.6019	0.1146	-5.2526	7.62E-06	***
	adult male - 6 mph control	-1.3687	0.1146	-11.9434	0	***
	adult male - 6 mph CT	-1.4496	0.1146	-12.6492	0	***
	adult male - 6 mph FT	-1.4257	0.1146	-12.441	0	***
	adult male - 6 mph HT	-1.3294	0.1146	-11.601	0	***
	adult male - adult female	-1.7843	0.106	-16.8336	0	***
sox9 PE1	12 mph control - 12 mph CT	0.2288	0.0867	2.637	0.198166254	
	12 mph control - 12 mph FT	0.2904	0.0907	3.2017	0.044750511	*
	12 mph FT - 12 mph CT	-0.0616	0.0904	-0.6816	0.999597713	
	12 mph HT - 12 mph control	0.1519	0.0878	1.7299	0.774597051	
	12 mph HT - 12 mph CT	0.3806	0.0875	4.3499	0.000515621	***
	12 mph HT - 12 mph FT	0.4422	0.0914	4.8378	7.75E-05	***
	6 mph control - 12 mph control	0.5471	0.0959	5.7039	2.36E-07	***
	6 mph control - 12 mph CT	0.7758	0.0956	8.111	1.09E-14	***
	6 mph control - 12 mph FT	0.8374	0.0992	8.4382	4.55E-15	***
	6 mph control - 12 mph HT	0.3952	0.0966	4.0916	0.001795264	**
	6 mph control - 6 mph CT	0.1096	0.0987	1.1099	0.983340073	
	6 mph control - 6 mph FT	-0.1755	0.0956	-1.8348	0.708482246	
	6 mph CT - 12 mph control	0.4375	0.0901	4.8534	4.46E-05	***
	6 mph CT - 12 mph CT	0.6662	0.0899	7.4141	1.52E-12	***
	6 mph CT - 12 mph FT	0.7279	0.0937	7.7698	1.45E-13	***
	6 mph CT - 12 mph HT	0.2856	0.0909	3.1435	0.05300711	.
	6 mph FT - 12 mph control	0.7225	0.0867	8.3292	4.44E-15	***
	6 mph FT - 12 mph CT	0.9513	0.0865	11.0026	0	***
	6 mph FT - 12 mph FT	1.0129	0.0904	11.2022	0	***
	6 mph FT - 12 mph HT	0.5707	0.0875	6.5223	9.70E-10	***
	6 mph FT - 6 mph CT	0.2851	0.0899	3.1725	0.048255962	*
	6 mph HT - 12 mph control	0.2946	0.0875	3.3673	0.026188438	*
	6 mph HT - 12 mph CT	0.5233	0.0872	6.0019	9.12E-08	***
	6 mph HT - 12 mph FT	0.585	0.0911	6.4194	1.14E-08	***
	6 mph HT - 12 mph HT	0.1427	0.0882	1.6176	0.836800092	
	6 mph HT - 6 mph control	-0.2525	0.0963	-2.6216	0.20553622	
	6 mph HT - 6 mph CT	-0.1429	0.0906	-1.5779	0.856133684	
	6 mph HT - 6 mph FT	-0.428	0.0872	-4.9084	3.93E-05	***
	adult female - 12 mph control	1.3296	0.1173	11.337	0	***
	adult female - 12 mph CT	1.5584	0.1171	13.3116	0	***
	adult female - 12 mph FT	1.62	0.12	13.4973	0	***

sox9 PE1	adult female - 12 mph HT	1.1778	0.1178	9.9948	0	***
	adult female - 6 mph control	0.7826	0.124	6.3105	4.99E-09	***
	adult female - 6 mph CT	0.8921	0.1196	7.4594	1.42E-11	***
	adult female - 6 mph FT	0.6071	0.1171	5.1856	9.88E-06	***
	adult female - 6 mph HT	1.035	0.1176	8.8006	5.55E-16	***
	adult male - 12 mph control	1.1572	0.0867	13.3392	0	***
	adult male - 12 mph CT	1.3859	0.0865	16.0293	0	***
	adult male - 12 mph FT	1.4475	0.0904	16.0086	0	***
	adult male - 12 mph HT	1.0053	0.0875	11.4894	0	***
	adult male - 6 mph control	0.6101	0.0956	6.3786	8.15E-09	***
	adult male - 6 mph CT	0.7197	0.0899	8.0091	2.11E-14	***
	adult male - 6 mph FT	0.4346	0.0865	5.0267	2.03E-05	***
	adult male - 6 mph HT	0.8626	0.0872	9.8929	0	***
	adult male - adult female	-0.1725	0.1171	-1.4731	0.900480615	