# ABSTRACT

Title of Document: EFFECTS OF TEMPERATURE ON GENE EXPRESSION AND SEX DETERMINATION IN THE MANGROVE RIVULUS, *KRYPTOLEBIAS MARMORATUS*

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Rivulus is a hermaphroditic, self-fertilizing fish species that possesses an ovotestis and, in the wild, exist as androdioecious populations comprised of hermaphrodites and males. Following embryonic incubation at an ambient temperature of 25 °C, rivulus develop normally as hermaphrodites. Embryos exposed to a lower temperature of 20 °C during a critical phase of embryogenesis develop as males. In this study, rivulus embryos were maintained at ambient (25 °C), low (20 °C), or high (31 °C) temperatures during various stages of embryogenesis. The expression of seven evolutionary conserved genes with known relevance to gonadal differentiation including *figα*, *foxl2*, *cyp19a1b*, *cyp19a1a*, *dmrt1*, *sox9a*, and *sox9b* was measured using real-time PCR. The expression of *cyp19a1a* was downregulated at 20 °C and the expression of ovarian-specific genes increased throughout embryogenesis. These results provide the first data documenting how temperature affects the expression of genes relevant to sex determination during embryogenesis in rivulus.

# EFFECTS OF TEMPERATURE ON GENE EXPRESSION AND SEX DETERMINATION IN THE MANGROVE RIVULUS, *KRYPTOLEBIAS MARMORATUS*

by

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# Dedication

To all my family and friends, thank you for your endless support.

## Acknowledgements

To my advisor, Dr. Edward Orlando, thank you for allowing me the opportunity to pursue my interest in fish reproduction.

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# Chapter 1: Literature Review

## GSD vs. ESD

The mechanisms of sex determination are highly variable across vertebrate species [1]. The two most common patterns of sex determination among vertebrates are genetic sex determination (GSD) and environmental sex determination (ESD). In vertebrate animals with GSD, two sex chromosomes are present and different combinations of these chromosomes determine whether the organism will develop as a male or a female. Animals that exhibit ESD often lack sex chromosomes and their sex is determined by environmental factors that include temperature, population structure, and exogenous hormones or other chemicals. Mammals and birds exhibit GSD, whereas fish, reptiles, and amphibians often exhibit ESD.

## Sex determination in mammals

In mammals, the male is heterogametic and sex is usually determined by the presence a Y chromosome (XY = male) or two X chromosomes (XX = female) [1]. The *Sry* gene (sex region Y-chromosome gene) linked to the Y chromosome of mammals is a transcription factor responsible for initiating the pathway of male development [2]. Without a functional *Sry* gene, ovaries will develop even if the individual is otherwise genetically XY. As a result of mutations that alter male development in mammals, several elements of the pathway downstream of *Sry* have been identified [3]. Thus, a great deal is known about sex determination and differentiation of the gonads in mammals.

## Sex determination in fish

In contrast to mammals, the underlying mechanisms that control fish sex determination and differentiation of the gonad remain largely elusive. Fish exhibit a variety of reproductive strategies [4]. Not surprisingly, the mechanisms of sex determination among fish species are also highly variable [5]. Most fish are gonochoristic, that is, males possess only testicular tissue and females possess only ovarian tissue for the duration of their lives. Most hermaphroditic fish develop first as male (protandrous) or female (protogynous) before undergoing a sex reversal and are deemed sequential hermaphrodites. Although uncommon, a few species develop functional ovotestes and are called simultaneous hermaphrodites.

## Environmental sex determination

Very few fish (~10%) are known to possess morphologically distinct chromosomes responsible for determining sex [5, 6]. Most fish species exhibit other methods of sex determination and sex may be influenced by certain environmental factors including temperature and population structure. Environmental sex determination is not uncommon among vertebrates. The bluehead wrasse, *Thalassoma bifasciatum*, exemplifies ESD based on population structure [7]. Populations are comprised of protogynous hermaphrodites and primary males. Primary males are easily distinguished from secondary males as primary males possess testes similar in appearance to gonochoristic species, whereas secondary males retain evidence of previous ovarian function. The development of primary males can be altered depending upon the structure of the population when the fish is a juvenile. Small populations contain few primary

males while large populations contain many primary males. When these fish are raised under laboratory conditions, individuals maintained in isolation develop as females but at least one individual would develop as a primary male when raised in groups of three. It has been suggested that this developmental strategy has some genetic basis but population size clearly plays a role in determining sex in bluehead wrasse.

The most prevalent form of environmental sex determination is temperature-dependent sex determination. For instance, very few turtles are known to have sex chromosomes and most rely heavily on environmental cues such as nest temperature for sex determination [8]. Additionally, recognizable sex chromosomes have not been identified in any crocodilian species in which nest temperature also plays a critical role in sex determination [8, 9]. Interestingly, among crocodilians there also appears to be a general pattern such that females develop following exposure to low and high temperatures while males develop following exposure to intermediate temperatures during the temperature-sensitive period [9].

Similar to many reptiles and amphibians, some fish species display temperature-dependent sex determination. Although these environmental factors play an important role in sex determination, sex is most likely determined by an interaction between genes and the environment [6]. For example, evidence for an XX-XY sex-determination system in flatfishes as abundant [10]. However, at least two species of the genus *Paralichthys* are known to exhibit TSD opposite that observed in crocodilians. In the Japanese flounder, *Paralichthys olivaceus,* and the southern flounder, *Paralichthys lethostigma,* low and high temperatures induce these fish to develop as males while an intermediate temperature produces females.

The interaction between a genetic system and the environment is also apparent in *Menidia* species [11]. In this case latitudinal location determines the strength of the genetic system. At intermediate latitudes, ESD is more prevalent whereas GSD is more prevalent at the northern and southern extremes for these fish. Additionally, higher temperatures result in a larger proportion of males while lower temperatures result in a larger proportion of females. It has also been suggested that this unique variability in the roles that GSD and ESD play in these species is adaptive.

The interaction between the environment and genetics in Tilapia is also multifaceted [12]. Tilapias also utilize an XX-XY sex determining system but temperature is known to override this system during the critical period of sex differentiation. In tilapias, phenotypic males develop following exposure to high temperatures during the critical period sex differentiation despite being genetically XX (female). Additionally, there is evidence that sensitivity to a temperature-dependent sex change is heritable; thus, the role of TSD in tilapias is not uniform.

**The mangrove rivulus**

The mangrove rivulus, *Kryptolebias marmoratus*, is a species of fish that exhibits environmental sex determination. Rivulus is a self-fertilizing hermaphrodite that was discovered in Cuba and originally described by Poey in 1880 [13]. Wild hermaphrodites are typically a dark brown or maroon color and exhibit a distinct ocellus on the caudal peduncle (Figure 1-1). As a self-fertilizing hermaphrodite, this fish possesses a bilobed ovotestis with discrete ovarian and testicular tissues that produce eggs and spermatozoa, which are released into a common lumen for fertilization (Figure 1-2). However, fish

that possess only testicular tissue have been observed in the laboratory as well as in the wild (Figure 1-1) [14]. Adult males are visually distinct from adult hermaphrodites with an orange body color and no caudal ocellus (Figure 1-1) [13].

Rivulus inhabits mangroves in the Caribbean, Gulf of Mexico, and western Atlantic [13]. Its range includes as far south as southeastern Brazil with a northern limit around Tampa Bay and Melbourne Beach in Florida [15]. Among these mangroves, rivulus reside in crab holes that are often subject to dry periods. It is evident that rivulus has evolved to live in environments that can change very quickly. The versatility of this species is most likely what has allowed it to be successful in the climates within its range. For instance, some individuals, including embryos, have also been found in damp logs and leaf litter. Rivulus can also tolerate salinities ranging from 0-68 ppt and temperatures from 7-38 °C. Despite its tolerance for a wide range of environmental conditions, it is hypothesized that rivulus will leave the water environment when conditions are not ideal and return when conditions improve. Amazingly, rivulus can survive under extended exposure to humid air enabling this fish to travel across land; hence, this fish is sometimes referred to as amphibious [16].

However, the most unique aspect of rivulus is its mode of reproduction. Although a majority of rivulus collected from the wild have been hermaphrodites, males have been observed in the laboratory and found in the wild on rare occasions [14]. Currently, no females have been found in the wild or observed in the laboratory. The existence of males in a hermaphroditic species that self-fertilizes is of particular interest. The reason for the existence of these males is often attributed to the need for outcrossing. Fertilization in rivulus occurs internally and embryos are emitted after various lengths of

time following fertilization and develop outside the body until hatching [13]. Therefore, it would be necessary for a hermaphrodite to emit viable unfertilized eggs in order for a male to execute external fertilization as rivulus males lack an intromittent device. While this process has been documented in the laboratory, it appears that this occurs in the wild as well [17, 18]. With the existence of some genetic variation in populations of rivulus, external fertilization by males of unfertilized eggs is a logical explanation for the prevention of inbreeding depression.

Harrington (1967) was the first to observe males in the laboratory. After ten generations, he observed less than 5% males develop among 350 fish at ambient temperature (25 °C). After experimenting with various combinations of temperature, light, and salinity, only temperature was found to play a role in determining whether or not an embryo would develop as a male or hermaphrodite [14]. Harrington determined that these males resulted from exposure to cold temperatures (~20 °C) during a critical phase of development, termed the phenocritical period [14]. These males were labeled as primary males. Secondary males refer to fish that are first functional, self-fertilizing hermaphrodites and become male as ovarian tissue regresses and testicular tissue proliferates. The upper limit of the threshold for producing primary males is 21.2 °C [14]. Embryos incubated in salt water (36 ppt) at 18.0°C suffered from high mortality [14]. Thus, 20 °C effectively produces male fish without incurring high rates of mortality.

A total of thirty-three stages of rivulus embryonic development have been described [19]. The stage of embryonic development during which rivulus are most susceptible to male-producing temperatures is stage 31b [20]. Although the phenocritical

period is just before hatching, the duration of this stage, which may be up to several days, is the longest of any of the embryonic stages [19]. Extended time in the phenocritical period may allow ample opportunity for gene expression and synthesis of proteins that comprise the sex determining gene network in rivulus. Embryos from Harrington's second experimental series were exposed to cold temperatures (20 °C) at stage 22 and were then removed from the cold prior to hatching (stage 32). Among these embryos, only hermaphrodites were observed. Another set of embryos were exposed to cold temperatures (20 °C) at various stages (stage 22-31) and remained exposed until they reached stage 32. Among these embryos approximately 80% were primary males. By exposing embryos to cold temperatures at stage 31 and continuing exposure for a various number of hours following stage 31, it was concluded that stage 31b was the phenocritical period at which rivulus primary males could be produced [20].

Similar to many other fish species, the sex of rivulus can also be manipulated with hormones [5]. For example, a potent androgen, 17α-methyltestosterone (MT), effectively produces primary male rivulus [21]. In this study, at approximately twelve days post fertilization (dpf), embryos were immersed in a solution of seawater and MT. This exposure to MT continued for ten days at which point the embryos were removed from the MT solution and placed in salt water only. Although not 100% effective, Kanamori et al. (2006) observed 97% of MT-treated fish as primary males (compared to 80% produced by exposure to cold temperatures during the phenocritical period). Kanamori et al. (2006) also tracked gonadal development in rivulus. Both the MT-treated and control fish followed similar paths of development until 39 dpf when the number of oocytes in MT-treated fish significantly decreased relative to control fish. MT-treated fish also

began spermatogenesis earlier than control fish. Interestingly, *figα* (factor in the germline α), an ovarian-specific gene, decreased, while *dmrt1* (doublesex and mab-3 related transcription factor 1), a testis-specific gene, increased in MT-treated fish at 39 dpf [21].

As a result of the unique ability of rivulus to self-fertilize and be manipulated in terms of sex determination via temperature and hormones, this species is a good model for learning how sex is determined The development of these tissues can be manipulated by exogenous hormone treatment. Many methods can then be used to examine gene expression, gonad morphology, hormone levels, and protein levels in order to better understand how the development of ovarian and testicular tissue is regulated.

**Sex determining genes**

Many of the genes that have been shown to play a role in sex determination appear to be highly conserved among vertebrates. Included in this set of genes are *dmrt1* and *sox9* (sex determining region Y-box 9) which are associated with the development of testicular tissue and *figα*, *cyp19a1a*, *cyp19a1b*, and *foxl2* which have been shown to be crucial for ovarian development. There is evidence that many of these genes function early in the sex determination pathways of vertebrates. For example, *sox9* and *foxl2* are often implicated for their roles early in sex determination [2, 22]. In medaka (*Oryzias latipes*), *dmrt1* and *figα* have also been implicated as playing important roles in early sex determination [23]. In some cases, there is also evidence that these genes directly repress or upregulate one another to coordinate the intricate mechanisms that are responsible for sex determination. For example, *dmrt1* represses *cyp19a1a*, *foxl2* represses *sox9*, and *foxl2* regulates *cyp19a1a* [24-26]. Therefore, the expression patterns of *dmrt1*, *sox9*, *figα*,

*cyp19a1a*, *cyp19a1b* and *foxl2* in rivulus embryos could provide important information on putative mechanisms that control gonad development in rivulus and other vertebrates.

Among fish species and other vertebrates, the role of *dmrt1* in gonad development and function has been relatively well characterized. However, the role of *dmrt1* is not necessarily conserved. The *dmrt1* gene encodes a protein with a zinc finger-like DNA binding motif (DM-domain). Although it was originally characterized in *Drosophila melanogaster* and *Caenorhabditis elegans*, *dmrt1* is present in a variety of vertebrates [2]. This gene is required for normal testis development and appears to be located downstream of primary sex determining genes, such as *Sry*, in mice [27, 28]. Being highly conserved, it is not surprising that *dmrt1* plays a similar role in testis development in many fish species. In a study where the expression of *figα* and *dmrt1* were compared between MT-treated and control fish, *dmrt1* was localized by *in situ* hybridization in the testis [21]. *dmrt1* has also been shown to repress transcription of the ovarian aromatase gene, *cyp19a1a*, in Nile tilapia [24]. Aromatase is responsible for converting androgens to estrogens. Sufficient concentrations of estrogens are necessary for the development (and maintenance) of ovarian tissue [5]. Inhibition of estrogen production via suppression of aromatase by *dmrt1* allows for development of male fish gonads [29].

The expression of *dmrt1* also appears to be regulated by temperature in vertebrate species with temperature-dependent sex determination. For example, red-eared slider turtle, *Trachemys scripta*, embryos displayed an increase in *dmrt1* expression in the genital ridge when exposed to cold temperatures which favored subsequent testis differentiation [30]. However, *dmrt1* is not likely to be the primary sex determining gene and appears to be downstream of other genes in the testis-determining pathway [27]. For

example, the expression of *dmrt1* in spermatogenic (germ) cells of the protogynous orange-spotted grouper, *Epinephelus coioides*, has been demonstrated [31]. Thus, *dmrt1* may be required for maintaining the testis in adult fish as well as producing spermatogonia and spermatocytes. In order to promote and maintain testis differentiation, it would seem likely that *dmrt1* suppresses ovarian development in some manner. **In turn, it is hypothesized that *dmrt1* expression will be elevated in embryos that are incubated at the male-producing temperature of 20 °C compared to the control (25 °C). It is also hypothesized that 31 °C will produce males and *dmrt1* will also be elevated in embryos incubated at 31 °C when compared to control embryos.**

It is clear that *dmrt1* is not solely responsible for the differentiation of the testis. Another testis-specific gene, *sox9*, is suspected to play a role early in the sex determination pathway [2]. In mammals, *sox9* has been proposed to be a direct target of the *SRY* gene on the Y chromosome of males [2]. In teleost fish, there was a duplication event of *sox9* during evolution resulting in two forms, *sox9a* and *sox9b*, that are homologous with the *sox9* gene of other vertebrates [32]. Although *sox9* is also highly conserved among vertebrates, the roles of the two forms in fish do not appear to be conserved. In fish, the two forms of *sox9* may be involved in maintenance of the gonads rather than early sex determination. In zebrafish, *Danio rerio*, *sox9a* is expressed in the adult testis whereas *sox9b* is expressed in previtellogenic oocytes of the ovary [32]. In contrast, *sox9b* is expressed in XY gonads of the Philippine medaka, *O. luzonensis*, but not in XX gonads [33]. Interestingly, the expression patterns of *sox9* have been correlated to temperature in the red-eared slider turtle as increased expression of these genes occurs during differentiation at male-producing temperatures [34]. **It is**

**hypothesized that expression of *sox9a* and *sox9b* will also be affected by temperature in rivulus, such that one *sox9* isoform will be expressed in embryos incubated at 20 °C and 31 °C while the other isoform will be expressed in embryos incubated at 25 °C.**

In order to better understand testis differentiation and development, it is also necessary to understand the differentiation of the ovary as the pathways that lead to development of a testis or ovary often oppose one another. In some fish species, such as zebrafish it appears that juvenile fish first develop an ovary regardless of the final sex of the fish [35]. Zebrafish are considered juvenile hermaphrodites as they pass through a stage in which they have an ovary before developing a testis and becoming an adult male [35]. Although the exact mechanism has yet to be determined, it appears that the ovarian tissue undergoes apoptosis and testicular tissue proliferates as the juvenile hermaphrodite morphs into an adult male [35].

Similar to zebrafish, rivulus also appear to pass through a juvenile stage in which the gonad contains strictly ovarian tissue. Kanamori et al. (2006) observed rivulus histologically and noted that MT-treated and control fish had germ cells that had entered oogenesis by the time of hatching. It was not until one week following hatching that histological differences could be observed between gonads of MT-treated fish that developed as primary males and control fish that developed as hermaphrodites. "Pure females" of rivulus have been reported from other histological studies [36]; however, it is likely that these fish were not yet mature hermaphrodites. Rather, these fish were likely to be immature hermaphrodites that had not yet begun to develop testicular tissue and were subsequently passing through the female stage.

Several genes have been shown to be important for development of the ovary. Some of the most commonly studied genes in ovarian determination and differentiation include *figα, foxl2, cyp19a1b* and *cyp19a1a*. It has been proposed that expression of aromatases in the brain, i.e., neuroestrogen synthesis, may be involved early in the pathway of sex determination in fish [37]. Yet, controversy remains over this hypothesis as sexual dimorphism in *cyp19a1b* expression was not observed during gonad differentiation in zebrafish [38]. In a study by Lee et al. (2006), *cyp19a1b* in rivulus was expressed during all stages of embryonic development measured, while *cyp19a1a* expression was not observed until the midpoint of embryonic development [39]. *cyp19a1a* expression was also highest in rivulus embryos at the midpoint of development; however, even the highest expression level of *cyp19a1a* was lower than *cyp19a1b* at all stages of embryonic development [39]. *cyp19a1b* expression was highest in embryos near the end of embryonic development around the phenocritical period but expression decreased significantly in recently hatched fry [39]. As brain development precedes that of the gonad in vertebrates, the spike in *cyp19a1b* expression around the phenocritical period in rivulus may help resolve some of the outstanding questions regarding the role of neuroestrogen synthesis on early gonad differentiation in fish. **It is hypothesized that (a) *cyp19a1b* expression will increase before *cyp19a1a* in control embryos (at 25 °C), (b) overall expression levels of *cyp19a1b* will be higher than *cyp19a1a* in rivulus embryos at ambient temperature (25 °C), and (c) it is predicted that these genes will be down-regulated in embryos incubated at 20 °C and 31 °C.**

In contrast to brain aromatase, the expression patterns of other genes relevant to sex determination are less controversial. In rivulus, expression of the *figα* gene in the

non-MT-treated (control) fish was shown to increase at 39 dpf and remained elevated throughout development and into adulthood [21]. *figα* and *cyp19a1a* have also been shown to be important regulators of oocyte development in medaka [40]. In medaka, *figα* is expressed as early as one day post hatching (dph). As an oocyte-specific marker, *figα* is not likely to be the first gene in this complex pathway nor is it the last. Evidence for this is observed in the expression of target genes of *figα,* including *zpc4* and *zpb* (ZP domain containing egg envelope genes), which are not expressed until 5 and 15 days respectively following the beginning of *figα* expression [40]. Like many other genes in the sex determination and differentiation pathways, *figα* appears to be conserved as it encodes a protein similar to that produced by *figα* in mice [40]. This gene is also expressed in many vertebrate species including mice, human, zebrafish, and medaka [41-44].

In zebrafish, *figα* is expressed later in development around 22 dpf [45]. However, *cyp19a1a* expression was highest almost immediately after hatching from 4-8 dpf [45]. The expression pattern of *figα* in zebrafish therefore resembles the expression pattern of *figα* in rivulus but not medaka. Thus, it remains that *figα* and *cyp19a1a* are sufficient indicators of ovarian differentiation. Note that the expression of these genes late in development may suggest a more powerful role in sex differentiation rather than determination in zebrafish. Accounting for the expression pattern of *figα* observed by Kanamori et al. in rivulus, *figa* may also play a more important role in gonad development in juvenile or adult rivulus rather than embryos [40]. **Thus, it is hypothesized that *figa* will be expressed in very low concentrations in rivulus**

**embryos incubated at ambient temperature (25 °C) and will not be expressed in embryos incubated at 20 °C and 31 °C.**

In contrast to the late expression of *figα* and *cyp19a1a*, the early expression of *foxl2* (forkhead box L2) in many vertebrate species provides evidence for its role in sex determination. In mammals and fish, *foxl2* is suspected to be a regulator of *cyp19a1a* [25, 29]. This gene may also be directly responsible for down regulation of *dmrt1* in order to prevent testis differentiation and promote ovarian differentiation. Recently, *foxl2* has been shown to not only be important in sex determination in mice but also for maintaining a functional ovary [25]. FOXL2 represses *sox9,* which functions at the beginning of the sex determination pathway and is involved with initiating testis development. Thus, expression of *foxl2* ultimately represses the formation of testicular tissue.

While *foxl2* has long been suspected to play a role in early sex determination, the study by Uhlenhaut et al. (2009) showed that *foxl2* expression is necessary to maintain suppression of testicular development. By deleting *foxl2* in XX mice, granulosa cells transformed into Sertoli-like cells and theca cells were transformed to Leydig-like cells [25]. While sex reversal in adult fish is not an uncommon event, sex reversal in adult mammals with differentiated gonads has not yet been demonstrated. With this new insight on mammalian sex determination and differentiation, Uhlenhaut et al. (2009) suggest that the sex reversal phenomenon in lower vertebrates (including fish) may be explained by mechanisms which include *foxl2* and its regulators and targets. **As one of the known conserved ovarian genes, it is hypothesized that *foxl2* will be expressed in**

**rivulus embryos incubated at ambient temperature (25 °C) and expression of this gene will be lower or nonexistent in rivulus embryos incubated at 20 °C and 31 °C.**

There are many genes known to play important roles in the development of ovarian and testicular tissue. These genes are highly conserved throughout the vertebrate lineages; however, the specific roles of these genes in sex determination appear to be species specific in some cases. In general, the development of the ovary is dependent upon genes that include *cyp19a1a, cyp19a1b, figα,* and *foxl2*. These genes have been identified as markers for ovarian development in fish and other vertebrates and will be used as markers for ovarian development in rivulus embryos in this thesis research. The expression of genes such as *dmrt1* is highly correlated with development of testicular tissue and will be used to mark development of testicular tissue in rivulus as well. The relatively recent discovery of two *sox9* isoforms may make it difficult to interpret the functional significance of *sox9a* and *sox9b* in rivulus; however, expression patterns that correlate with ovarian or testicular gene markers may provide insight into the roles that these genes play in rivulus sex determination and differentiation.

The genes used to study sex determination in this study are not exclusive and the involvement of other genes should be noted. Unfortunately, the role of other genes in sex determination and differentiation may be highly variable among different classes of vertebrates. For example, *sf1,* steroidogenic factor 1, is expressed in the testis of rats and mice but is expressed in ovaries of the chicken [46, 47]. Even among vertebrates with temperature-dependent sex determination, tissue-specific expression of *sf1* is variable. In American alligators, *Alligator mississippiensis, sf1* expression is limited to the ovaries, whereas *sf1* expression is confined to testes of turtles [48, 49].

The sexual dimorphism of other genes associated with sex determination and differentiation is also questionable. An example of such a case can be found in the American alligator where *dax1* (dosage sensitive sex reversing- adrenal hypoplasia congenita critical region of the X chromosome) and *wt1* (Wilms' tumour suppressor gene) expression levels did not differ between males and females [48]. It has also been shown that DAX1 is not necessary for ovarian development in mice, and it has been suggested that WT1 is not directly involved in sex determination [50, 51]. As the expression patterns and roles of other genes such as *sf1*, *dax1*, and *wt1* are more variable among vertebrates, *cyp19a1a*, *cyp19a1b*, *figα*, and *foxl2*, *dmrt1*, *sox9a* and *sox9b* were deemed appropriate gene markers for ovarian and testicular tissue in rivulus this study.

**Overview and objectives**

The objective of this study is to examine the effect of temperature on the expression of seven sex determination genes during rivulus embryogenesis. Other studies provide evidence that sexual development is regulated in part by temperature's effects on gene expression, heat-shock protein function, enzyme action, and possibly through epigenetic regulation of genes in the sex determination pathway [12, 52, 53]. In rivulus, low temperatures (20 °C) result in the development of primary males while ambient temperatures (25 °C) result in the development of hermaphrodites [14]. Developmental fates of rivulus embryos incubated at high temperatures (31 °C) have yet to be clearly described. **Accordingly, it is hypothesized that embryos incubated at each temperature during the phenocritical period will exhibit various gene expression patterns such that 1) embryos at 20 °C will express *dmrt1* and will not express**

**ovarian-specific genes, 2) embryos at 25 °C will express both testis- and ovarian-specific genes (*dmrt1, sox9a, sox9b, figα, cyp19a1b, cyp19a1a,* and *foxl2*), and 3) embryos at 31 °C will express *dmrt1* and will not express ovarian-specific genes.** The results of this study will provide additional information to what is known about development of gonads in fish and provide insight into the mechanism that allows an ovotestis to develop from pathways that typically oppose one another.

Figure 1-1. Hermaphrodite (top) and male (bottom) rivulus.

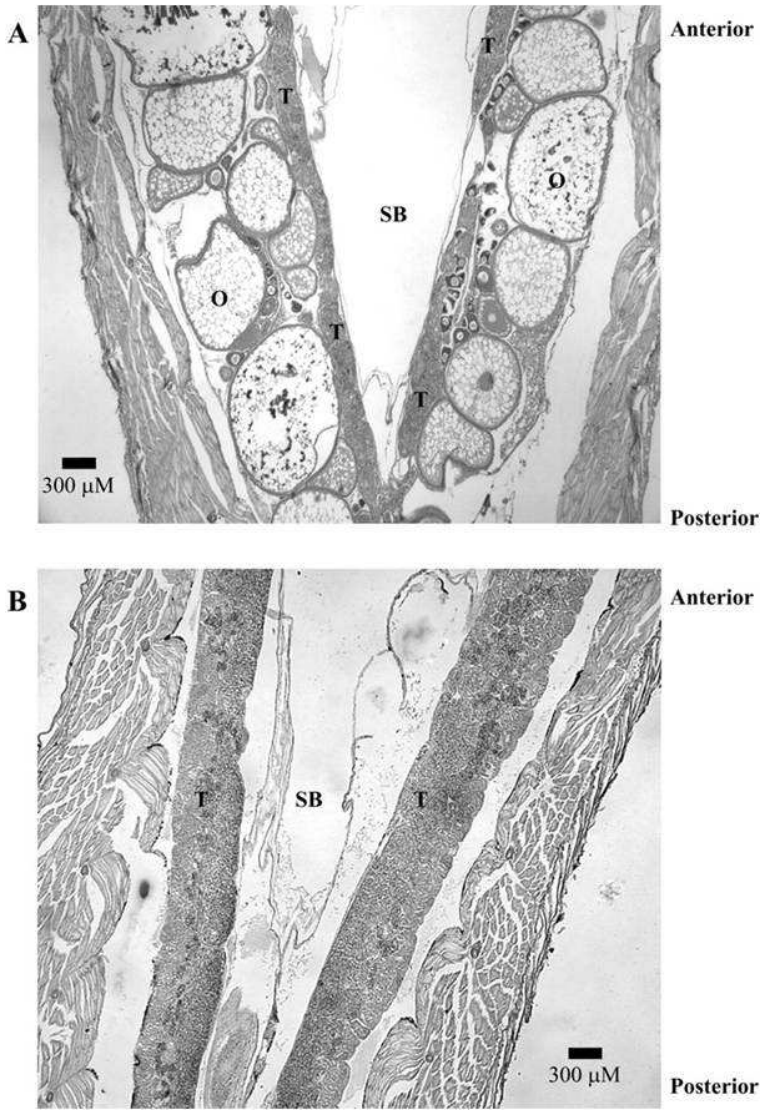



Figure 1-2. Rivulus gonad histology. Ventral sections of the rivulus hermaphrodite (A) and male (B) ovarian tissue (O), testicular tissue (T), and swim bladder (SB) [54].

# Chapter 2: Materials and Methods

## Fish

Adult, hermaphroditic fish from an isogenic strain were kept in individual glass bowls with a Teflon net on the bottom containing pores large enough to allow released embryos to fall through and prevent the parent from consuming the embryos. This isogenic strain (03-RhlC) was established from a fish collected by Dr. William P. Davis, USEPA, from Reckley Hill Lake, San Salvador, Bahamas. Isogenicity of this strain has been confirmed by microsatellite analysis [55]. Each bowl contained 200 mL of salt water (15 ppt, comprised of deionized water plus synthetic sea salts). The holding room was maintained at 25 °C (+/- 0.5 °C). Adult hermaphrodites were generously fed newly hatched brine shrimp (*Artemia* sp.) *ad libitum*.

## Embryos

Embryos were collected and held in groups of ten in 25 mL salt water (15 ppt, comprised of deionized water plus synthetic sea salts). Water temperature was maintained at 25 °C (+/- 0.05 °C) until treatment. Developmental stage of embryos was determined based on a modification of stages described by Koenig and Chasar (1981). The 33 stages of embryonic development were consolidated into seven broader categories, which have easily recognized characteristics. In Stage I, one to several cells or a blastula can be observed (Figure 2-1). In Stage II, the epiblast covers a majority of the yolk (Figure 2-2). Stage III is defined by the presence of the embryonic keel that has no distinct features (Figure 2-3). Stage IV is characterized by the presence of eyes without any pigmentation in the optic cup or body (Figure 2-4). When pigmentation is present solely in the body

and not yet in the optic cup, the embryo is classified as Stage V (Figure 2-5). In Stage VI, both the optic cup and body are pigmented and no caudal fin is apparent (Figures 2-6). In the final stage before hatching (Stage VII), which contains the phenocritical period defined by Harrington (31b), a caudal fin with pigmented rays can be observed (Figure 2-7) [20] . Upon reaching the appropriate stage of development, embryos were transferred to their respective treatment. Treatments consisted of incubation at 20 °C, 25 °C, and 31 °C. Embryos normally held at 25 °C were exposed to a treatment temperature for one embryonic developmental stage and then snap-frozen in liquid nitrogen and subsequently stored at -80 °C.

**Validation of Quantitative Real-Time Polymerase Chain Reaction (QPCR) Assays**

Total RNA was extracted from whole embryos or brain and gonad (ovotestis or testis) tissue from fish according to the Trizol RNA Isolation Protocol (Invitrogen). Total RNA quantity and quality was determined using a Nanodrop ND-1000 and agarose gel electrophoresis. Isolated RNA was then used to synthesize cDNA using the SuperScript$^{TM}$ III First-Strand Synthesis System for a reverse transcriptase polymerase chain reaction (RT-PCR) kit (Invitrogen). cDNA was used for PCR with the appropriate primers, which were designed with Beacon Designer (BioRad) with the exception of primers for *cyp19a1b* and *rpl8* which were provided by Dr. Edward Orlando [54] and *dmrt1* which were provided by Dr. Akira Kanamori [21]. Following ligation into PCR® II-TOPO® vector (Invitrogen) or pPrime Cloning Vector (5 Prime) and transformation into One Shot Chemically Competent *E. coli* bacteria cells (Invitrogen) which were cultured, the vector containing the rivulus gene was isolated using a Qiagen mini-prep

kit. DNA was sequenced using the Applied Biosystems 3730xl Genetic Analyzer (University of Maryland Genomics Core), and sequences were compared to known sequences using NCBI BLAST. Primer sequences for each gene can be found in Table 2-1.

**Optimization of QPCR assays**

The optimal melting temperature ($T_m$) and concentration for each set of primers was determined [54] (Table 2-2). QPCR was performed using a temperature gradient and three different concentrations of primers. For each set of primers, the combination of temperature and primer concentration that yielded the lowest $C_t$ value was determined and was used for all assays for each respective primer set. Standard curves were made from plasmid DNA for each gene and had acceptable efficiency (90-105%) and goodness of fit ($r \geq 0.99$).

**Temporal gene expression**

To observe any effects of temperature on gene expression during embryonic development, embryos began treatment at Stages I, II, III, IV, V, VI or VII and were removed from treatment upon reaching the subsequent stage. Immediately after removal from their treatments, embryos were snap-frozen in liquid nitrogen and stored at -80 °C until further processing. Total RNA was extracted (as described above) from individual embryos (n=5 per temperature and embryonic stage except Stage I where n=3 at 25 °C) and treated with DNase (Qiagen; 5Prime). 42.5 µl RNA was combined with 5 µl RDD buffer and 2.5 µl DNase and incubated at room temperature for 30 minutes. Following

this incubation period, 2 µl EDTA was added and each sample was incubated in a 75 °C water bath for 10 minutes and then placed on ice immediately. Total RNA quantity was determined using a Nanodrop ND-1000 and quality was determined using an Experion™ System and Experion RNA HighSens Analysis Kit (Bio-Rad). DNase-treated RNA was used to create cDNA as described above. The amount of total RNA used for each cDNA reaction was 271.2 ng. To run QPCR, SYBR Green PCR Master Mix, forward and reverse primers (see Table 2-2), and water were combined and gently vortexed. A standard curve with six points was made from a ten-fold serial dilution of plasmid DNA that contained the amplicon of interest. In cases where expression of genes in the sample was lower than the last point on the standard curve, values for data points were extrapolated to three additional points at the low end of the standard curve. Into each well of a PCR 96-well reaction plate (Bio-Rad), 15 µl of this mixture was pipetted in triplicate for standards, samples, and a no-template control. QPCR cycles were as follows: 95.0 °C for 3 min and 50 cycles of 95.0 °C for 10 sec and optimal $T_m$ (see Table 2-2) for 30 sec. A melt curve analysis was used to confirm the amplification of a single product. Plates were run on a MyiQ$^{TM}$ Single-Color Real-Time PCR Detection System (Bio-Rad).

**Statistical Analysis**

Development

Differences in mortality rates among each stage between temperatures were analyzed using a Chi-square test (SigmaStat 3.5). A Kruskal-Wallis test was performed to compare developmental time for each stage of development at the three temperatures.

A post-hoc Dunn's test was used to analyze differences in time spent at each stage of embryonic development between temperatures with $p<0.05$ indicating a significant difference.

Gene expression

To check for the presence of interplate variance, standard curves for each gene were analyzed using a general linear model (SYSTAT 13). Data for *cyp19a1b* were transformed by taking the square root of the copy number to meet the ANOVA assumption of a normal distribution. A two-way ANOVA was used to check for an interaction between temperature and stage (SigmaStat 3.5). The ANOVA assumption of residual normality was not met for *figα*, *foxl2*, *cyp19a1a*, *rpl8*, and *sox9a*. Data for *foxl2*, *rpl8*, and *sox9a* were log transformed in order to meet the assumption of equal variances. A permutation test was performed on data sets for *figα*, *foxl2*, *cyp19a1a*, *rpl8*, and *sox9a* and *P* values were generated based on 10,000 permutations (SAS). A post-hoc Tukey's test was used to assess differences across temperatures and stages. For all statistical analyses, $p<0.05$ was considered significant.

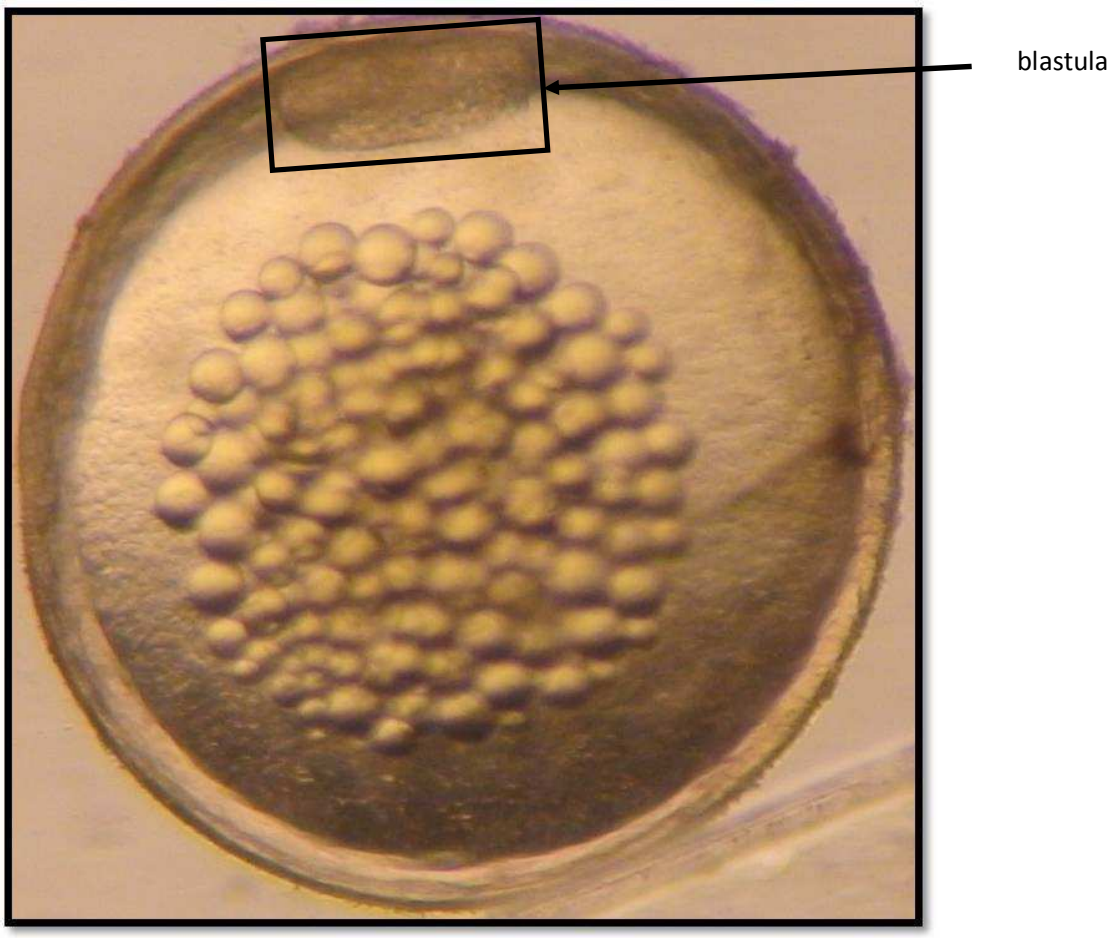



Figure 2-1. Rivulus Stage I embryo.

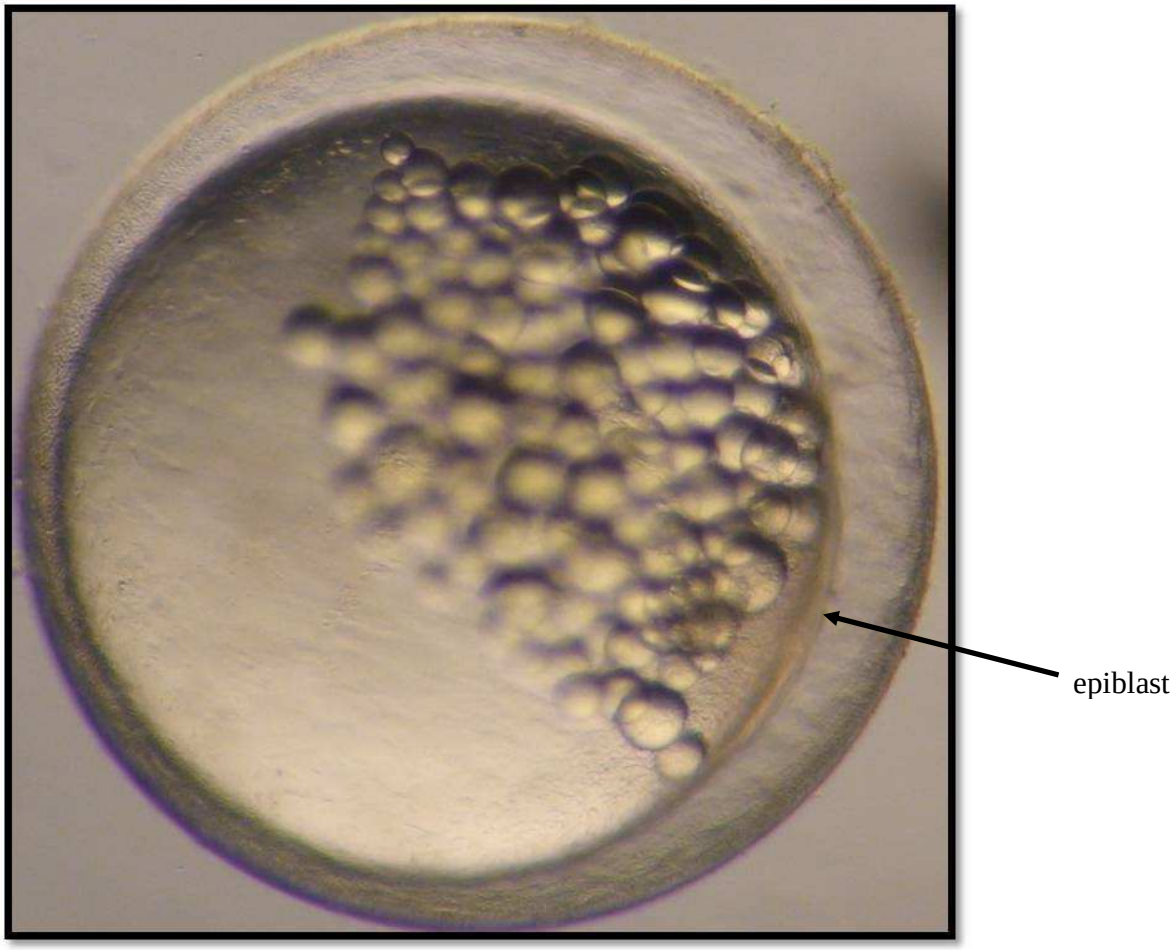



Figure 2-2. Rivulus Stage II embryo.

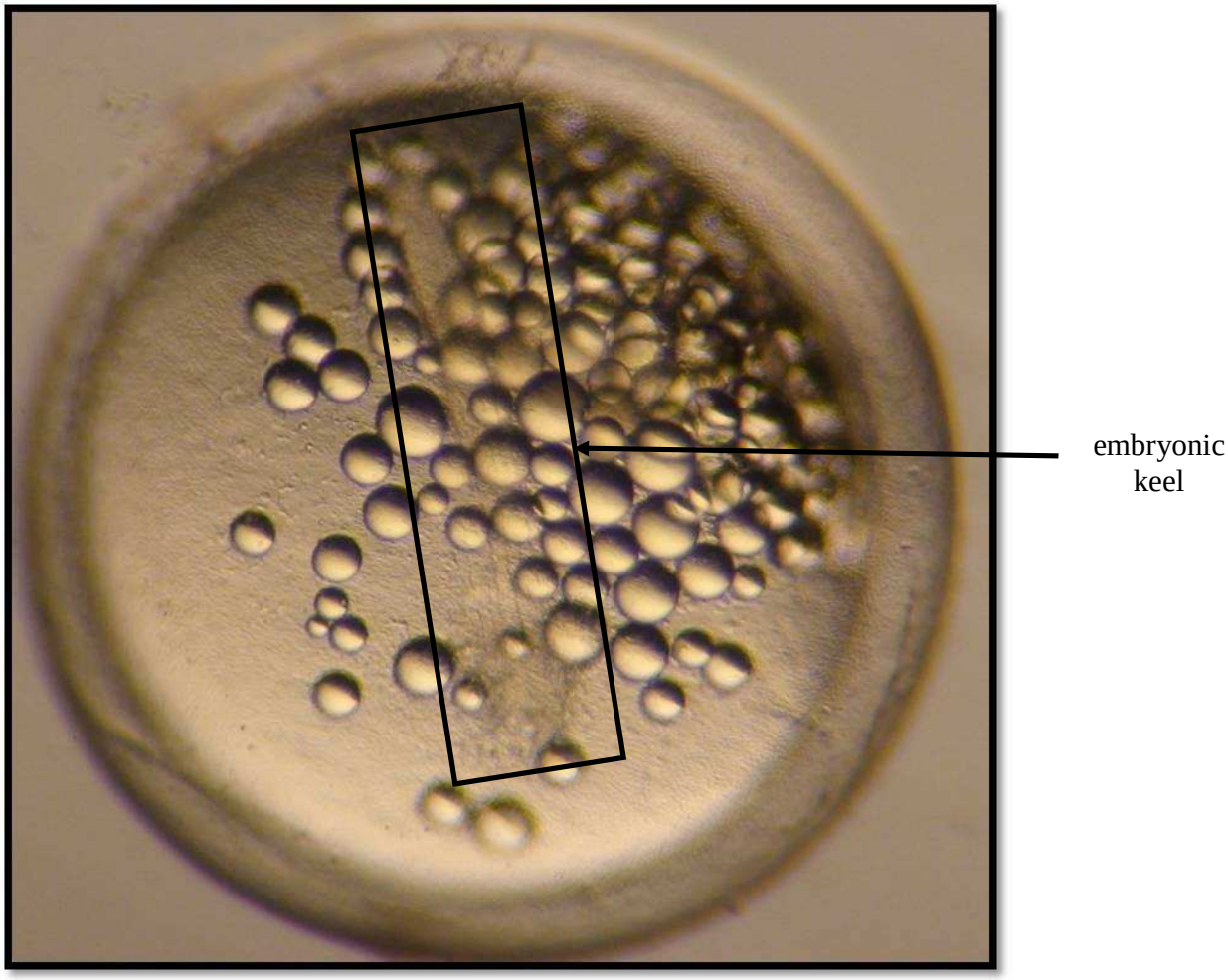



Figure 2-3. Rivulus Stage III embryo.

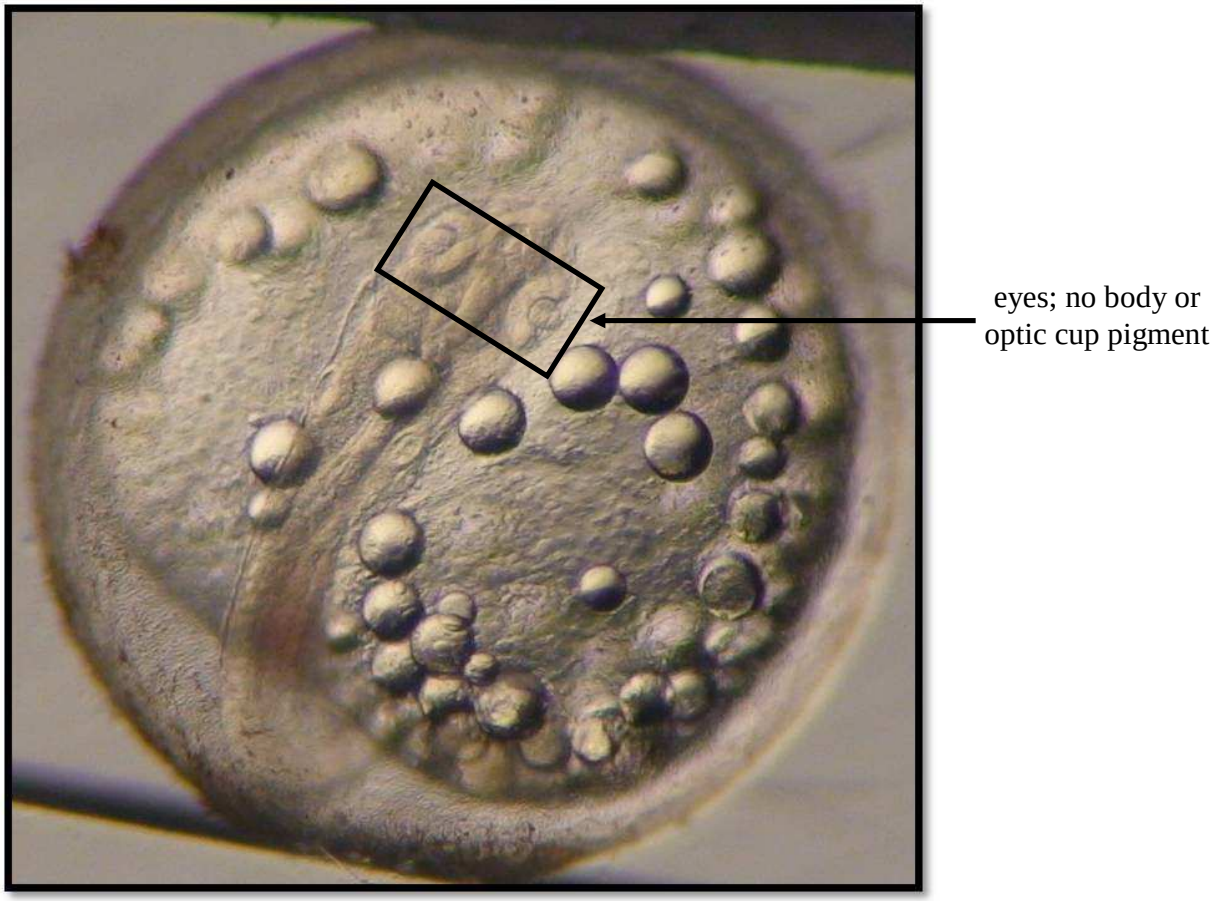



Figure 2-4. Rivulus Stage IV embryo.

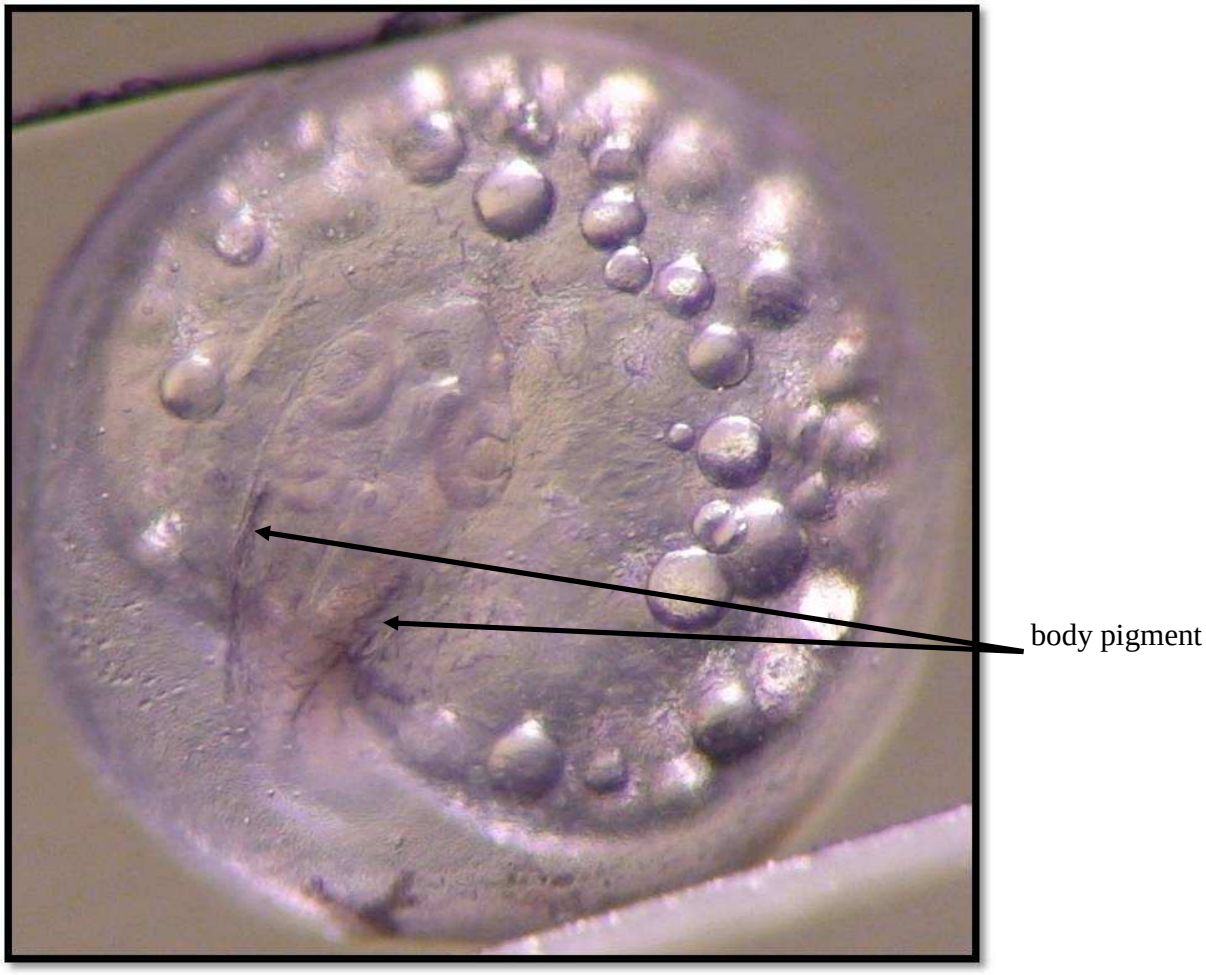



Figure 2-5. Rivulus Stage V embryo.

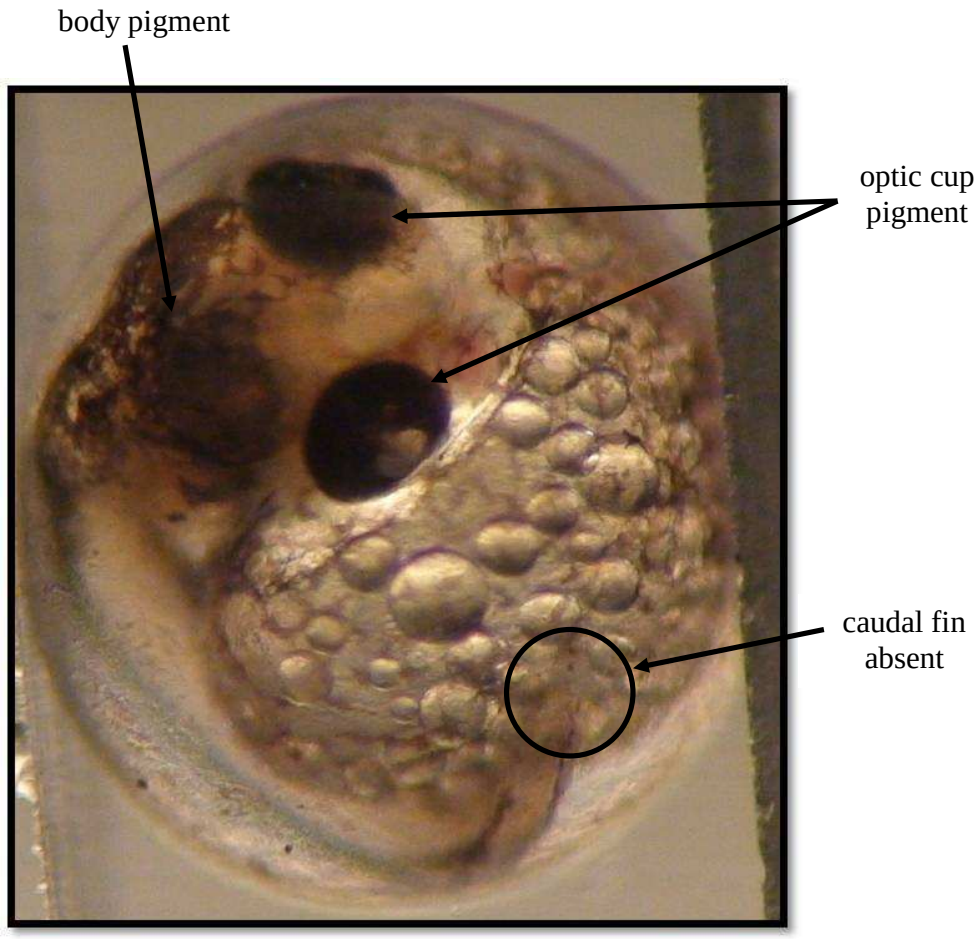


Figure 2-6. Rivulus Stage VI embryo.

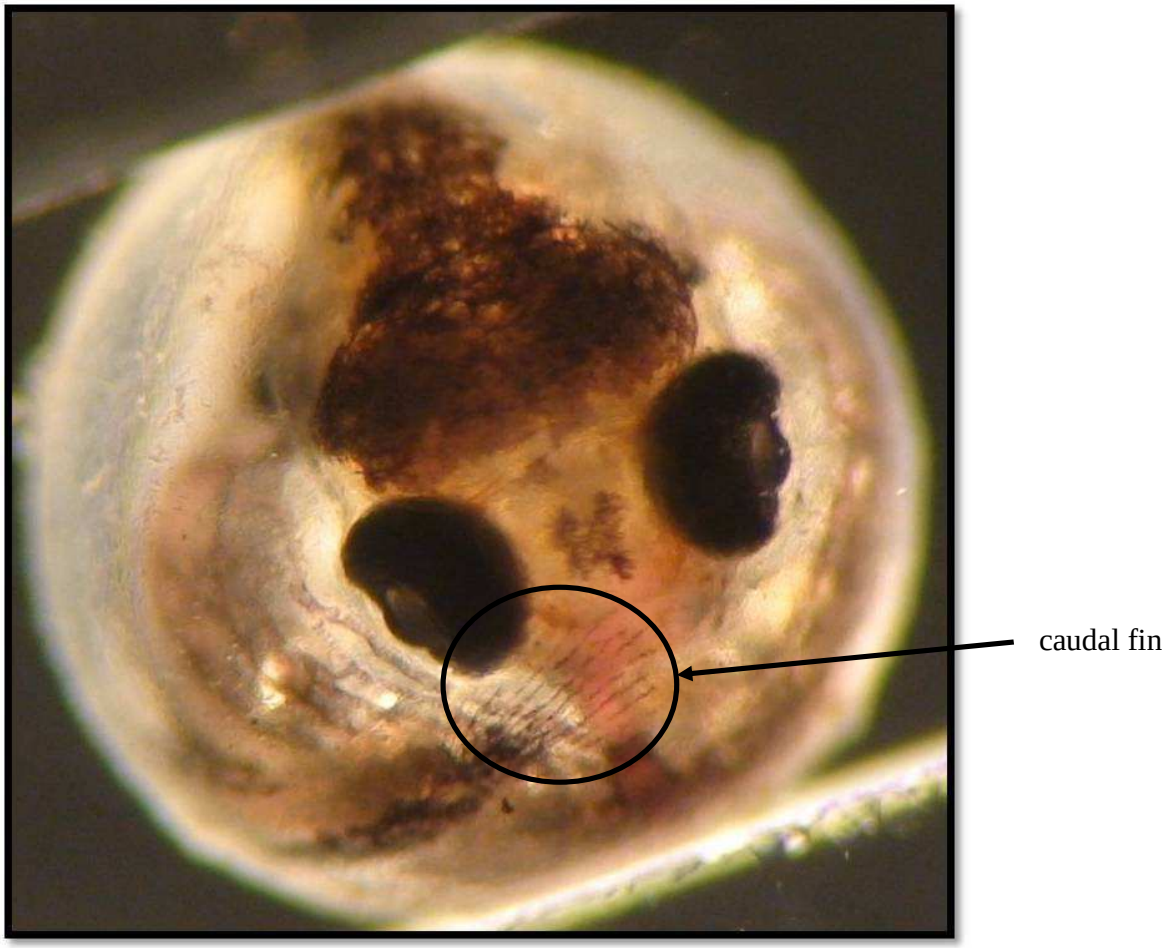



Figure 2-7. Rivulus Stage VII embryo.

| Gene | Forward Primer | Reverse Primer | GenBank Accession # |
|---|---|---|---|
| *cyp19a1a* | 5'-TCGGCATGAATGAGAGAGG-3' | 5'GGGCTTTGGCGAAATACG-3' | AB251460 |
| *cyp19a1b* | 5'-GTGGTTGACTTCACGATGCGT-3' | 5'-TGCATGAGGCCAAGGTTGA-3' | AB251459 |
| *dmrt1* | 5'-CAACTTCTACCAGCCGTCACGCTAC-3' | 5'-CGAATGCATTCGGTAC TGAGAGGACA3' | DQ683742 |
| *fig α* | 5'-CAACTTGAATGATGAACTGATACC-3' | 5'-CCACAGAGGACAGCGATA-3' | DQ683743 |
| *foxl2* | 5'-TACATCATCAGCAAGTTCC-3' | 5'-CTTGATGAAGCACTCGTT-3' | DQ683738 |
| *rpl8* | 5'-TGACAAGCCCATCCTGAAGGC-3' | 5'-GGCTATGAATCCTGTTGAGCA-3' | NM_001104909.1 |
| *sox9a* | 5'-ATCAGTACCCACATCTGC-3' | 5'-GCTTCTCTACCTCGTTGA-3' | DQ683739 |
| *sox9b* | 5'-AATCTGTCAAGAACGGTCA-3' | 5'-CTTTGAAGATCGCATTTGGA-3' | DQ683740 |

Table 2-1. QPCR primers used to amplify each gene.

| Gene | Optimal Tm (°C) | Optimal primer concentration (nM) |
|---|---|---|
| *cyp19a1b* | 54.4 | 350 |
| *dmrt1* | 59 | 250 |
| *fig* α | 58.5 | 350 |
| *foxl2* | 60 | 350 |
| *cyp19a1a* | 60.6 | 350 |
| *sox9a* | 58.3 | 350 |
| *sox9b* | 59.3 | 150 |
| *rpl8* | 59 | 350 |

Table 2-2. The optimal melting temperature ($T_m$) and primer concentration for each set of primers.

# Chapter 3: Results

## Development

During this study, mortality rates were highest at 25 °C during Stages VI and VII which were also the longest stages. During Stage I the mortality rates at 20 °C, 25 °C and 31 °C were 18%, 24%, and 18%, respectively (Figure 3-1). A chi-square test revealed that these rates were not significantly different from one another ($P = 0.724$). It should be noted that the power of this test was low (0.098). The mortality rates during Stage II at 20 °C, 25 °C and 30 °C were 33%, 17%, and 14%, respectively. There were no differences in mortality rates at the three temperatures for Stage II ($p=0.189$). However, the power for this test was also low (0.339). . All of the embryos treated at 20 °C, 25 °C and 31 °C during Stage III survived. When exposed to different temperatures during Stage IV, all embryos survived at 20 °C and 31 °C. Only 4% of embryos died at 25 °C during Stage IV. The rate of mortality during Stage IV is not different among the three temperatures ($p=0.336$). However, the power of this chi-squared test was low (0.231). . At 20 °C and 31 °C, all embryos survived Stage V but 6% of embryos at 25 °C died during Stage V. There were no differences in mortality at this stage across all three temperatures ($p=0.130$) but the power of this test was low (0.409). During Stage VI, the proportion of embryos that died was 9% at 20 °C, 34% at 25 °C, and 8% at 31 °C. A chi-square test revealed a significant difference in mortality rate during Stage VI across the three temperatures ($p<0.05$). It appears that there was a higher incidence of death at 25 °C than at 20 °C and 31 °C during Stage VI. During Stage VII, 17% of the embryos died at 20 °C, 74% of embryos died at 25 °C, and 42% of embryos died at 31 °C. The differences in mortality across the three temperatures during Stage VII are significant

($p<0.05$). It appears that the incidence of death was higher at 25 °C than at 31 °C and 20 °C. Also, at 31 °C, the incidence of death appears higher than that at 20 °C.

The median time in hours in Stage I at 20 °C, 25 °C, and 31°C was 11, 24, and 16 hours, respectively (Figure 3-2). The median time in Stage I at 25 °C is significantly higher than that at 20 °C and 31°C ($p<0.05$). The median time in Stage II at 20°C, 25 °C, and 31°C was 22, 23.5, and 24.5 hours, respectively. The median time in this Stage at each temperature is not different ($p=0.191$). The median time in Stage III at 20°C, 25 °C, and 31°C was 17, 18, and 19 hours, respectively. The median time in this stage is not different across the three temperatures ($p=0.6$). The median time in Stage IV at 20°C, 25 °C, and 31°C was 87, 44, and 20 hours, respectively. The median time in Stage IV at these temperatures is significantly different ($P<0.05$). As temperature increases, the median time in Stage IV decreases. The median time in Stage V at 20°C, 25 °C, and 31°C was 45.5, 24, and 18 hours, respectively. The median duration of Stage V at these temperatures is significantly different ($P<0.05$). As temperature increases, the median time in Stage V decreases. The median time in Stage VI at 20°C, 25 °C, and 31°C was 208, 119, and 71 hours, respectively. The median duration of Stage VI at these temperatures is significantly different ($P<0.05$). As temperature increases, the median duration of Stage VI decreases. The median time of Stage VII at 20°C, 25 °C, and 31°C was 356, 453, and 268 hours, respectively. The median duration of Stage VII at these temperatures is significantly different ($P<0.05$). As temperature increases, the length of Stage VI decreases. The median time spent in Stage VII at 31 °C is significantly shorter than that of 25 °C and 20 °C.

**Gene expression**

*cyp19a1b*

It is important to note that the original values for 76% of the data points were extrapolated. There was no significant interaction between temperature and stage ($p=0.142$) so analyses for temperature and stage consider only the overall effect. There was no effect of temperature on expression of *cyp19a1b* ($p=0.057$). However, the expression of *cyp19a1b* did vary by stage ($p<0.05$). Embryos from Stages VI and VII had a significantly higher number of *cyp19a1b* transcripts than embryos from Stages I-V (Figure 3-3). However, the number of *cyp19a1b* transcripts in embryos from Stages VI and VII were not different from each other.

*dmrt1*

The *dmrt1* gene was not expressed in any samples or the number of transcripts present was below the detectable level of this assay.

*sox9b*

The *sox9b* gene was not expressed in any samples or the number of transcripts present was below the detectable level of this assay.

*figα*

It is important to note that the original values for 99% of the data points were extrapolated. There was a significant interaction between stage and temperature ($p<0.05$). Temperature only affected the expression of *figα* during Stages I and IV

(Figure 3-4). Among Stage I embryos, the expression of *figα* was significantly higher in embryos incubated at 20 °C than at 30 °C, but there was no difference in expression levels between embryos incubated at 20 °C and 25 °C. There was also no difference in *figα* expression levels between embryos incubated at 31 °C and 25 °C. In Stage IV embryos, the mean number of *figα* transcripts was significantly higher in embryos incubated at 31 °C than those at 25 °C. There was no difference in the mean number of *figα* transcripts in embryos incubated at 31 °C compared to those at 20 °C and embryos incubated at 20 °C compared to those at 25 °C. Temperature had no effect on *figα* expression during Stages II, III, V, VI, and VII.

The number of *figα* transcripts also varied during embryogenesis. Among embryos incubated at 20 °C, there was a significantly higher number of *figα* transcripts present in Stage I embryos when compared to Stage II embryos (Figure 3-5). However, the number of *figα* transcripts in Stage I did not differ from any of the other stages and the number of *figα* transcripts Stage II did not differ from any other stages. The expression *figα* is elevated during Stage I and there is a decrease in *figα* during the remainder of embryogenesis. Interestingly, the expression of *figα* was not different among any embryos incubated at 25 °C (data not shown). Among embryos incubated at 31 °C, the mean number of *figα* transcripts during Stage IV was significantly higher when compared to Stages I, II, III and V but there were no differences between Stage IV and Stages VI or VII (Figure 3-6). When embryos are incubated at 31 °C, *figα* expression appears to be highest during the middle of embryogenesis.

*foxl2*

It is important to note that the original values for 77% of these data points were extrapolated. There was a significant interaction between stage and temperature ($p<0.05$). Temperature only had an effect on *foxl2* expression during Stage II (Figure 3-7). The mean log number of *foxl2* transcripts present in Stage II embryos incubated at 31 °C was significantly higher than those present in Stage II embryos incubated at 20 °C ($p<0.05$). There was no difference in *foxl2* expression between Stage II embryos incubated at 20 °C and 25 °C as well as between Stage II embryos incubated at 25 °C and 30 °C. During all other stages, there were also no significant differences in *foxl2* expression across the three temperatures.

Expression of *foxl2* also varied by stage at each temperature. As there was a significant interaction between stage and temperature, the expression patterns of *foxl2* during embryogenesis must be analyzed at each temperature separately. Among embryos incubated at 20 °C, the mean log number of transcripts was significantly higher during Stages IV, V, VI, and VII than at Stages I, II, and III ($p<0.05$) (Figure 3-8). It appears that at 20 °C, *foxl2* transcription is upregulated during Stage IV and the number of transcripts remained elevated until hatching.

Embryos incubated at 25 °C exhibited a similar pattern of *foxl2* expression to embryos incubated at 20 °C (Figure 3-9). The mean log number of transcripts was significantly higher during Stages IV, V, VI, and VII than at Stages I, II, and III ($p<0.05$). Similar to embryos at incubated at 20 °C, it appears that at 25 °C, *foxl2* transcription is upregulated during Stage IV and the number of transcripts remained elevated until hatching.

During embryogenesis at 31 °C, *foxl2* transcription appears to steadily increase from Stage I-VI and then decreases slightly before hatching (Figure 8-10). Stage I embryos possessed the fewest number of transcripts while embryos from Stage VI possessed the largest number of *foxl2* transcripts. The expression pattern of *foxl2* during embryogenesis at 31°C was not similar to the patterns observed at 20 °C and 25 °C.

*cyp19a1a*

It is important to note that 100% of the original values for these data points were extrapolated. There was no significant interaction between stage and temperature ($p=0.3917$) so analyses for temperature and stage consider only the overall effect. Temperature had an effect on the expression of *cyp19a1a* ($p<0.05$) (Figure 3-11). The mean number *cyp19a1a* transcripts present among embryos incubated at 20 °C was significantly less than embryos incubated at 25 °C and 31 °C. The number of *cyp19a1a* transcripts did not differ between embryos incubated at 25 °C and 30 °C.

The expression of *cyp19a1a* varied during embryogenesis. There appears to be a trend in *cyp19a1a* expression such that the number of transcripts decreases during the middle of embryogenesis and then rises until hatching (Figure 3-12). There was only a significant difference between the mean number of transcripts in Stage IV and VII ($p<0.05$). Significant differences in transcript number were not present between any other stages of embryogenesis.

*sox9a*

It should be noted that about 8% of the original values for these data points were extrapolated. There was no significant interaction between stage and temperature ($p=0.63$) so analyses for temperature and stage consider only the overall effect. There was no effect of temperature on the expression of *sox9a* (data not shown). There were significant differences in the mean log number of *sox9a* transcripts between some stages of embryogenesis ($p<0.05$) (Figure 3-13). The mean log number of *sox9a* transcripts was lowest during early embryogenesis and highest during late embryogenesis. There appears to be a trend in *sox9a* expression such that the mean log number of transcripts increased steadily throughout embryogenesis.

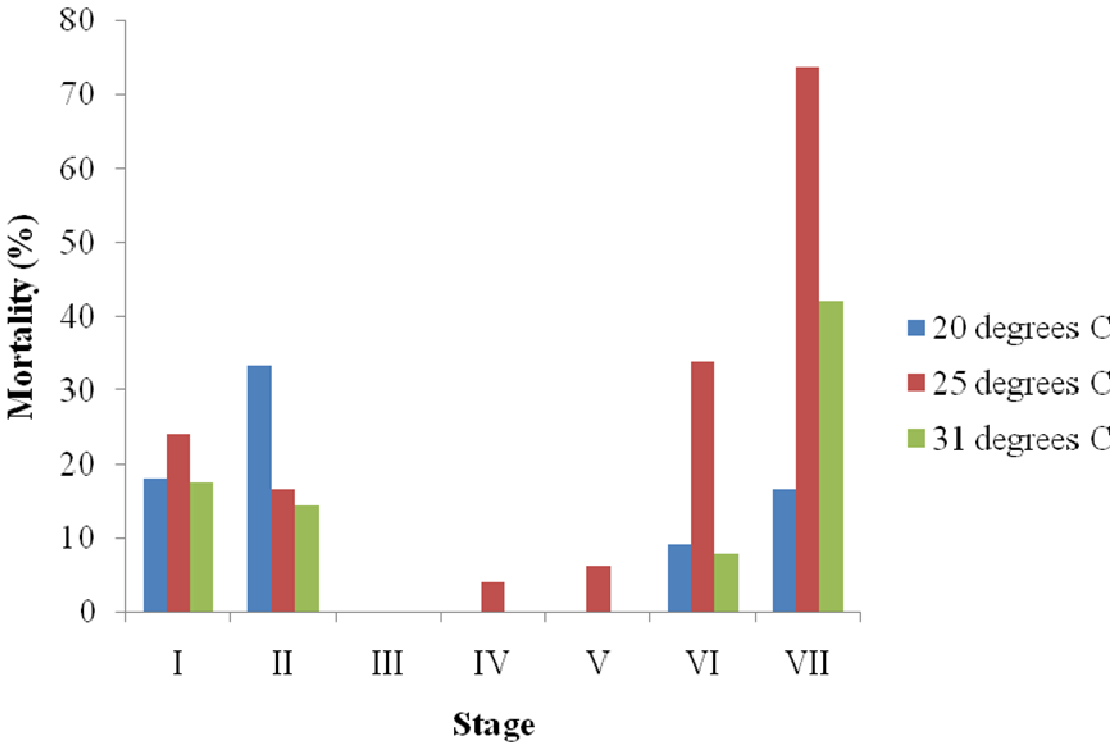
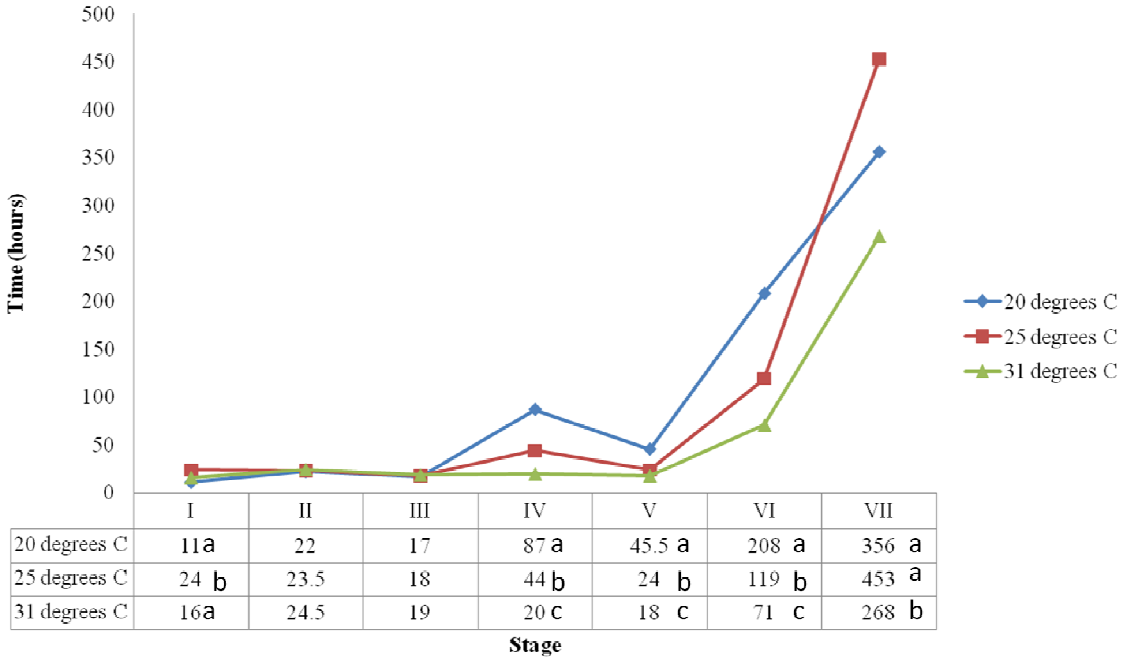



Figure 3-1. Mortality rates during embryogenesis. The percent of embryos that died during each stage at each temperature is presented. There is a significant difference in mortality rate across the three temperatures during Stage VI and VII.




| | I | II | III | IV | V | VI | VII |
|---|---|---|---|---|---|---|---|
| 20 degrees C | 11a | 22 | 17 | 87 a | 45.5 a | 208 a | 356 a |
| 25 degrees C | 24 b | 23.5 | 18 | 44 b | 24 b | 119 b | 453 a |
| 31 degrees C | 16a | 24.5 | 19 | 20 c | 18 c | 71 c | 268 b |

Stage

Figure 3-2. Rivulus embryonic development. The median time, in hours, of each stage at each temperature is presented. Among each stage, values that contain the same letter are not significantly different from one another.

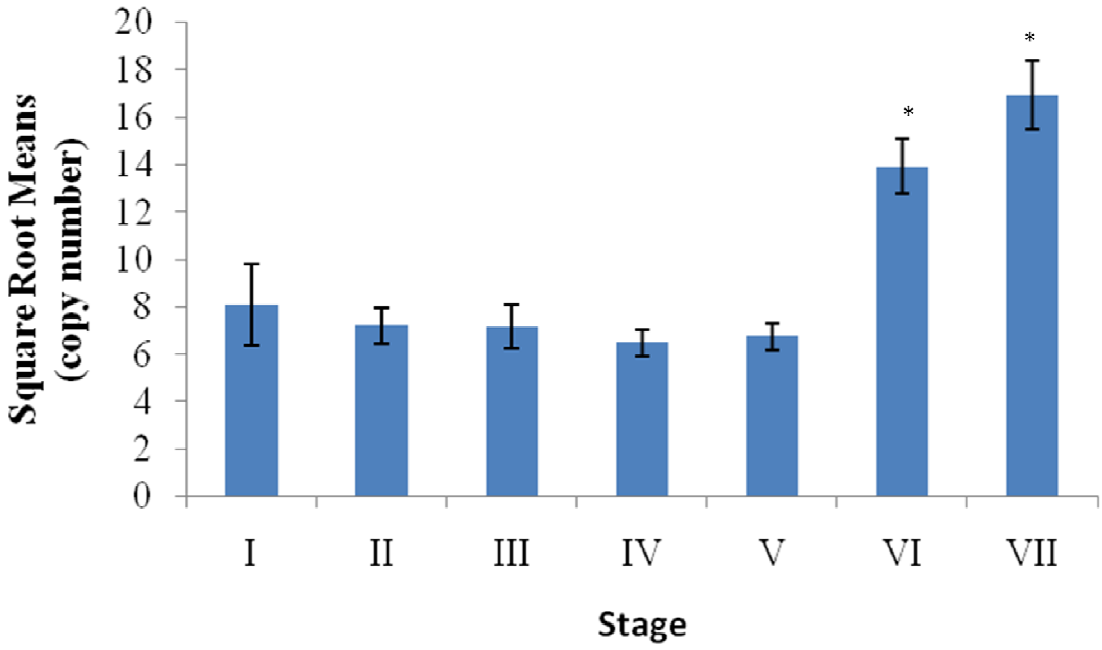



Figure 3-3. The expression patterns of *cyp19a1b* during embryogenesis. The square root-transformed mean number of *cyp19a1b* transcripts is presented for each stage. Error bars represent the standard error of the mean. An * indicates that the number of *cyp19a1b* transcripts present at that stage are significantly different from the other stages ($p<0.05$). Stages VI and VII are not significantly different from one another. n=15 for all stages except for Stage I where n=13.

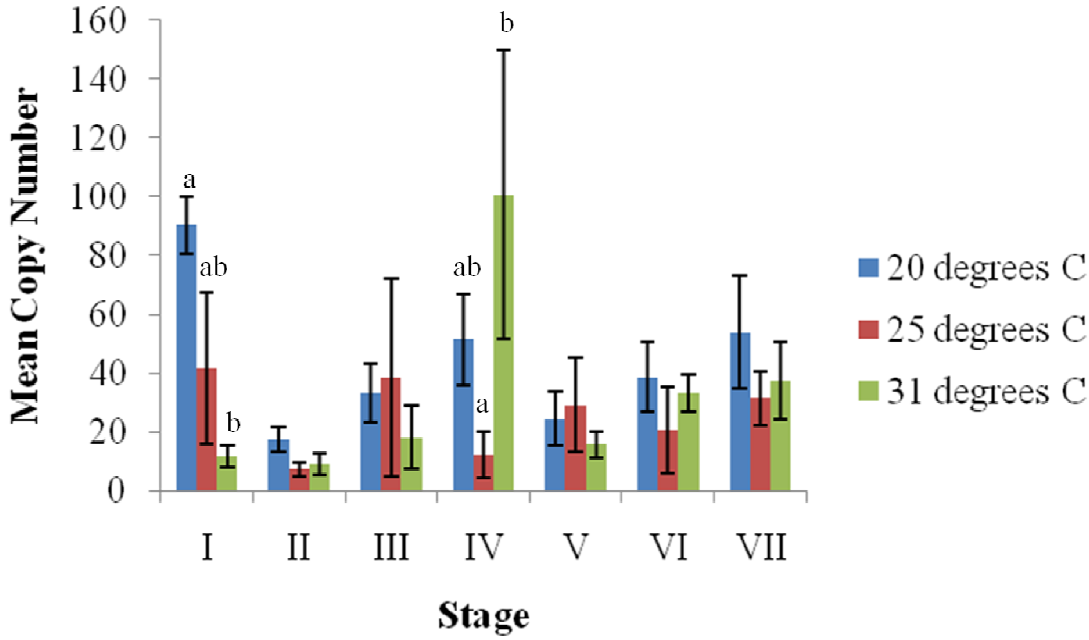



Figure 3-4. The expression of *figα* is affected by temperature during Stage I and IV. Within stages, bars that have the same letter are not different from one another. Stages without letter labels represent the lack of any difference in *figα* expression across temperatures. Error bars represent the standard error of the mean. n=5 for all treatment groups except for Stage I at 25 °C where n=3.

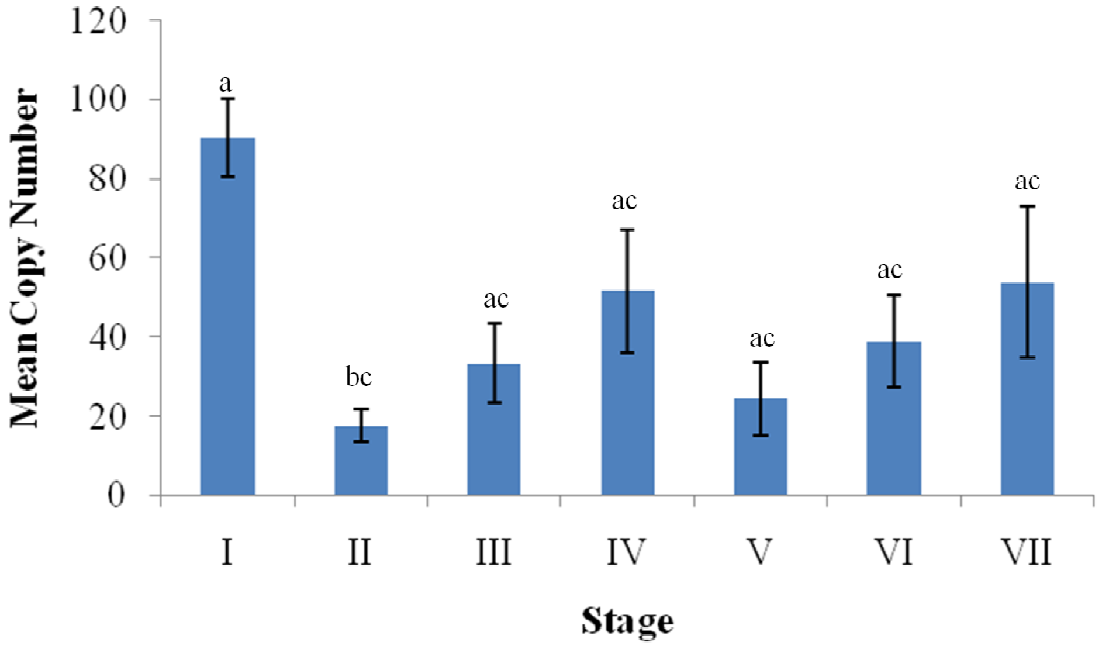



Figure 3-5. The mean number of *figα* transcripts present in embryos at 20 °C. Means having the same letter are significantly different from one another. Error bars represent the standard error of the mean. n=15 per stage.

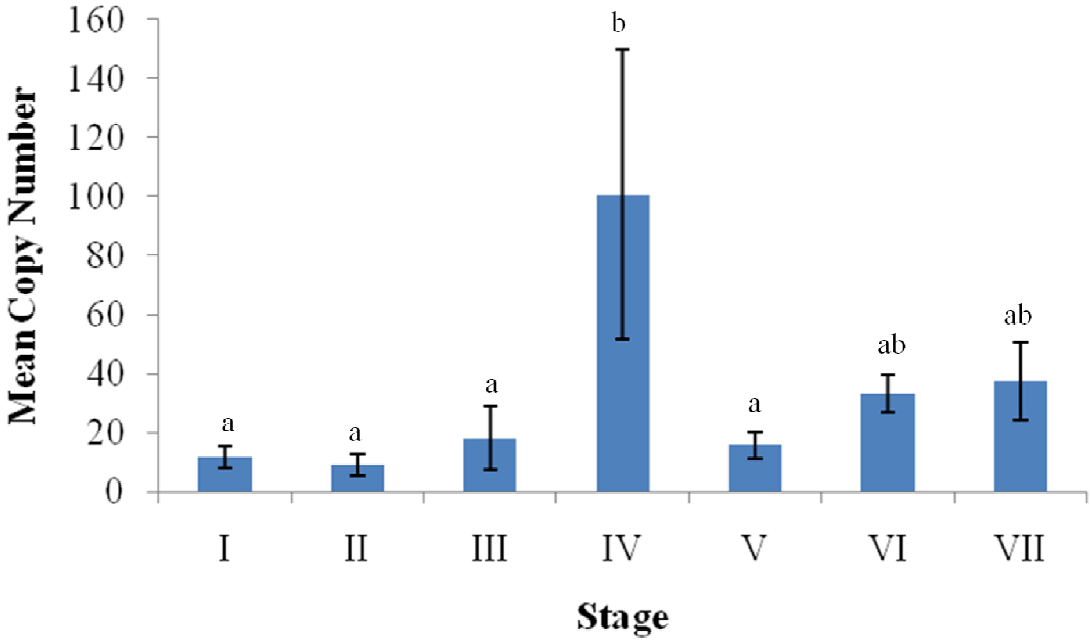



Figure 3-6. Expression pattern of *figα* during embryogenesis at 31 °C. Means with the same letter are not different from another. Error bars represent the standard error of the mean. n=15 per stage.

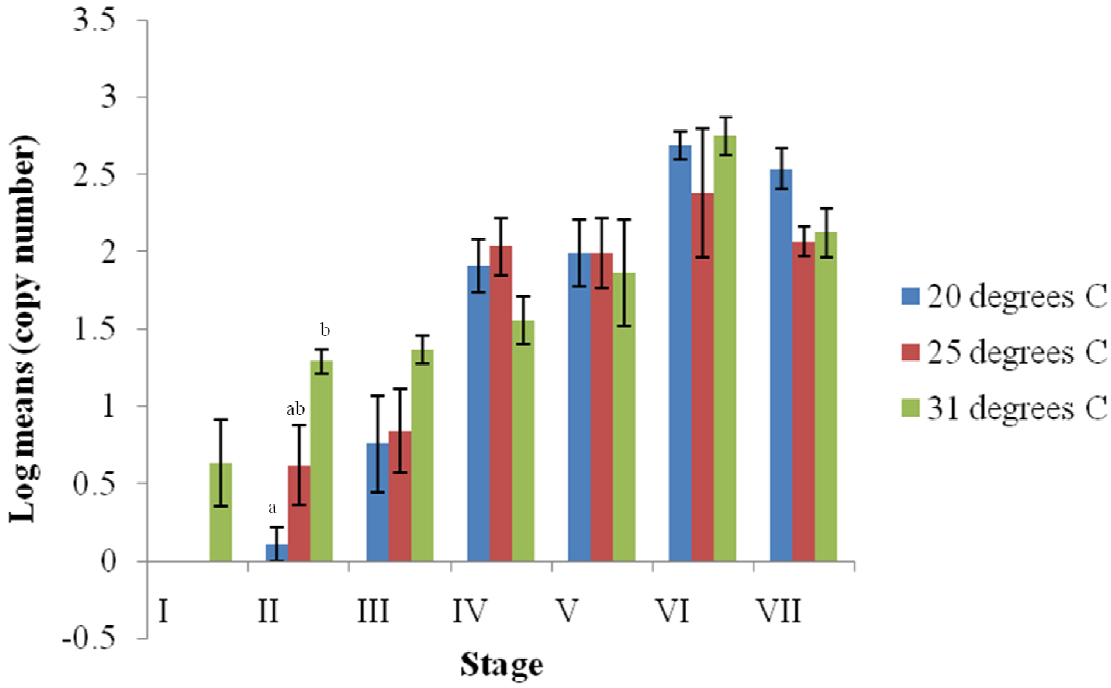



Figure 3-7. Effects of temperature on *foxl2* expression. Data were log-transformed to meet the assumption of equal variances and the means for this transformed data are presented. Temperature only had an effect on *foxl2* expression during Stage II. Means with the same letters are not different from one another. Stages without letters represent a lack of any differences across temperatures. Error bars represent the standard error of the mean. n=5 for all treatment groups except for Stage I at 25 °C where n=3.

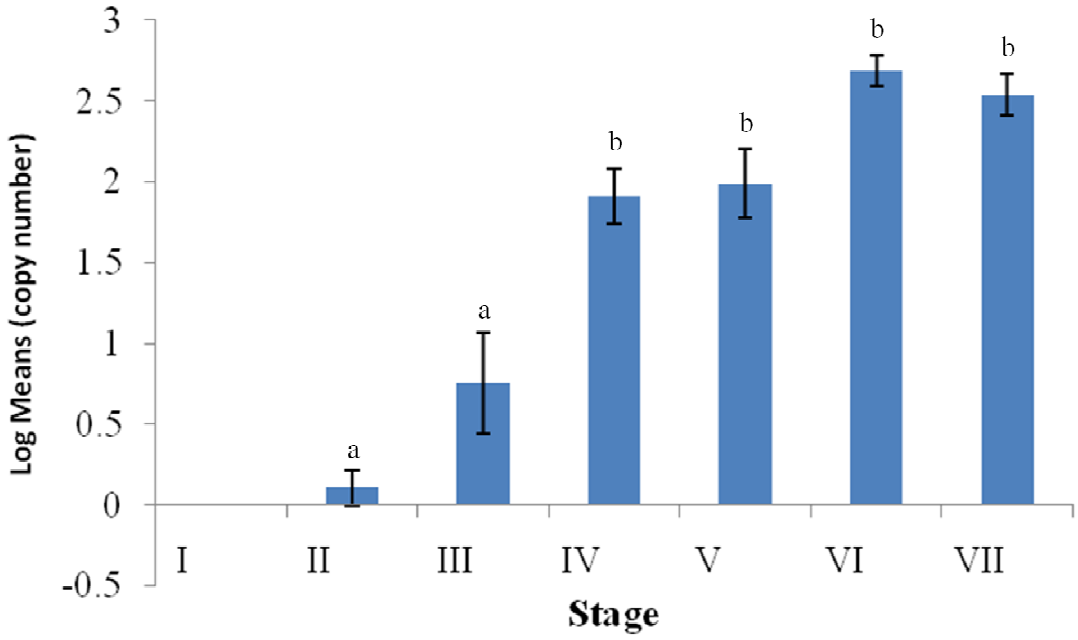



Figure 3-8. Expression pattern of *foxl2* during embryogenesis at 20 °C. Data are presented as the means of the logs of the number of transcripts. Means with the same letters are not different from one another. Error bars represent that standard error of the mean. n=15 per stage.

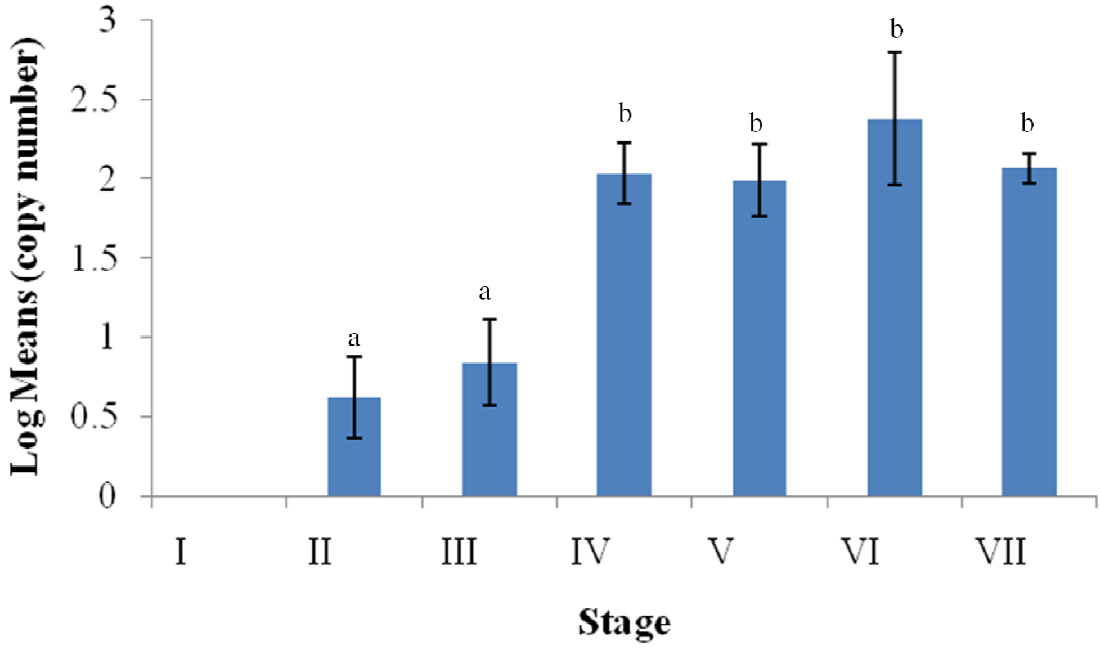



Figure 3-9. Expression pattern of *foxl2* during embryogenesis at 25 °C. Data are expressed at the mean log number of transcripts. Means with the same letter are not different from one another. Error bars represent the standard error of the mean. n=15 per stage except for Stage I where n=13.

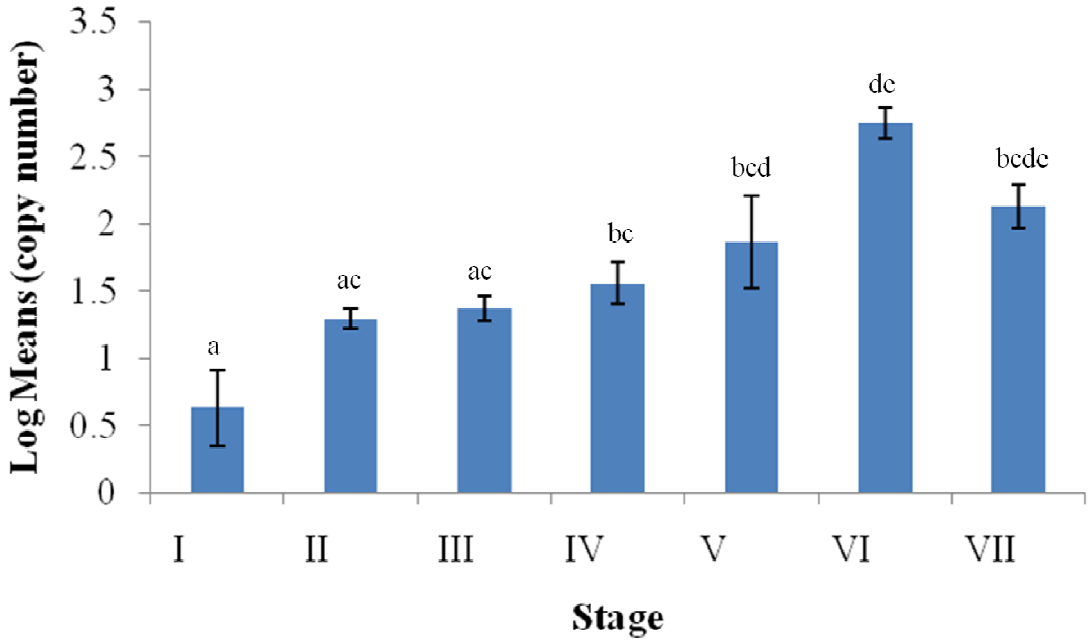



Figure 3-10. Expression pattern of *foxl2* during embryogenesis at 31 °C. Data are expressed as the mean log number of transcripts. Means with the same letter are not different from one another. Error bars represent the standard error of the mean. n=15 per stage.

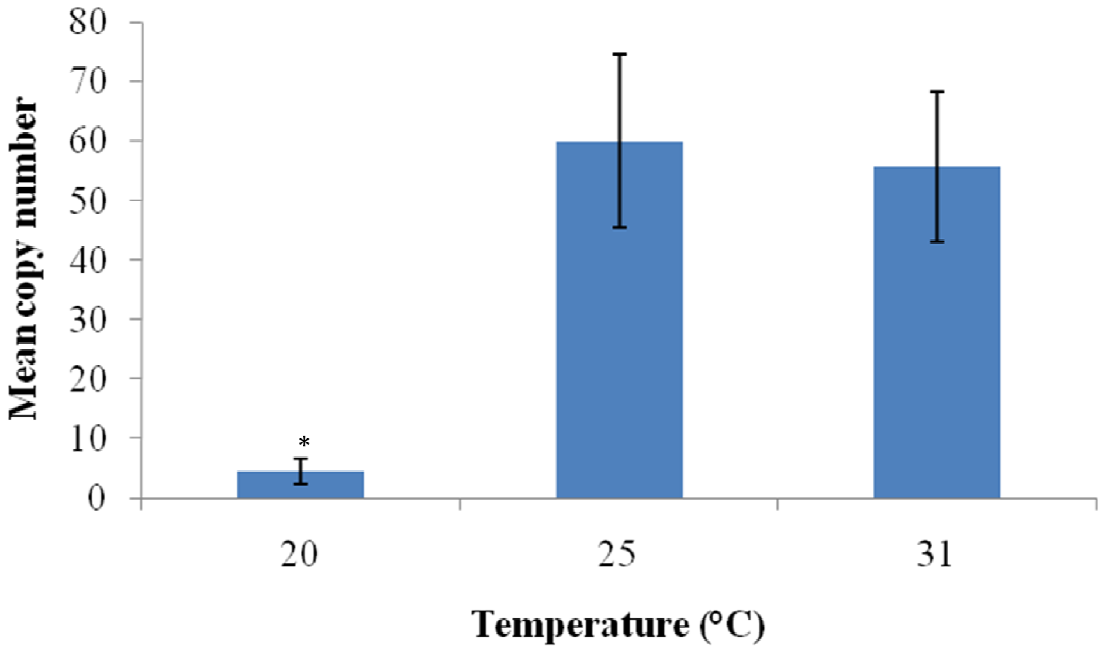



Figure 3-11. Effect of temperature on *cyp19a1a* expression. Data are presented as mean copy number. Means marked with an * are significantly different. Error bars represent the standard error of the mean. n=35 for 20 °C and 31 °C; n=33 for 25 °C.

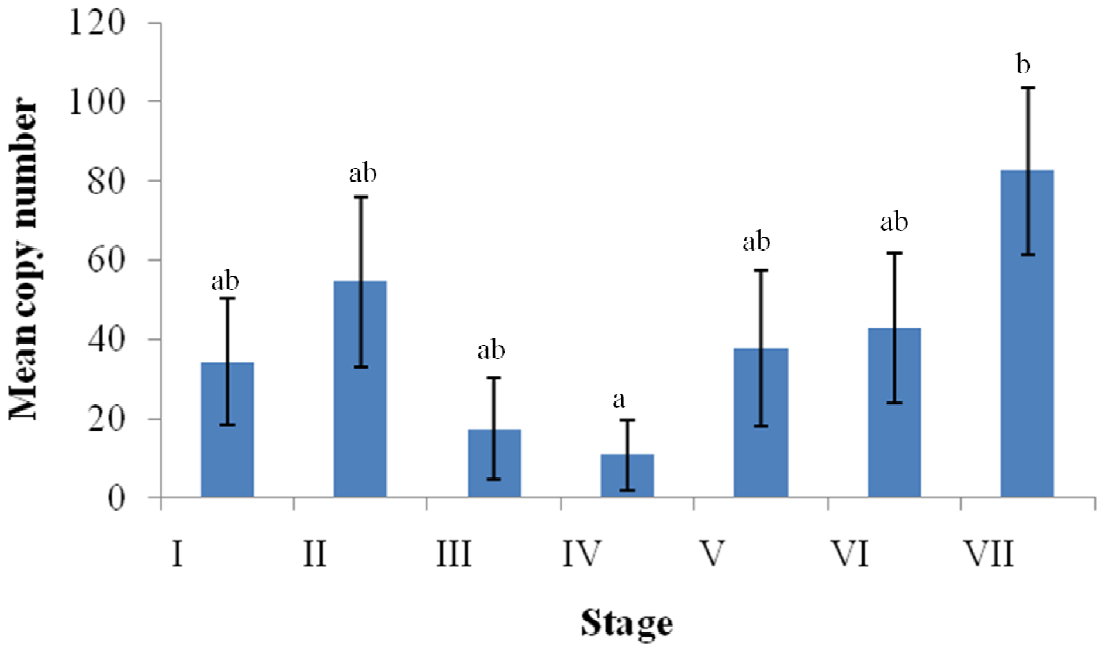



Figure 3-12. *cyp19a1a* expression during embryogenesis. Data are shown as mean *cyp19a1a* transcript number present during each stage. Means with the same letter are not different from one another. Error bars represent the standard error of the mean. n=15 for all stages expect Stage I where n=13.

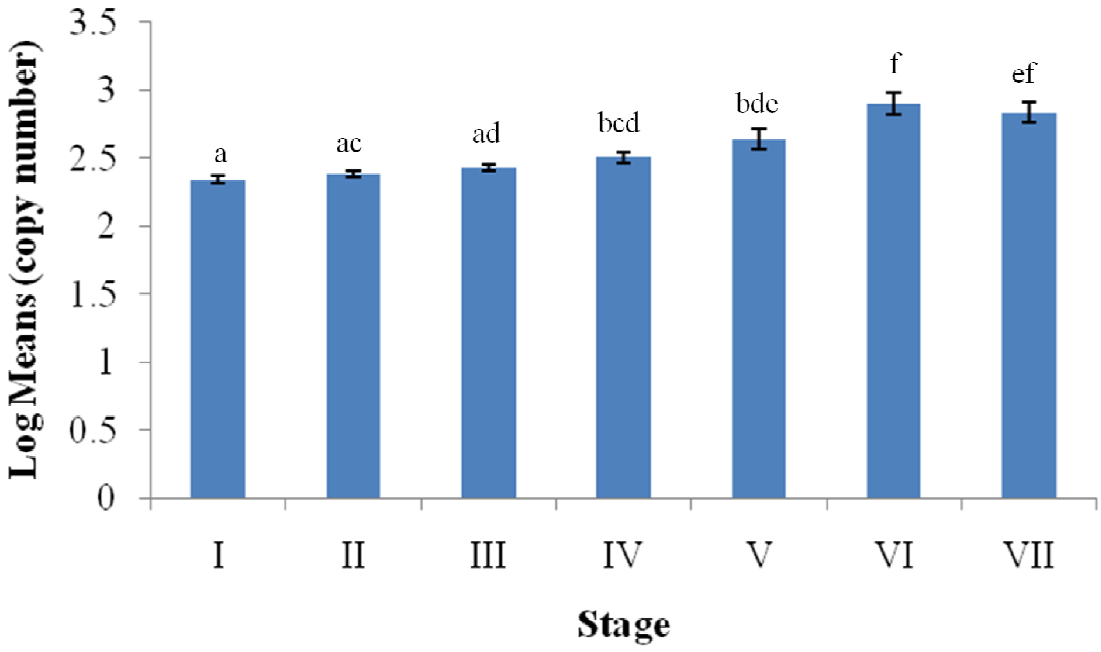



Figure 3-13. Expression pattern of *sox9a* during embryogenesis. Data are shown as the mean log number of transcripts. Means with the same letter are not different from one another. Error bars represent the standard error of the mean. n=15 for all stages expect Stage I where n=13.

## Chapter 4: Discussion

To my knowledge, this is the first study to examine the expression of sex determining genes across all of embryogenesis (from fertilization to hatching) in rivulus. This is also the first study in rivulus that has examined the effect of temperature on gene expression in rivulus. The results of this study show minimal effects of temperature on expression of the seven genes measured in this study. However, the expression levels of these seven genes did exhibit various patterns across the period of embryogenesis. This study also provides information on the baseline levels of gene expression for *dmrt1, sox9a, sox9b, figα, cyp19a1b, cyp19a1a,* and *foxl2* during embryogenesis at three temperatures, which will help provide a better understanding of the basic biology of this unique vertebrate species that is growing in popularity as a model species. From this study, it also became apparent that future gene expression studies on individual rivulus embryos will have some limitations, and it is recommended that embryos be pooled in order to measure gene expression more accurately. Fortunately, variation that may arise from pooling embryos from this species is not of high concern as isogenic strains can be easily established.

Temperature did not appear to affect the rate of mortality except during Stage VI and VII (Figure 3-1). However, these stages are also the two longest stages out of all seven stages used in this study allowing ample time for the different temperature treatments to affect developmental processes that are critical for survival (Figure 3-2). It appears that the mortality rate was highest among embryos incubated at 25 °C during Stages VI and VII. It would be expected that mortality rates at 25 °C would be significantly lower than 20 °C and 31 °C as 25 °C is considered ambient for rivulus.

However, rivulus embryos are known to estivate and are more likely to do so during later stages of development. Thus, embryos that appeared dead may have been estivating and were discarded resulting in a skewed measurement of mortality rates. It is also possible that rivulus embryos do not estivate at temperatures that deviate from ambient temperature (for example, 20 °C and 31 °C). As a result, the higher mortality rates measured during stages VI and VII at 25 °C in this study may be an artifact of the experimental design. However, more information on this behavior among rivulus embryos is needed and may be an interesting focus of future research projects.

In this study, the expression of *cyp19a1b* was not affected by temperature. In general, the expression levels of *cyp19a1b* were nearly the same at 20 °C, 25 °C, and 31°C. *cyp19a1b* codes for the aromatase enzyme that is found predominately in the brain. As this enzyme is responsible for converting androgens to estrogens, and as estrogens are known to play important roles in the development of ovarian tissue, it might be expected that this gene would be sensitive to temperature in a species that possesses temperature-dependent sex determination. In this case, it would be expected that this gene would be downregulated in embryos at the male-producing temperature of 20 °C. However, there is evidence for the involvement of estrogens in neurogenesis [56]. If estrogens are vital for normal development and growth of the brain, the maintenance of *cyp19a1b* expression and function would be critical for survival of the organism. Thus, it is possible that *cyp19a1b* may be part of a mechanism that is resistant to alterations that may result from environmental factors in order to allow for survival of the animal.

The lack of apparent sexual dimorphism of *cyp19a1b* in rivulus embryos from this study also agrees with a lack of sexual dimorphism of *cyp19a1b* in zebrafish embryos and

adults [38, 56]. The pattern of rivulus *cyp19a1b* expression in this study also agrees with the pattern observed by Lee et al. (2006). Lee et al. (2006) observed a rise in *cy19a1b* expression just before hatching. Although the classification of developmental stages between this study and that done by Lee et al. differ, a significant rise in *cy19a1b* expression was also observed in this study (Figure 3-3). Although, *cy19a1b* expression does not differ between male- (20 °C) and hermaphrodite- (25 °C) producing temperatures, the consistency in expression patterns of this gene provide valuable information regarding normal development in rivulus and can be used as a marker for deviations from normal embryogenesis in rivulus.

In this study, *dmrt1* was not expressed in any rivulus embryos. Although this gene has been identified as a male-specific marker, the lack of *dmrt1* expression in rivulus embryos is not surprising. Previous studies in rivulus show that *dmrt1* expression increased at 39 daf among fish that were treated with methyltestosterone [21]. Therefore, *dmrt1* expression increased only in fish that had hatched and this relatively early expression was likely a result of treatment with an exogenous hormone. With the exception of these MT-treated fish, *dmrt1* was otherwise only expressed in adult testicular tissue.

Studies in rivulus have shown expression of *dmrt1* in testicular somatic cells of adult ovotestis and testes [21]. In some species, such as medaka, *dmrt1* has been associated with early testicular differentiation [23]. However, this does not appear to be the case in rivulus, as *dmrt1* was not expressed in any embryos. As rivulus also appear to pass through a juvenile “female” stage regardless of final sexual outcome, similar to what has been observed in zebrafish, it is possible that the embryos had not yet completed this

transition making detection of any male-specific gene markers unlikely [35]. The lack of involvement of *dmrt1* early in the pathway of sex determination in rivulus is likely as temporal expression of *drmt1* does vary among teleosts [57]. In this case, *dmrt1* may play an important role in testis development during juvenile stages or may be more important for maintenance of testicular tissue in adult rivulus.

In this study, *sox9b* was also not expressed in any embryos. Similar to *dmrt1*, it is likely that *sox9b* may play a more important role in gonad development and/or maintenance following embryogenesis. Teleost fish are also unique in comparison to mammals, birds, and reptiles as they possess two *sox9* isoforms that are the result of a whole-genome duplication event following the divergence of ray-finned and lobe-finned fishes [58]. It has been suggested that the *sox9a* isoform retained its function in the testis in catfish, *Clarias gariepinus*, while *sox9b* evolved a function in the ovary [59]. However, in other fish species, such as carp, *Cyprinus carpio*, and the Philippine medaka, *sox9b* expression has been localized to testicular tissue [33, 60]. Therefore, involvement of *sox9a* and *sox9b* in the development of ovarian and testicular tissue may vary depending upon the species.

Regardless of this potential sexual dimorphism, *sox9b* was not expressed in rivulus embryos. Most other studies that have measured expression levels of *sox9b* in fish used adult tissues. A study that observed differences in *sox9a* and *sox9b* expression in medaka embryos utilized whole-mount in situ hybridization [61]. Unfortunately, this study could not provide information regarding the involvement of *sox9b* in the differentiation of the gonad. Thus, it appears that *sox9b* expression may be difficult to measure in embryos, especially if individuals are used. However, we may again

speculate that *sox9b* is involved in testicular differentiation and/or maintenance as its expression was not detected in rivulus embryos that may pass through a juvenile "female" stage similar to zebrafish. Future studies on *sox9b* expression in juvenile and adult rivulus may provide valuable information regarding this hypothesis.

Temperature had an effect on *figα* expression only during Stages I and IV. Interestingly, the expression of *figα* was significantly higher among embryos at 20 °C than embryos incubate at 31°C. If 20 °C is the temperature at which males develop, we would expect *figα* expression to be low in these embryos. One possible explanation for this observation is maternal transfer of mRNA. At this cold temperature, the breakdown or inhibition of maternally transferred genes may be slowed. Currently, it is not known when the mid-blastula transition occurs in rivulus embryos, so the *figα* expression that was detected may be RNA that was transferred from the parent. Additionally, almost all of the data points for *figα* expression were extrapolated and may be slightly inaccurate.

Although, *figα* expression remained constant at 25 °C, its expression was highest during the middle of embryogenesis at 20 °C and 31 °C (Figures 3-5 and 3-6). *figα* is an oocyte-specific marker, so its expression in rivulus embryos is interesting. In the Kanamori et al. study, *figα* was shown to increase at 39 daf [21]. Yet again, this study did not measure *figα* expression during embryogenesis. In a study on zebrafish sex differentiation, a peak in *figα* expression occurred at the time of expected gonadal differentiation of the ovary [45]. Unfortunately, the peak in *figα* expression in this study does not correspond with the phenocritical period during rivulus embryogenesis. However, the involvement of *figα* in rivulus sex determination cannot be eliminated. It is possible that *figα* is critical for oocyte development in rivulus and morphological effects

resulting from this alteration in gene expression may be observable in juveniles and adults.

Similar to *figα*, the effect of temperature on *foxl2* expression was also limited. In this case, temperature only affected *foxl2* expression during Stage II where expression of *foxl2* was significantly higher among embryos incubated at 31 °C than those incubated at 20 °C. As a majority of the original values for these data points were extrapolated, this difference may be an artifact of the assay. However, at all three temperatures, *foxl2* appeared to increase during embryogenesis. *foxl2* is known to play a major role in the development of ovarian tissue and has been identified as an early factor in the pathway to ovarian differentiation [57]. As a transcription factor, early expression of *foxl2* would be expected in order for downstream effects to occur. In turn, it is not surprising that, in catfish, genes such as *foxl2* were expressed earlier than genes directly related to steroidogenesis [62]. Thus, increased expression of *foxl2* during embryogenesis would be necessary if ovarian-specific genes downstream of *foxl2* are to be regulated.

The lack of effect of temperature on *foxl2* expression may again be attributed to the passage of all rivulus through a juvenile "female" stage. With all embryos, including those at the known male-producing temperature, exhibiting similar *foxl2* temporal expression patterns, the hypothesis for development of ovarian tissue before development of any testicular tissue is supported. Furthermore, in another hermaphroditic fish species, the protogynous wrasse, *Halichoeres trimaculatus*, *foxl2* expression was not sexually dimorphic [63]. Future studies on gene expression of sex determining genes may reveal differences in *foxl2* expression in juvenile or adult rivulus. It is also possible that in hermaphroditic species, the regulation or role of *foxl2* differs from that of gonochores.

There was also no effect of temperature on *sox9a* expression in rivulus embryos. In Nile tilapia, *Oreochromis niloticus*, *sox9a* was also measured using QPCR and expression was not different between males and females until histological differences could be observed [64]. It is possible that a similar situation occurs in rivulus. As ovarian tissue in rivulus cannot be observed histologically until 28 dph, any sexual dimorphism in *sox9a* expression may not occur until after embryogenesis. At this point, the organ(s) to which *sox9a* is localized in rivulus is unknown and discriminating between expression in ovarian or testicular tissue was not possible in this study. However, as this gene is expressed all throughout embryogenesis, it is hypothesized that, in rivulus, *sox9a* expression may be more highly expressed in ovarian tissue. As the other ovarian-specific genes measured in the study were present in most embryos and no testicular-specific genes could be detected, further validation for this hypothesis exists. If rivulus passes through a "female" stage regardless of final sexual outcome, ovarian tissue will develop first before regressing in male fish and the concomitant expression of *sox9a* would then be correlated with ovarian development.

However, *sox9a* is also known to play an important role in chondrogenesis [65]. Therefore, the *sox9a* expression observed throughout embryogenesis may be a result of the formation of cartilage. An inability to form cartilage would likely result in the demise of the embryo making *sox9a* expression crucial for survival. A gene that is absolutely necessary for survival may then be resistant to alterations that could result from changes in the environment, including temperature. The expression of *sox9a* increased slightly during embryogenesis and expression was highest before hatching which may also provide evidence for involvement of *sox9a* in the development of cartilage in rivulus.

The only gene that was affected by temperature in this study was *cyp19a1a*. The expression of *cyp19a1a* was significantly down-regulated among embryos incubated at 20 °C. It was predicted that the transcription of *cyp19a1a* would be down-regulated at the male producing temperature as this gene codes for an enzyme that ultimately makes estrogen. At a male-producing temperature, one might expect that estrogens would be lower in abundance. This data may also indicate that the higher temperature in this study (31 °C) does not effectively create males and rivulus do not display a similar temperature-dependent sex determination pattern as is found in some flatfish [10]. However, it is important to note that the original values for all these data points were extrapolated and the effects seen here may be artifacts of the assay.

Additionally, the temporal expression pattern of *cyp19a1a* in this study does not agree with the expression patterns of *cyp19a1a* observed in other studies on rivulus; however, this may be due to genetic differences among rivulus strains used in each study. In this case, we can only compare expression patterns for the later stages of embryogenesis. Previous studies demonstrated a rise in *cyp19a1a* expression during the middle of embryogenesis with a quick decline in expression until hatching and slightly beyond hatching [39]. In this study, *cyp19a1a* expression appeared lowest during the middle of embryogenesis and expression appeared to rise from the middle of embryogenesis until hatching and was highest just before hatching.

Interestingly, this rise in *cyp19a1a* expression just before hatching correlates with the fact that *foxl2* is known to bind to and upregulate *cyp19a1a* [26]. As *foxl2* expression increased before that of *cyp19a1a*, it is likely that *foxl2* upregulates *cyp19a1a* in rivulus. As temperature did not have an effect on *foxl2*, ovarian differentiation may be

altered as a result of effects downstream of *foxl2*. A study that measures gene expression and gonad morphology of juveniles and adults will provide more insight into details of ovarian differentiation in rivulus.

The expression of a potential housekeeping gene, *rpl8*, was measured in this study (Appendix A). Temperature did affect *rpl8* expression; however, an effect could only be seen during Stage I. Expression of *rpl8* appeared to increase steadily during embryogenesis at all three temperatures. Therefore, for this experiment, *rpl8* does not act as a housekeeping gene.

In this study, the same amount of total RNA was loaded into each cDNA reaction and although *rpl8* expression varied during Stage I at different temperatures, expression was only higher at 31 °C and this difference was restricted to Stage I. Therefore, it is reasonable to speculate that equal amounts of total RNA were accurately added to each cDNA reaction and the results observed in this study represent an accurate measure of gene expression in rivulus. Many sources will also argue that housekeeping genes are unreliable and more than one housekeeping gene may be necessary and that housekeeping genes are specific to each experiment. Although *rpl8* may not be an appropriate housekeeping gene for this study, future studies looking at the effects of temperature on gene expression in rivulus should consider the possibility of other genes that may serve as housekeeping genes. As a housekeeping gene for a developmental study will also likely be difficult to find, other methods of validating normalization of RNA samples or analyzing QPCR data should be considered.

In this study, gene expression of conserved sex-determining genes was measured in rivulus embryos that were incubated at 20 °C, 25 °C, and 31 °C. Only *cyp19a1a*

expression appeared to be altered by temperature. The other genes chosen for this study did not exhibit any changes in expression levels between the three temperatures. Most importantly, there were no changes in expression levels during the phenocritical period defined by Harrington which was contained in Stage VII of this study [20]. However, this does not exclude the possibility that temperature has an effect on gene expression in rivulus which ultimately results in the development of hermaphrodites and males. This study only measured gene expression in rivulus embryos. It is possible that the effect of temperature would not be observable until later in development following embryogenesis. It is also possible that temperature affects other genes not measured in this study (ex. *dax1*, *sf1*, and *wt1*) and these genes may be more directly involved in early sex determination in rivulus. Additionally, temperature may exert effects on other factors such as enzyme activity or protein levels, which were not measured in this study. Further research on how sex is determined in this unique organism will provide a better understanding of the intricate balance that allows for the development of an ovotestis in rivulus, and how this balance may be altered to produce males among a species that consists predominantly of hermaphrodites.

## Appendix A: *rpl8*, a potential housekeeping gene.

### QPCR Results

Only one data point from this set of data was extrapolated. There was a significant interaction between stage and temperature ($p<0.05$). Temperature had an effect on *rpl8* transcription only during Stage I of embryogenesis ($p<0.05$) (Figure A-1). The mean log number of transcripts was significantly higher among Stage I embryos incubated at 30 °C than at 20 °C and 25 °C. The mean log number of transcripts did not vary between Stage I embryos incubated at 20 °C and 25 °C and there were no differences in the mean log number of transcripts across temperatures during Stages II-VII.

Among embryos incubated at 20 °C, there appears to be a slight trend in the number of *rpl8* transcripts such that *rpl8* expression increases from Stage I-IV and then the number of transcripts remained constant until hatching (Figure A-2). A similar trend for *rpl8* expression was observed for embryos incubated at 25 °C (Figure A-3). The mean log number of transcripts during Stages II and III was significantly higher than Stage I ($p<0.05$). The mean log number of transcripts during Stages IV-VII was significantly higher than Stages I-III ($p<0.05$). At 25 °C, *rpl8* expression seemed to increase from Stage I-IV and then the number of transcripts remained constant until hatching. Embryos incubated at 31 °C did not exhibit an *rpl8* expression pattern similar to embryos incubated at 20 °C and 25 °C. The number of *rpl8* transcripts appears to be relatively consistent over development at 31 °C (Figure A-4). However, there was a significant difference in the number of *rpl8* transcripts present early in development

(Stages I and II) compared to the later stages of embryonic development (Stages VI and VII) ($p<0.05$).

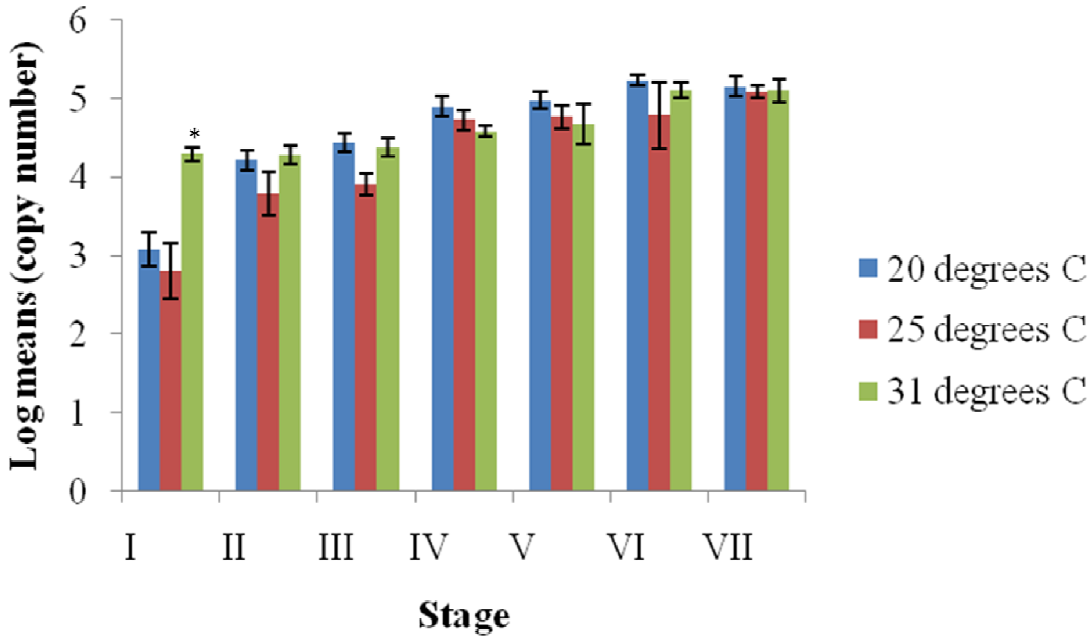



Figure A-1. Effect of temperature on *rpl8* expression. Data are shown as the mean log transcript number present during each stage at each temperature. An * indicates a significant difference. Comparisons were made only within stages. Error bars represent the standard error of the mean. n=5 per treatment group except for Stage I where n=3 at 25 ℃.

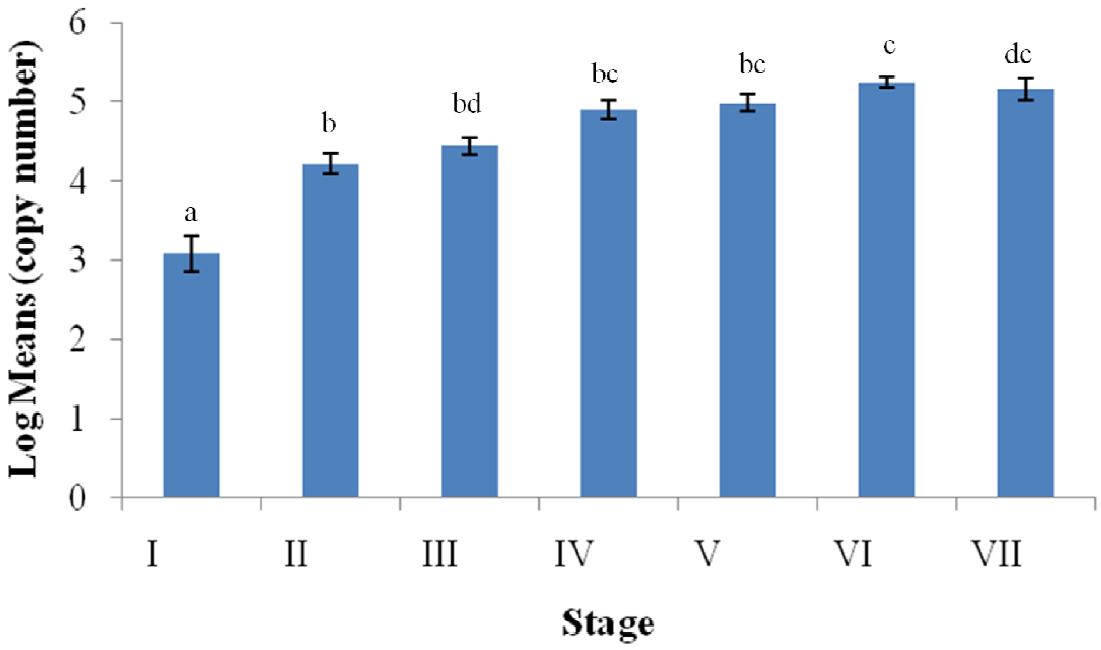



Figure A-2. Expression pattern of *rpl8* during embryogenesis at 20 °C. Data are shown as the mean log number of transcripts. Means with the same letter are not different from one another. Error bars represent the standard error of the mean. n=5 per stage.

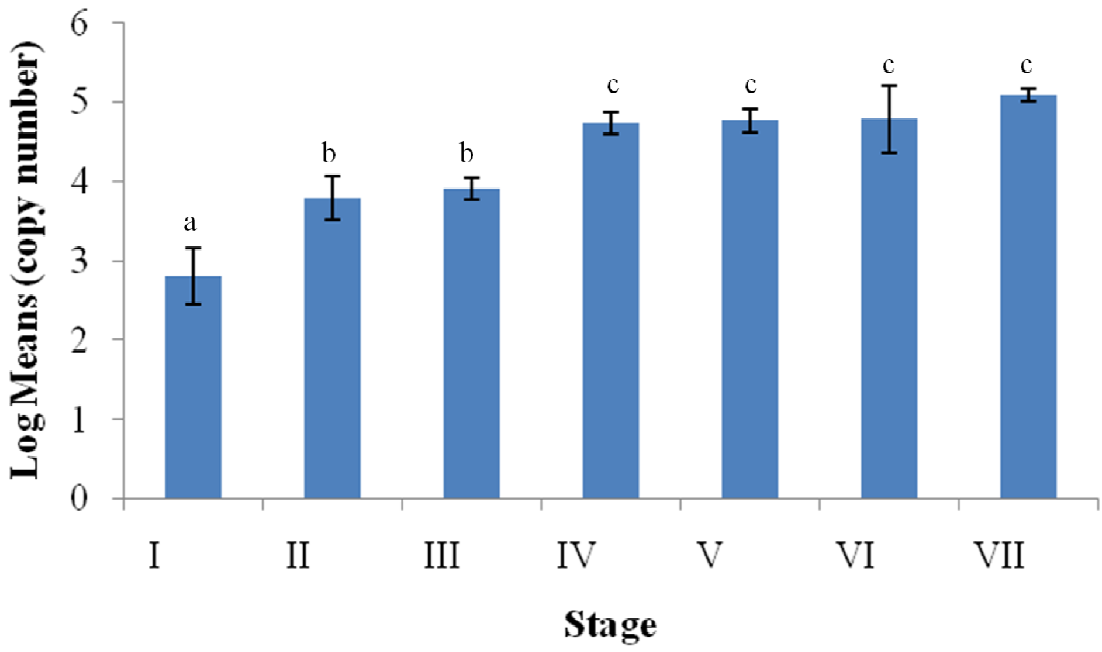



Figure A-3. Expression pattern of *rpl8* during embryogenesis at 25 °C. Data are shown as the mean log number of transcripts. Means with the same letter are not different from one another. Error bars represent the standard error of the mean. n=5 per stage except Stage I where n=3.

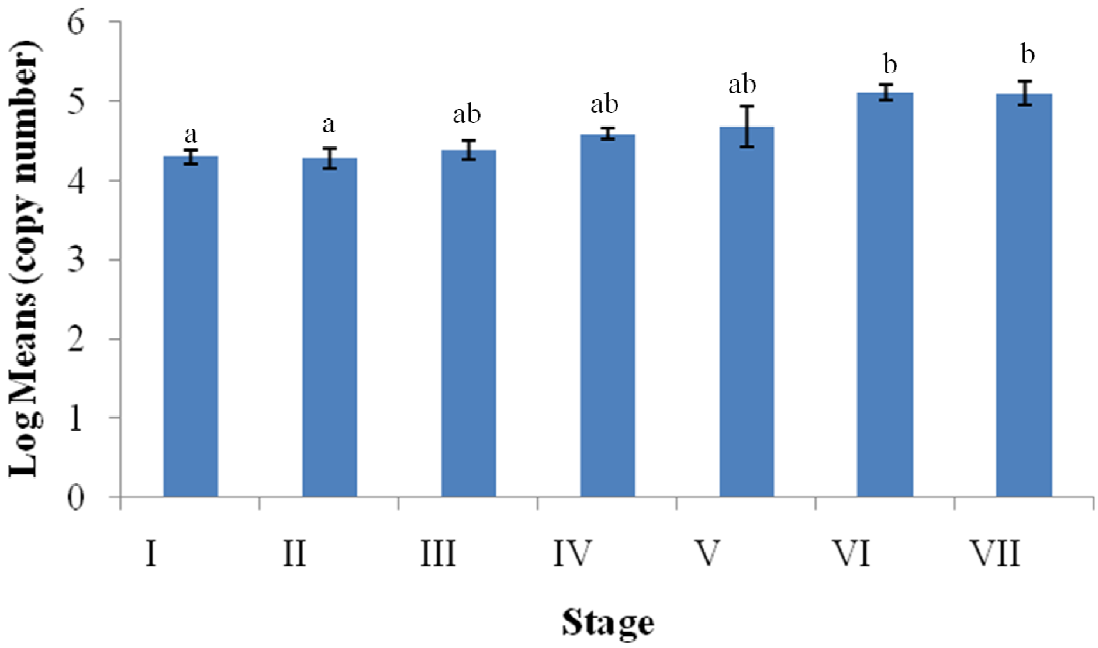



Figure A-4. Expression pattern of *rpl8* during embryogenesis at 31 °C. Data are shown as the mean log number of transcripts. Means with the same letter are not different from one another. Error bars represent the standard error of the mean. n=5 per stage.

## Appendix B: Raw data

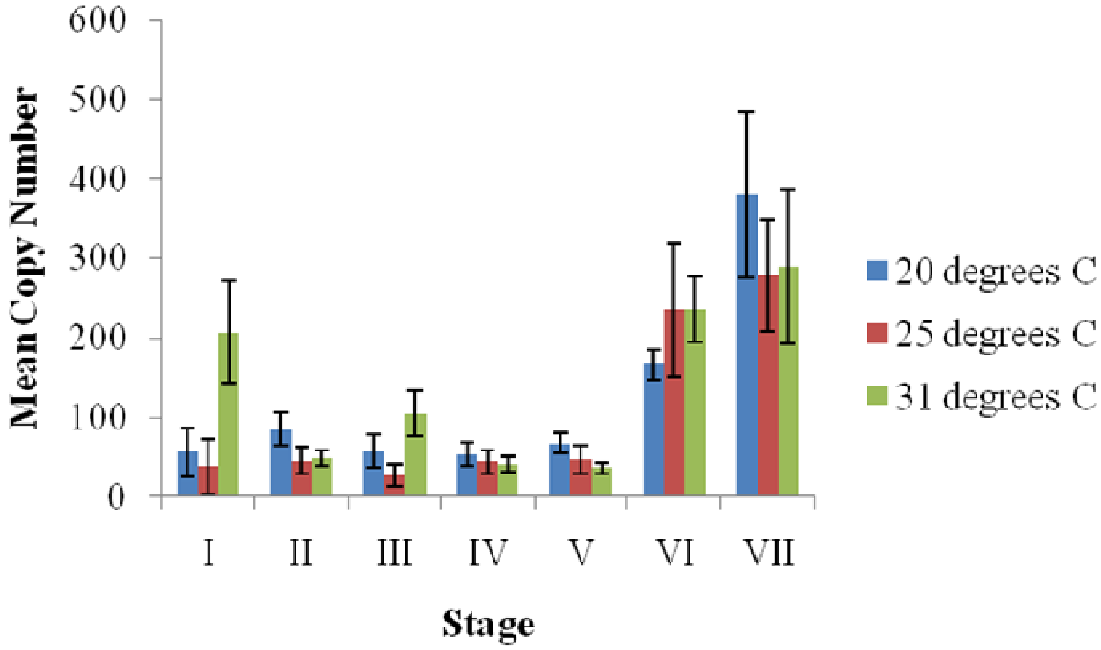



Figure B-1. *cyp19a1b* expression during each stage at each temperature. Error bars represent the standard error of the mean. n=5 per treatment group except for Stage I where n=3 at 25 °C.

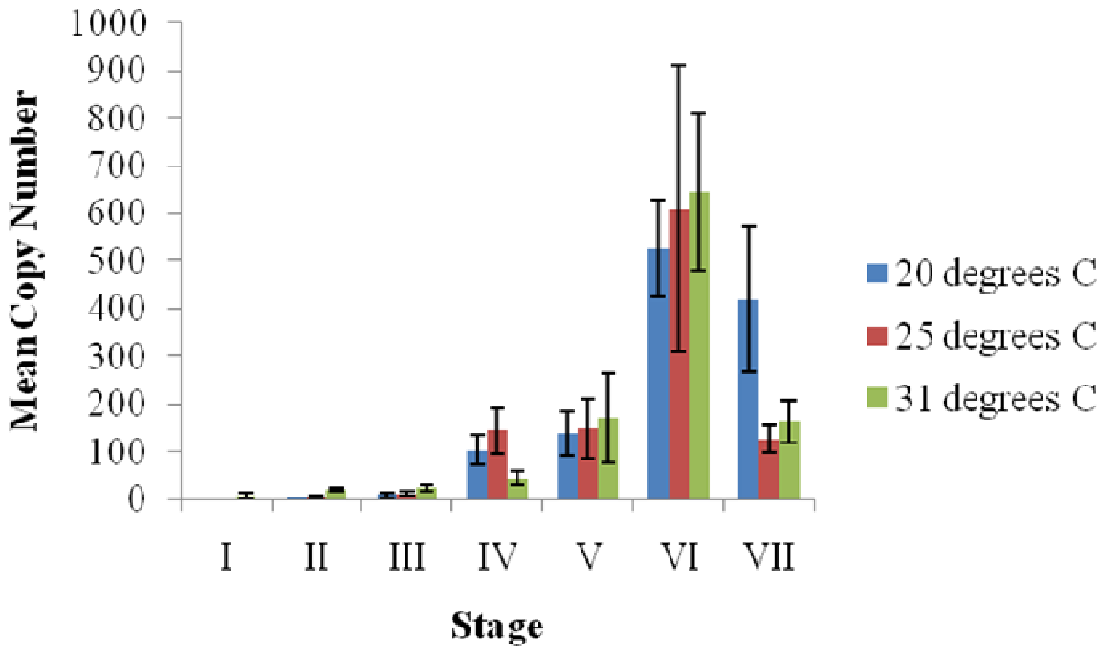



Figure B-2. *foxl2* expression during each stage at each temperature. Error bars represent the standard error of the mean. n=5 per treatment group except for Stage I where n=3 at 25 °C.

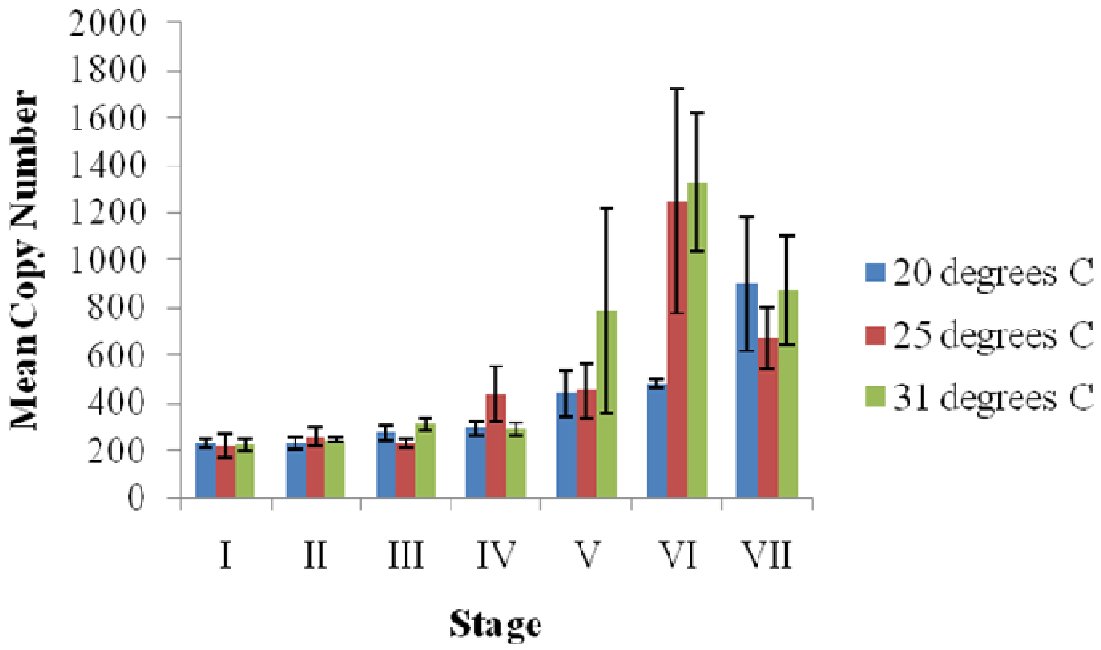



Figure B-3. *sox9a* expression during each stage at each temperature. Error bars represent the standard error of the mean. n=5 per treatment group except for Stage I where n=3 at 25 °C.

## Appendix C: Gel images

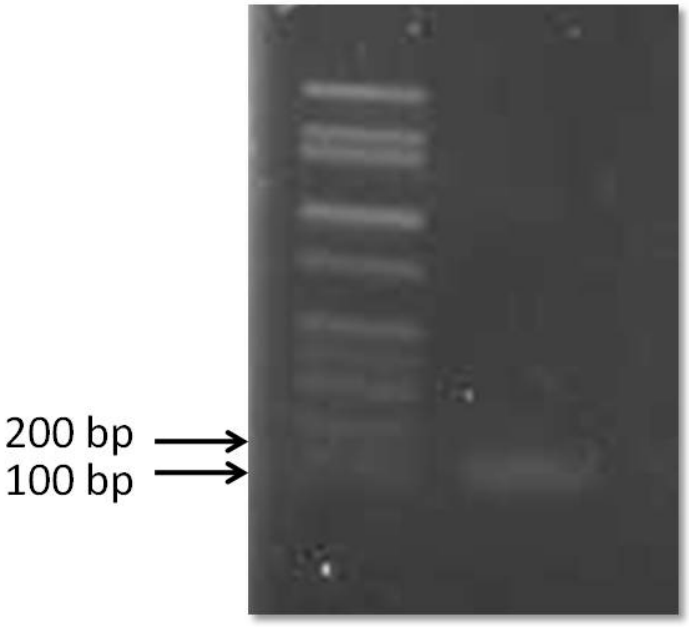



Figure C-1. *cyp19a1b* PCR product on an agarose gel stained with ethidium bromide.

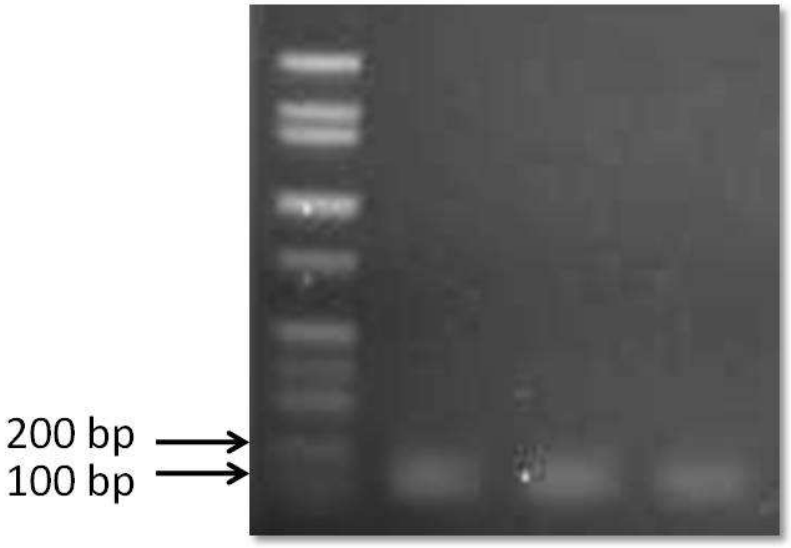



Figure C-2. *dmrt1* PCR product on an agarose gel stained with ethidium bromide.

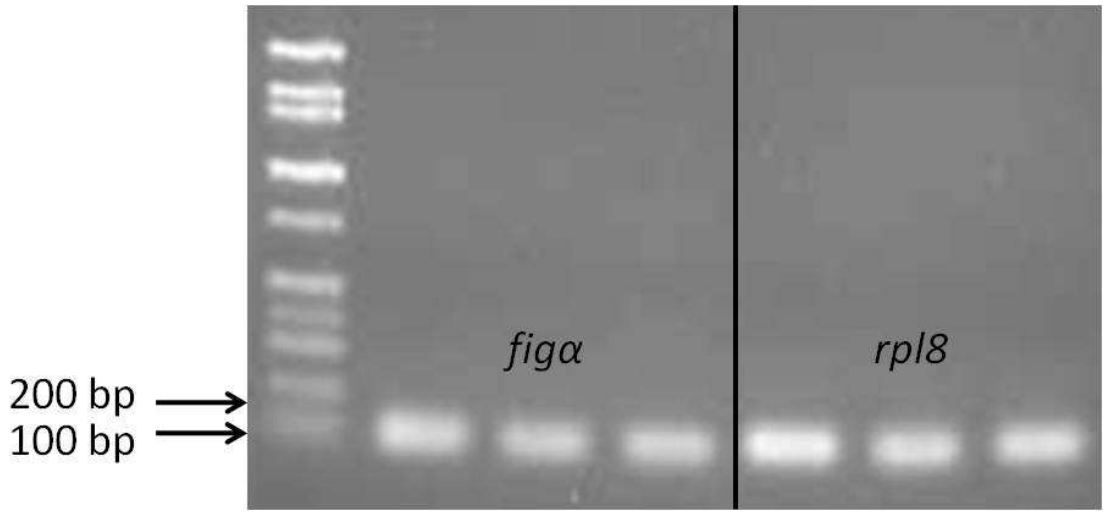



Figure C-3. *figα* and *rpl8* PCR products on an agarose gel stained with ethidium bromide.

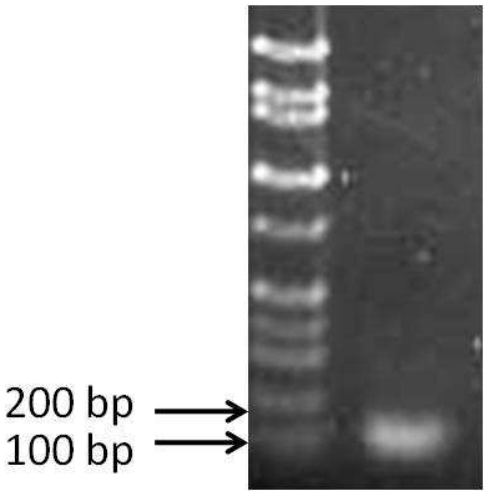



Figure C-4. *foxl2* PCR product on an agarose gel stained with ethidium bromide.

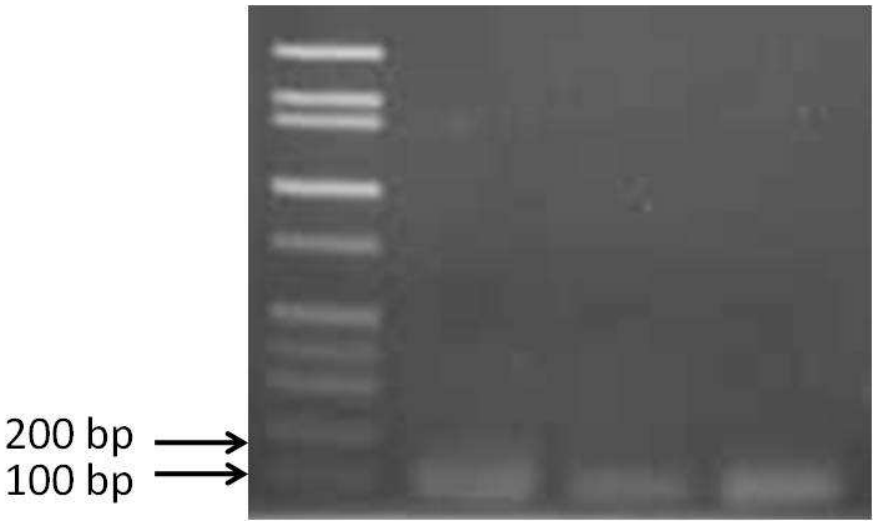



Figure C-5. *cyp19a1a* PCR product on an agarose gel stained with ethidium bromide.


Figure C-6. *sox9a* and *sox9b* PCR products on an agarose gel stained with ethidium bromide.

## References


1. Gilbert SF. Developmental Biology. Sunderland: Andrew D. Sinauer; 2006.
2. Brennan J, Capel B. One tissue, two fates: molecular genetic events that underlie testis versus ovary development. Nature Reviews. Genetics 2004; 5:509 - 521.
3. DiNapoli L, Capel B. SRY and the Standoff in Sex Determination. Molecular Endocrinology 2008; 22:1-9.
4. Baroiller JF, Guiguen Y, Fostier A. Endocrine and environmental aspects of sex differentiation in fish. Cellular and Molecular Life Sciences 1999; 55:910-931.
5. Devlin RH, Nagahama Y. Sex determination and sex differentiation in fish: an overview of the genetic, physiological, and environmental influences. Aquaculture 2002; 208:191-364.
6. Orlando EF, Guillette LJ, Jr. Sexual dimorphic responses in wildlife exposed to endocrine disrupting chemicals. Environmental Research 2006; 104:163-173.
7. Munday PL, White JW, Warner RR. A social basis for the development of primary males in a sex-changing fish. Proceedings of the Royal Society B-Biological Sciences 2006; 273:2845-2851.
8. Bull JJ. Sex determination in reptiles. Quarterly Review of Biology 1980; 55:3-21.
9. Lang JW, Andrews HV. Temperature-dependent sex determination in crocodilians. Journal of Experimental Zoology 1994; 270:28-44.
10. Luckenbach JA, Borski RJ, Daniels HV, Godwin J. Sex determination in flatfishes: Mechanisms and environmental influences. Seminars in Cell and Developmental Biology 2009; 20:256-263.
11. Yamahira K, Conover DO. Interpopulation variability in temperature-dependent sex determination of the tidewater silverside Menidia peninsulae (Pisces: Atherinidae). Copeia 2003:155-159.
12. Baroiller JF, D'Cotta H, Saillant E. Environmental Effects on Fish Sex Determination and Differentiation. Sexual Development 2009; 3:118-135.
13. Taylor DS. Biology and ecology of *Rivulus marmoratus*: new insights and a review. Florida Scientist 2000; 63:242-255.
14. Harrington J, R W. Environmentally controlled induction of primary male gonorchorists from eggs of the self-fertilizing hermaphroditic fish *Rivulus marmoratus* Poey. Biological Bulletin 1967; 132:174-199.
15. Davis WP, Taylor DS, Turner BJ. Does the autecology of the mangrove rivulus fish (*Rivulus marmoratus*) reflect a paradigm for mangrove ecosystem sensitivity? Bulletin of Marine Science 1995; 57:208-214.
16. Frick NT, Wright PA. Nitrogen metabolism and excretion in the mangrove killifish *Rivulus marmoratus* II. Significant ammonia volatilization in a teleost during air-exposure. 2002; 205:91-100.
17. Mackiewicz M, Tatarenkov A, Perry A, Martin JR, Elder JF, Bechler DL, Avise JC. Microsatellite documentation of male-mediated outcrossing between inbred laboratory strains of the self-fertilizing mangrove killifish (*Kryptolebias marmoratus*). Journal of Heredity 2006; 97:508-513.
18. Harrington J, R W. How ecological and genetic factors interact to determine when self-fertilizing hermaphrodites of *Rivulus marmoratus* change into functional

secondary males, with a reappraisal of the modes of intersexuality among fishes. Copeia 1971; 3:389-432.
19. Koenig CC, Chasar MP. Usefulness of the hermaphroditic marine fish, *Rivulus marmoratus*, in carcinogenicity testing. In: Hoover KL (ed.) Use of Small Fish in Carcinogenicity Testing, vol. 65. Lister Hill Center, Bethesda, Maryland: National Institutes of Health; 1981: 15-33.
20. Harrington J, R W. Delimitation of the thermolabile phenocritical period of sex determination and differentiation in the ontogeny of the normally hermaphroditic fish *Rivulus marmoratus* Poey. Physiological Zoology 1968; 41:447-460.
21. Kanamori A, Yamamura A, Koshiba S, Lee J-S, Orlando EF, Hori H. Methyltestosterone efficiently induces male development in the self-fertilizing hermaphrodite fish, *Kryptolebias marmoratus*. Genesis: the Journal of Genetics and Development 2006; 44:495 - 503.
22. Loffler KA, Zarkower D, Koopman P. Etiology of Ovarian Failure in Blepharophimosis Ptosis Epicanthus Inversus Syndrome: FOXL2 Is a Conserved, Early-Acting Gene in Vertebrate Ovarian Development. Endocrinology 2003; 144:3237-3243.
23. Scholz S, Rösler S, Schäffer M, Hornung U, Schartl M, Gutzeit HO. Hormonal induction and stability of monosex populations in the medaka (*Oryzias latipes*): expression of sex specific marker genes. Biology of Reproduction 2003; 69:673-678.
24. Wang D-S, Zhou L-Y, Kobayashi T, Matsuda M, Shibata Y, Sakai F, Nagahama Y. Doublesex- and Mab-3-Related Transcription Factor-1 Repression of Aromatase Transcription, a Possible Mechanism Favoring the Male Pathway in Tilapia. Endocrinology 2010; 151:1331-1340.
25. Uhlenhaut NH, Jakob S, Anlag K, Eisenberger T, Sekido R, Kress J, Treier A-C, Klugmann C, Klasen C, Holter NI, Riethmacher D, Schütz G, et al. Somatic Sex Reprogramming of Adult Ovaries to Testes by FOXL2 Ablation. Cell 2009; 139:1130-1142.
26. Pannetier Ml, Fabre Sp, Batista F, Kocer A, Renault L, Jolivet Gv, Mandon-PÃpin B, Cotinot C, Veitia R, Pailhoux E. FOXL2 activates P450 aromatase gene transcription: towards a better characterization of the early steps of mammalian ovarian development. Journal of Molecular Endocrinology 2006; 36:399-413.
27. DeFalco T, Capel B. Gonad Morphogenesis in Vertebrates: Divergent Means to a Convergent End. In, vol. 25; 2009: 457-482.
28. Raymond CS, Murphy MW, O'Sullivan MG, Bardwell VJ, Zarkower D. Dmrt1, a gene related to worm and fly sexual regulators, is required for mammalian testis differentiation. Genes and Development 2000; 14:2587-2595.
29. Guiguen Y, Fostier A, Piferrer F, Chang C-F. Ovarian aromatase and estrogens: A pivotal role for gonadal sex differentiation and sex change in fish. General and Comparative Endocrinology 2010; 165:352-366.
30. Kettlewell JR, Raymond CS, Zarkower D. Temperature-dependent expression of turtle Dmrt1 prior to sexual differentiation. Genesis 2000; 26:174-178.
31. Xia W, Zhou L, Yao B, Li C-J, Gui J-F. Differential and spermatogenic cell-specific expression of DMRT1 during sex reversal in protogynous hermaphroditic groupers. Molecular and Cellular Endocrinology 2007; 263:156-172.

32. Chiang EFL, Pai C-I, Wyatt M, Yan Y-L, Postlethwait J, Chung B-c. Two Sox9 Genes on Duplicated Zebrafish Chromosomes: Expression of Similar Transcription Activators in Distinct Sites. Developmental Biology 2001; 231:149-163.
33. Nakamoto M, Muramatsu S, Yoshida S, Matsuda M, Nagahama Y, Shibata N. Gonadal sex differentiation and expression of *Sox9a2*, *Dmrt1*, and *Foxl2* in *Oryzias luzonensis*. Genesis 2009; 47:289-299.
34. Shoemaker C, Ramsey M, Queen J, Crews D. Expression of Sox9, Mis, and Dmrt1 in the gonad of a species with temperature-dependent sex determination. Developmental Dynamics 2007; 236:1055-1063.
35. Orban L, Sreenivasan R, Olsson P-E. Long and winding roads: Testis differentiation in zebrafish. Molecular and Cellular Endocrinology 2009; 312:35-41.
36. Cole KS, Noakes DLG. Gonadal development and sexual allocation in mangrove killifish, *Rivulus marmoratus* (Pisces: Atherinomorpha). Copeia 1997:596 - 600.
37. Trant JM, Gavasso S, Ackers J, Chung B-C, Place AR. Developmental expression of cytochrome P450 aromatase genes (CYP19a and CYP19b) in zebrafish fry (*Danio rerio*). Journal of Experimental Zoology 2001; 290:475-483.
38. Kallivretaki E ER, Neuhauss SC, Kah O, and Segner H. The zebrafish, brain-specific, aromatase cyp19a2 is neither expressed nor distributed in a sexually dimorphic manner during sexual differentiation. Developmental Dynamics 2007; 236:3155-3166.
39. Lee Y-M, Seo JS, Kim I-C, Yoon Y-D, Lee J-S. Endocrine disrupting chemicals (bisphenol A, 4-nonylphenol, 4-tert-octylphenol) modulate expression of two distinct cytochrome P450 aromatase genes differently in gender types of the hermaphroditic fish *Rivulus marmoratus*. Biochemical and Biophysical Research Communications 2006; 345:894 - 903.
40. Kanamori A, Toyama K, Kitagawa S, Kamehara A, Higuchi T, Kamachi Y, Kinoshita M, Hori H. Comparative genomics approach to the expression of figα, one of the earliest marker genes of oocyte differentiation in medaka (*Oryzias latipes*). Gene 2008; 423:180-187.
41. Liang L, Soyal SM, Dean J. FIGalpha, a germ cell specific transcription factor involved in the coordinate expression of the zona pellucida genes. Development 1997; 124:4939-4947.
42. Huntriss J, Gosden R, Hinkins M, Oliver B, Miller D, Rutherford AJ, Picton HM. Isolation, characterization and expression of the human Factor In the Germline alpha (FIGLA) gene in ovarian follicles and oocytes. Molecular Human Reproduction 2002; 8:1087-1095.
43. Onichtchouk D, Aduroja K, Belting H-G, Gnügge L, Driever W. Transgene driving GFP expression from the promoter of the zona pellucida gene zpc is expressed in oocytes and provides an early marker for gonad differentiation in zebrafish. Developmental Dynamics 2003; 228:393-404.
44. Kanamori A. Systematic identification of genes expressed during early oogenesis in medaka. Molecular Reproduction and Development 2000; 55:31-36.

45. Jorgensen A, Morthorst J, Andersen O, Rasmussen L, Bjerregaard P. Expression profiles for six zebrafish genes during gonadal sex differentiation. Reproductive Biology and Endocrinology 2008; 6.
46. Hatano O, Takayama K, Imai T, Waterman MR, Takakusu A, Omura T, Morohashi K. Sex-dependent expression of a transcription factor, Ad4BP, regulating steroidogenic P-450 genes in the gonads during prenatal and postnatal rat development. Development 1994; 120:2787-2797.
47. Smith CA, Smith MJ, Sinclair AH. Gene expression during gonadogenesis in the chicken embryo. Gene 1999; 234:395-402.
48. Western PS, Harry JL, Graves JAM, Sinclair AH. Temperature-dependent sex determination in the American alligator: expression of SF1, WT1 and DAX1 during gonadogenesis. Gene 2000; 241:223-232.
49. Fleming A, Wibbels T, Skipper JK, Crews D. Developmental Expression of Steroidogenic Factor 1 in a Turtle with Temperature-Dependent Sex Determination. General and Comparative Endocrinology 1999; 116:336-346.
50. Iyer AK, McCabe ERB. Molecular mechanisms of DAX1 action. Molecular Genetics and Metabolism; 83:60-73.
51. Rhee J-S, Kim B-M, Lee CJ, Yoon Y-D, Lee Y-M, Lee J-S. Bisphenol A modulates expression of sex differentiation genes in the self-fertilizing fish, *Kryptolebias marmoratus*. Aquatic Toxicology 2011; 104:218-229.
52. Blázquez M, Navarro-Martín L, Piferrer F. Expression profiles of sex differentiation-related genes during ontogenesis in the European sea bass acclimated to two different temperatures. Journal of Experimental Zoology Part B: Molecular and Developmental Evolution 2009; 312B:686-700.
53. Kohno S, Katsu Y, Urushitani H, Ohta Y, Iguchi T, Jr. LG. Potential contributions of heat shock proteins to temperature-dependent sex determination in the American alligator. Sexual Development 2010; 4:73-87.
54. Orlando EF, Katsu Y, Miyagawa S, Iguchi T. Cloning and differential expression of ER-α, ER-ß, aromatase-a, and aromatase-b in the brain, liver, and gonad of self-fertilizing hermaphroditic and male reproductive morphs of the mangrove rivulus, *Kryptolebias marmoratus*. Journal of Molecular Endocrinology 2006 37:353 - 365.
55. Tatarenkov A, Ring BC, Elder JF, Bechler DL, Avise JC. Genetic Composition of Laboratory Stocks of the Self-Fertilizing Fish *Kryptolebias marmoratus*: A Valuable Resource for Experimental Research. PLoS ONE 2010; 5:art. no. e12863, pp. 12861-12869.
56. Menuet A, Pellegrini E, Brion F, Gueguen MM, Anglade I, Pakdel F, Kah O. Expression and estrogen-dependent regulation of the zebrafish brain aromatase gene. Journal of Comparative Neurology 2005; 485:304 - 320.
57. Leet JK, Gall HE, Sepúlveda MS. A review of studies on androgen and estrogen exposure in fish early life stages: effects on gene and hormonal control of sexual differentiation. Journal of Applied Toxicology 2011:n/a-n/a.
58. Amores A, Force A, Yan YL, Joly L, Amemiya C, Fritz A, Ho RK, Langeland J, Prince V, Wang YL, Westerfield M, Ekker M, et al. Zebrafish hox clusters and vertebrate genome evolution. Science (New York, N.Y.) 1998; 282:1711-1714.

59. Raghuveer K, Senthilkumaran B. Isolation of sox9 duplicates in catfish: localization, differential expression pattern during gonadal development and recrudescence, and hCG-induced up-regulation of sox9 in testicular slices. Reproduction 2010; 140:477-487.
60. Du Q-Y, Wang F-Y, Hua H-Y, Chang Z-J. Cloning and study of adult-tissue-specific expression of Sox9 in *Cyprinus carpio*. Journal of Genetics 2007; 86:85-91.
61. Klüver N, Kondo M, Herpin A, Mitani H, Schartl M. Divergent expression patterns of Sox9 duplicates in teleosts indicate a lineage specific subfunctionalization. Development Genes and Evolution 2005; 215:297-305.
62. Raghuveer K, Senthilkumaran B, Sudhakumari CC, Sridevi P, Rajakumar A, Singh R, Murugananthkumar R, Majumdar KC. Dimorphic Expression of Various Transcription Factor and Steroidogenic Enzyme Genes during Gonadal Ontogeny in the Air-Breathing Catfish, *Clarias gariepinus*. Sexual Development 2011.
63. Kobayashi Y, Horiguchi R, Nozu R, Nakamura M. Expression and localization of forkhead transcriptional factor 2 (Foxl2) in the gonads of protogynous wrasse, *Halichoeres trimaculatus*. Biology of Sex Differences 2010; 1:1-9.
64. Kobayashi T, Kajiura-Kobayashi H, Guan G, Nagahama Y. Sexual dimorphic expression of DMRT1 and Sox9a during gonadal differentiation and hormone-induced sex reversal in the teleost fish Nile tilapia (*Oreochromis niloticus*). Developmental Dynamics 2008; 237:297-306.
65. Yan Y-L, Miller CT, Nissen R, Singer A, Liu D, Kirn A, Draper B, Willoughby J, Morcos PA, Amsterdam A, Chung B-c, Westerfield M, et al. A zebrafish sox9 gene required for cartilage morphogenesis. Development 2002; 129:5065-5079.