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Genomic and Hormonal Components of Altered Developmental Pathways in the

Annual Killifish, *Austrofundulus limnaeus*

by

Benjamin M. Pri-Tal

a thesis submitted in partial fulfillment of the
requirements for the degree of

Master of Science
in
Biology

Thesis Committee:
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Abstract

The annual killifish, *Austrofundulus limnaeus*, may enter embryonic diapause at three distinct points of development, termed diapause I, II, and III. Previous studies suggest a role for steroid hormones in the regulation of diapause in annual killifish. This study concerns the hormonal and genomic components involved in the developmental decision to enter or escape diapause II from both a maternal and embryonic perspective. Steroid hormone levels were measured in tissues isolated from adult female fish that were producing either high or low proportions of escape embryos. Levels of steroid hormones were also measured in new fertilized embryos that were known to be on either an escape or diapausing developmental trajectory. In addition, cDNA microarray gene expression analysis was used to identify gene sequences that may be associated with the regulation of entry into diapause in this species. Decreases in maternal estrogen levels associated with aging are correlated with decreasing escape embryo production, but there is no direct association between measured steroid hormone levels and escape embryo production. However, maternal production of escape embryos is correlated with increased ratios of 17 β -estradiol to testosterone in ovary tissue, and cDNA microarray gene expression analysis indicates differentially regulated sequences associated with escape embryo production in maternal tissues. Both of these independent measures suggest hormonal involvement in the regulation of diapause. Embryonic levels of steroid hormones in newly fertilized embryos are not correlated with entry or escape from diapause II, although

incubation in exogenous cortisol and 17 β -estradiol causes an increase in the proportion of escape embryos. Gene expression analysis again suggests hormonal involvement. Interestingly, genes involved in epigenetic control of gene expression through chromatin condensation are differentially regulated in both maternal tissues producing escape embryos, and in embryos on the different developmental trajectories. These data suggest that hormonal control of gene expression through alterations of chromatin condensation may regulate the decision to enter or escape diapause II.

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Introduction

The uniting factor of most environments is variability. Even in the most stable environments, such as Antarctic waters that are constantly at the freezing point of sea water, seasonal changes in food availability and photoperiod may perturb an organism's function. Often, environmental variation challenges an animal from a metabolic, thermal, or hydration perspective. In an aquatic environment, a lack of water availability typically leads to death of the organism and can lead to local population extinctions. Vertebrates have developed different strategies for coping with extremes in environmental variation; some develop tolerance or adapt to thrive in these conditions, while others avoid periods of unfavorable environmental conditions by migration or by entering a state of metabolic dormancy. Those organisms that use dormancy typically exhibit strategies that consist of lowering metabolic and physiological set points to reduce energy consumption and thus prolong survival in the absence of active resource acquisition. Diapausing embryos of the annual killifish are one of the most extreme examples of metabolic dormancy among vertebrates. However, very little is known about the physiological or biochemical underpinnings that control entry into dormancy in this species. The focus of this thesis is to explore these mechanisms.

Metabolic depression has long fascinated physiologists; Anton van Leeuwenhoek noted the dormant state of rotifers found in the gutters of house roofs over 300 years ago (in Guppy, 2004). Dormancy takes many forms in vertebrates, and

is mobilized in reaction to different environmental challenges. In the extreme seasonality of latitudes approaching the poles, animals may enter hibernation for the cold season, sleeping away the bitter cold in a den or burrow. For example, when winter arrives the Ranid frog, *Rana sylvatica*, undergoes biochemical changes that allow for the survival of freezing temperatures. At more tropical latitudes, escape from heat or lack of water through estivation is a common strategy, such as the Malagasy fat-tailed dwarf lemurs that rest away the hot season in dens (Dausmann et. al., 2004). The African lungfish covers itself in a desiccation resistant cocoon to rest through the dry periods of its ephemeral aquatic habitat (Janssens, 1965).

The unifying factor in all of these different strategies of avoiding unfavorable conditions is depressed metabolism; to quote the comparative physiologist Kjell Johanssen, these animals are capable of “turning down to the pilot light” (in Hochachka et. al, 1997). Through metabolic depression animals “wait out” periods of extreme environmental challenge and escape from “biological time”, decreasing their needs for water, oxygen, or heat (Hochachka and Somero, 2002). Indeed, this is an effective strategy as states of developmental arrest are found in species of all major animal phyla (Hand, 1991; Yuan et al., 2007).

Environmental physiology is the study of how animals manifest different responses to their environment, from the molecular and cellular level to that of the entire organism. These responses may be realized across various time scales ranging from evolutionary time to almost immediate adjustments. On the more immediate time scales (less than the lifetime of an organism) the interaction between genotype

and its external manifestation under different conditions (phenotype) can be adjusted in response to environmental variation. Phenotypic plasticity allows an individual to manifest varied responses to different environmental conditions. In many situations, developmental arrest in diapause is manifested as a form of phenotypic plasticity (Marcus, 1979; Denlinger, 1979; Caceres, 1998; Hairston et al., 1996; Drinkwater and Crowe, 2005; Gerisch and Antebi, 2004). This is also the case for embryos of *Austrofundulus limnaeus* that have recently been shown to exhibit plasticity in developmental pathways controlled by both environmental and maternal cues (Podrabsky et al., 2010). The unique life history and the amount of scientific information available on *A. limnaeus* make it an ideal candidate for studies on the factors involved in, and influencing, embryonic diapause.

The Biology of Annual Killifish

Annual killifish persist in an inhospitable environment, eking out a precarious existence where few other fish could survive. Annual killifish live in savanna and desert regions in Africa and South America that experience great seasonal variation in rainfall (Wourms, 1972a). Ephemeral ponds inhabited by the South American annual killifish, *Austrofundulus limnaeus*, exhibit large diurnal fluctuations in temperature, dissolved oxygen content, and pH values (Podrabsky et. al., 1998). Upon desiccation of the pools, adult and juvenile fish perish (Myers, 1952). Populations in a given location persist through the dry season as diapausing embryos buried in the soil. When rains return and pools refill, fish hatch and repopulate the new aquatic habitat.

Annual killifish are named as such due to their short life spans; typically around a year or less. In the laboratory, *A. limnaeus* become senescent at approximately one year of age with a maximum life span of about 1.5 years (Podrabsky, 1999). This is consistent with selection for quick growth and attainment of sexual maturity with a tradeoff for longevity, as would be expected in an animal from such a harsh and temporally limited environment. A species of African annual killifish, *Nothobranchius furzeri*, has one of the shortest life spans reported, with animals in the laboratory living less than 12 weeks (Valdesalici and Cellerino, 2003), and wild type a maximum of 25-32 weeks (Terzibasi et al., 2008). However, the study reporting the shorter lifespan was performed on extremely inbred laboratory fish, as determined by homozygosity of typically polymorphic loci (Terzibasi et al., 2008). The extremely short lifespan of these fish has made them a proposed model in vertebrate aging research, as well as in studies of experimental evolution and the effect of lifelong pharmaceutical treatments (reviewed in Genade et al., 2005). Vertebrates exhibiting such naturally short life spans can be extremely useful as a model for other more long-lived vertebrate species, that are significantly more difficult to study in a multigenerational context due to the constraints of typical human time frames.

Diapause in Annual Killifish

Unique among vertebrates, annual killifish exhibit three distinct developmental stages in which embryonic development may be arrested; these stages

of diapause have been termed diapause I, II, and III (Wourms, 1972a). Each of these three stages confers increased resistance to environmental insult. In various combinations, the three different stages of developmental arrest can produce eight different developmental trajectories. Ecologically, this staggering of developmental progression likely provides security in a varied environment where early or untimely emergence could otherwise lead to population extinction (Wourms, 1972c).

Diapause I is a developmental arrest interjected between epiboly and embryogenesis, a time that is typically associated with gastrulation in most species of fish. During epiboly, blastomeres form two distinct populations; deep blastomeres that consolidate as a mass and then migrate outwards as amoeboid cells, and outer blastomeres that form the enveloping cell layer. Epiboly commences in annual killifish with a low number of blastomeres (200-1000) compared to other teleosts such as *Fundulus* that begins epiboly with around 27,500 blastomeres (Wourms, 1972b). Diapause I occurs following dispersion of the embryonic blastomeres shortly after the completion of epiboly, but prior to the reaggregation of cells that constitute the embryonic axis. In *Austrofundulus*, arrest at diapause I is facultative and can be of variable duration, being composed of developmental arrest between stages 20 and 21 (Wourms, 1972c). A comparative study by Wourms (1972b) including 43 species of both annual and non-annual killifish, found that dispersion and reaggregation of the blastomeres and diapause I are only common to the killifish exhibiting an annual life cycle (i.e. dispersion and reaggregation are missing from species that do not exhibit diapause). Three benefits to the dispersion-reaggregation developmental

pattern have been hypothesized: (1) a period of developmental arrest can be interjected without disruption of neurulation, (2) the enveloping cell layer may become specialized to confer resistance to desiccation and osmotic stress, and (3) dispersed cells (if totipotent) can be sacrificed without affecting overall embryo survival (Wourms, 1972b).

Arrest at diapause II has been the most extensively studied of the embryonic diapause stages in annual killifish. The physiology of anoxia tolerance (Duerr and Podrabsky, 2010; Podrabsky et. al., 2007; Fergusson-Kolmes and Podrabsky, 2007), desiccation tolerance (Podrabsky et al., 2001), salinity tolerance (Machado and Podrabsky, 2007), bioenergetics (Duerr and Podrabsky, 2010; Podrabsky and Hand, 1999), protein synthesis (Podrabsky and Hand, 2000), and induction of molecular chaperones (Podrabsky and Somero, 2007), have all been studied in *A. limnaeus* entering diapause II. This stage is initiated at approximately 24 days post fertilization (dpf), in the long-somite embryo stage (Wourms, 1972a; Podrabsky and Hand, 1999). At this diapause stage, embryos have 38-40 pairs of somites, a beating heart, optic cups, and other basic elements of the central nervous system (Podrabsky and Hand, 1999). Diapause II embryos exhibit a depression of up to 90% of the pre-diapause oxygen consumption and heart rate. Both metabolism and heart rate increase during early development, and then begin to decline several days prior to cessation of development in diapause II (Podrabsky and Hand, 1999). Thus, it appears that the molecular events that signal for entry into diapause likely occur many days prior to

the cessation of morphological development associated with diapause II. This is an important point to consider when searching for the signals that induce diapause II.

A small proportion of the embryos from a given spawning event will not enter diapause II but instead develop through an alternate pathway (Podrabsky et al., 2010). Wourms (1972c) named these “escape embryos” because they escape from arrest of development in diapause II. Escape embryos can be distinguished morphologically from diapausing embryos when they reach stages that possess about 18-20 pairs of somites. Escape embryos exhibit a number of developmental characteristics that do not form until several days after diapause II breakage in arresting embryos. For example, escape embryos are covered on the dorsal aspect of the head and trunk with melanocyte cells, hemoglobin is expressed in circulating red blood cells, the vasculature of the yolk sac is greatly increased, chondrocyte condensations (otolith primordia) can be observed in the developing otic vesicles, and the early vestiges of the gut have formed. Escape embryos possess lower anaerobic and aerobic capacity at the termination of development than do embryos that have entered diapause II (Chennault and Podrabsky, 2010). This may indicate a tradeoff in development, where the rapidly developing escape embryos reach hatching at faster rates, but with lower metabolic capabilities when compared to embryos that have arrested development at diapause II (Chennault and Podrabsky, 2010).

Diapause III is a state of arrested development that occurs directly prior to hatching, when the embryos are fully developed. *Austrofundulus* embryos entering diapause III may remain dormant for over 100 days (Wourms, 1972c). This

developmental arrest is thought to be obligate in most embryos of *A. limnaeus*, although small subpopulations ($\approx 10\%$) bypass developmental arrest at diapause III and hatch immediately when the embryos are reared in a laboratory environment (Wourms, 1972c). Like diapause II, diapause III is also characterized by significant metabolic depression (Podrabsky and Hand, 1999). Interestingly, while metabolism decreases during this stage of developmental arrest, anaerobic and aerobic capacities increase (Chennault and Podrabsky, 2010). This may indicate different control mechanisms compared to diapause II. Diapause III may be rapidly terminated, and the immediate capacity to reverse metabolic arrest is likely necessary for post-hatching success (Chennault and Podrabsky, 2010).

Environmental Tolerance During Diapause

Anoxia tolerance in embryos of *A. limnaeus* is substantial during early development, and increases to the highest levels during diapause II. At 4 dpf, LT₅₀ values for anoxia are near 20 days. At 32 dpf, about a week after the induction of diapause II, these values reach over 65 days. This anoxia tolerance continues through 4 days post-diapause II (dpd), but is largely lost by 8 dpd. Anaerobic metabolic capacity is high throughout development in embryos of *A. limnaeus*, as evidenced by high lactate dehydrogenase (LDH) to citrate synthase (CS) ratios (Chennault and Podrabsky, 2010). At all examined stages of development, embryos entered anoxia induced quiescence, demonstrated by the cessation of morphological development, and physiological activity (Podrabsky et. al., 2007). During this anoxia induced quiescence, embryos in early developmental stages (diapause II and 4 dpd) may cease heart contractility, while later embryonic stages suffer severe bradycardia (Fergusson-Kolmes and Podrabsky, 2007). Early embryos demonstrate higher resistance and recovery rates from anoxic conditions than do later embryos, suggesting the cessation of heart contractility plays a role in tolerance of anoxia (Fergusson-Kolmes and Podrabsky, 2007). Embryos in diapause II show extremely low levels of aerobic capacity, as measured by CS enzymatic activity. This may indicate active suppression of mitochondrial metabolism in embryos arresting development at this stage (Chennault and Podrabsky, 2010).

The ability to resist dehydrating conditions is one of the most pronounced adaptations that permit the unique lifestyle of annual killifish. Annual killifish may

spend the majority of their lives in embryonic diapause under desiccated conditions (Wourms, 1972c). This tolerance to dehydration reaches its highest levels during diapause II (Podrabsky et al., 2001). *A. limnaeus* survive dehydrating conditions by reducing evaporative water loss to undetectable levels after 32 days of exposure to dehydrating conditions. Embryos show an initial loss of water from the perivitelline space (around 50% of the total embryo water), and also an increase in β -sheet contacts in the egg envelope with dehydration. These embryos show unprecedented desiccation tolerance for an aquatic vertebrate, with greater than 40% survival after 113 days at 75.5% relative humidity (Podrabsky et. al., 2001).

Consistent with the overall depression of metabolic and physiological turnover, protein synthesis is decreased in embryos during diapause II (Podrabsky and Hand, 1999). Prior to diapause, 36% of ATP utilization is diverted to protein synthesis; this approaches zero during diapause II (Podrabsky and Hand, 2000). The decreased protein synthesis during diapause II suggests that proteins conferring the environmental tolerance of embryos may be produced during the metabolically active period leading up to the cessation of growth. In support of this theory, an inducible 70 kDa-class heat shock protein is constitutively expressed during early development and at the highest levels in diapause II embryos (Podrabsky and Somero, 2007). Heat shock proteins can act as molecular chaperones, aiding proteins in retaining proper structure under environmentally challenging regimens. The induction of heat shock proteins was found to be integral to environmental stress tolerance in the diapause of the fleshfly *Sarcophaga crassipalpis* (Rinehart et al., 2007), the moth *Sesamia*

nonagrioides (Gkouvitsas et al., 2009), and the brine shrimp *Artemia franciscana* (Willsie and Clegg, 2001), suggesting the initiation of environmentally protective gene products may be a common factor in developmental arrest.

Regulation of Diapause Induction

Substantial variation exists in the relative ratios of diapause to escape embryos produced, both between females under constant conditions, and throughout an individual's life (Podrabsky et al., 2010). Despite a population-level pattern for the reduced production of escape embryos as a female ages, some females may exclusively produce either escape or diapausing embryos, while others produce a mixture of the two types (Podrabsky et al., 2010). In addition, embryos incubated under identical conditions following fertilization consistently show different developmental strategies. Thus, there appears to be significant maternal control over induction of diapause II. The mode of maternal transfer is currently unknown, but may include the packaging of proteins or mRNAs into embryos that control entry into diapause II.

The specific signaling molecules governing the induction of embryonic diapause in annual killifish are unknown. However, some evidence suggests a water-borne factor produced by both male and female adult fish inhibits development at diapause I and II in the East African annual killifish *Nothobranchius guentheri* (Inglima et al., 1981; Levels, 1988). The factor produced by the adult *N. guentheri* did not affect the development of a closely related non-annual killifish *Aphyosemion*

australe when the embryos were incubated together. However, incubation of annual killifish embryos with adults of different non-annual fish species resulted in arrest at diapause I and II, though the effect was diminished. Taken together this evidence suggests that some control over the initiation of embryonic diapause I and II is exercised through a specialized sensor or increased sensitivity in annual killifish to a common factor produced by fish. The decreased effect of this common factor produced in non-annual adults suggests that adult annual killifish may produce these compounds at higher levels. Alternately, it may be less easily degraded and/or metabolized, is specialized, or a combination of these factors (Inglima et al., 1981). Another study found that homogenates of ovary, and to a lesser magnitude testis, prolonged dispersion-reaggregation in *N. korthause*, without affecting incidence of diapause II. Embryo homogenates also inhibited development, though the effect was lost in homogenates prepared from hatchling fish. Boiling the homogenates increased the magnitude of the inhibitory effect by an unclear mechanism (Levels, 1988). Further work found that the factor prolonging dispersion-reaggregation was a polar, hydrophilic agent, although size determination was inconclusive. Interestingly, the apolar and hydrophobic purification fractions from adult ovary also contained a bioactive substance that in this case inhibited diapause II (Levels, 1988).

The biochemical nature of the substance inhibiting diapause II led the researchers to assess the influence of steroid hormones on the different stages of embryonic diapause. Embryonic incubation in progesterone spiked media ($>4 \mu\text{g/ml}$) significantly increased the duration of dispersion-reaggregation. All hormones tested

had an inhibitory effect on diapause II, although only β -estradiol had an effect at concentrations as low as 0.001 $\mu\text{g/ml}$. Significantly, of the hormones tested only β -estradiol and testosterone had inhibitory effects on diapause II without resulting in developmental anomalies in treated embryos (Levels, 1988).

Hormones are very likely candidates for regulation of diapause in annual killifish, especially considering their importance in regulating dormancy in other systems. Regulation of dormancy in nematodes is an excellent example. Dormancy in dauer stage larvae of the nematode *C. elegans*, is regulated through the insulin signaling cascade via the insulin receptor-like protein encoded by the gene *Daf-2* (Kimura et al., 1997). The endocrine system in *C. elegans* integrates environmental and endogenous signals to affect responses that can include the decision to enter or bypass the dauer stage diapause (Tatar et al., 2003; Gerisch and Antebi, 2004). In stressful environments, the Forkhead transcription factor, DAF-16, remains unphosphorylated, localizes to the nucleus, and affects downstream gene regulation resulting in induction of the dauer dormancy. In beneficial environments this factor is phosphorylated, which favors a cytoplasmic localization and thus the absence of DAF-16 induced gene expression (Henderson and Johnson, 2001; Ogg et al., 1997). Evidence suggests that this pathway is mediated by *Daf-9*, a cytochrome P450 steroidogenic hydroxylase that produces a hormone integrating the system (Gerisch and Antebi, 2004). Further studies have identified *Daf-9* as one of the most important aspects of the biosynthetic pathway involved in the production of the lipophilic steroid hormone involved in the dauer stage, although hormone production is affected

through a multistep process (Rottiers et al., 2009). *Daf-9* produces steroidal acid ligands (dafachronic acids) that when present inactivate the nuclear hormone receptor DAF-12; when ligands are absent dauer formation is initiated (Martin et al., 2010).

Hormones clearly play an important role in the regulation of diapause in other systems as well. Peptidergic neurons in the subesophageal ganglion of the silkworm, *Bombyx mori*, produce diapause hormone (DH), the initiator of embryonic diapause (Sato et al., 1993). Diapause hormone consists of 24 amino acid residues amidated at the carboxyl terminal, with a molecular weight of 2645 daltons, (Imai et al., 1991), encoded in a polyprotein precursor mRNA that also encodes four other hormones (Sato et al., 1993). This hormone acts on the ovaries, initiating gene transcription that ultimately results in the accumulation of glycerol and sorbitol, characteristics only of eggs in embryonic diapause (reviewed in Yamashita, 1995). Duration of diapause is regulated through the ERK and P38 MAP kinase cascades (Fujiwara et al., 2006). Activation of these cascades through extended low temperature terminates embryonic diapause (Iwata et al., 2005).

In the fleshfly, *Sarcophaga crassipalpis*, a failure to synthesize ecdysone is thought to arrest development. Concurrently, rhythmic pulses of juvenile hormone (JH) herald the onset of embryonic diapause. These JH pulses continue through developmental arrest, and a pulse directly precedes the increase in molting hormone that terminates embryonic diapause (Walker and Denlinger, 1980). The interplay of these hormones modifies their physiological effects, possibly in a regulatory fashion; this is evidenced by changes in duration and/or onset of developmental arrest, under

exogenous hormone treatments at different developmental time points (Denlinger, 1976; Zdarek and Denlinger, 1975). High temperature may also terminate diapause, through the ERK/MAP kinase cascade (Fujiwara and Denlinger, 2007), a likely candidate for the control of embryonic diapause in *A. limnaeus*. Furthermore, as in annual killifish, maternal photoperiod affects diapause incidence in progeny of the fleshfly *S. bullata*, although by an unknown mechanism (Rockey et al., 1989).

Global control of major life history transitions are often controlled by hormones; these compounds are structurally well conserved and powerful effector molecules that act to coordinate organism-wide responses. The role of hormones has been suggested in the regulation of embryonic diapause in annual killifish, and exogenous hormone treatment found to significantly affect developmental pathways in embryos of *N. korthause* (Levels, 1988). To elucidate factors involved in the control of embryonic diapause in *A. limnaeus*, I have investigated the role of steroid hormones in both maternal and embryonic control of diapause, and screened for changes in gene expression using a cDNA microarray approach. I hypothesize that hormone levels in escape and diapause embryos will differ through early development leading up to dormancy, and the hormonal environment *in utero* will influence the decision to bypass or enter embryonic diapause. This variation in hormone levels will be accompanied by differences in hormone receptors and their downstream gene products. Through these processes, ultimate developmental pathway will be determined, and the suite of metabolic and biochemical traits essential to different trajectories effected.

Materials and Methods

Husbandry of Adults and Collection of Embryos

Experimental animals were obtained from an in-house aquatic facility, as previously described (Podrabsky, 1999). Briefly, breeding pairs of *A. limnaeus* were housed in 3.8 L aquaria attached to shared sumps (21 aquaria per sump). Pairs were kept separate using an internal divider until spawning. Water was changed twice daily (25% each time) weekdays, and once daily on weekends (25%), using City of Portland tap water that was charcoal filtered, UV treated, and supplemented with salt (Coralife synthetic sea salt, 0.115%). Animals were fed on the same schedule as the water changes, primarily on Bio-pure frozen bloodworms (Hikari-US Koi, LLC, Cat# 30221). Chopped red wiggler worms (Timberline Live Pet Food) were fed on days immediately prior to spawning. Photoperiod was maintained on a 14 hr of light and 10 hr of dark (14:10 L:D) regimen, and ambient temperature kept at $\approx 26^{\circ}\text{C}$. System water temperatures normally ranged between 24-26 $^{\circ}\text{C}$.

Embryos were collected twice-weekly, also as described in Podrabsky (1999), and maintained in embryo medium (10 mM NaCl, 0.1424 mM KCl, 2.15 mM MgCl_2 , 0.0013 mM MgSO_4 , 0.792 mM CaCl in Nanopure water) containing methylene blue (0.0001%) until 4 dpf to inhibit fungal and bacterial growth. The embryos were then bleached in a mild sodium hypochlorite solution (0.03%, 2 x 5 min) as described in Podrabsky (1999). To ensure neutralization of the bleach, the embryos were rinsed in

0.005% sodium thiosulfate for 10 min. Following the bleaching treatment, embryos were transferred into embryo medium containing gentamicin sulfate (10 mg L⁻¹) to inhibit bacterial growth. Embryos were maintained in 100 mm x 15 mm petri plates (VWR, Cat#25384-342) at densities of 50-100 embryos per dish. Embryo medium was changed daily for the first four dpf, and then 6 times a week following. Dishes of embryos were incubated at constant temperature (25 or 30°C depending on the experiment) in the dark (VWR growth chambers, model 2015).

Developmental pathway of embryos at 25°C was scored at 18-22 dpf with bright phase microscopy (Leica DMIRB inverted microscope). The proportion of escape embryos was quantified through a representative sample of each female's clutch allowed to develop normally (10-12 embryos). Females were monitored for multiple spawning events (>3) before tissue harvesting.

Extraction of Steroid Hormones

Adult tissues

Adult fish were anesthetized in ice water for several minutes, followed by cervical dislocation. Ovary tissues were quickly harvested, placed into 14 ml polypropylene tubes, and flash frozen in liquid nitrogen. Tissues were stored at -80°C until immediately prior to hormone extraction. Hormones were extracted from whole tissues by homogenization in 5 ml of ice cold 100% ethanol (Sigma-Aldrich,

Cat#493546) using an IKA rotary homogenizer (IKA Works, Ultra-Turrax S10). After homogenization, the dispersing element was rinsed into the sample tube with an additional 2 ml absolute ethanol. Samples were spun at 0°C in a refrigerated centrifuge (Sorvall, Superspeed RC2-B) for 10 min at 3020 x g. The supernatant was collected into baked borosilicate tubes, capped, and stored at -20°C until hormone quantification.

Whole Blood was collected from adult fish through an incision made at the caudal peduncle, and whole blood from the caudal vein and artery collected into micro hematocrit tubes (75 µl, heparinized). The tubes were chilled on ice for a maximum of one hour before centrifugation for 5 min at full speed in a hematocrit centrifuge (Adams Autocrit). The plasma fraction was isolated, weighed, and flash frozen in liquid nitrogen. Plasma samples were stored at -80°C until extraction and analysis.

Whole Embryos

Embryos were collected onto a mesh screen using a wide-mouth transfer pipette, blotted dry with kimwipes, weighed, and then flash frozen in liquid nitrogen. Frozen embryos were stored at -80°C until immediately prior to processing. Embryos were homogenized and frozen by the same protocol as previously described for tissues, in either 5 ml absolute ethanol for groups larger than 40, or in 3 ml for groups

of 20, with the dispersing element rinsed in both cases with 2 ml of absolute ethanol.

Isolation and Quantification of Steroid Hormones

Steroid hormone levels were quantified according to standard protocols by the Endocrine Technology and Support Core Lab at the Oregon National Primate Research Center/Oregon Health & Science University (Rasmussen et al., 1984). Briefly, ethanol extracts were dried under forced air, then extracted with 300 μ l of 0.1% bacto-gelatin in 1X PBS and 5 ml redistilled diethyl ether to separate aqueous and ether soluble materials. The ether soluble fractions were decanted and then dried under forced air. The extract was then re-dissolved in 200 μ l of column solvent (Hexane:benzene:methanol = 62:20:13). Steroid hormones were separated by column chromatography in a 1 x 6 cm glass column containing 1 g Sephadex LH-20, and individual hormone fractions collected and dried under forced air. Each hormone was quantified by specific radioimmunoassay (Antibodies: 17 β -estradiol- Dr. G. Niswender, GDN#344; Androstenedione- ICN Pharmaceuticals, 07109016; Testosterone- W. Ellinwood, WEE-23; Progesterone- Sandoz Pharmaceuticals, Surve 12; Corticosterone-ICN Pharmaceuticals, 07120017). Final values were corrected to account for recovery efficiency as determined by addition of a radioactive tracer to the sample (Tracers: 17 β -estradiol-NEN Radiochemicals, NET317250UC; Androstenedione-NEN Radiochemicals, NET469250UC; Testosterone-NEN Radiochemicals, NET370250UC; Progesterone-NEN Radiochemicals, NET381250UC; Corticosterone-NEN Radiochemicals, NET399250UC). Recovery efficiencies were typically 60-80%. The limit of sensitivity for 17 β -estradiol was 3

pg/ assay tube, and 5 pg/ assay tube for androstenedione, progesterone, corticosterone, and testosterone.

Treatment of Embryos with Exogenous Steroid Hormones

17 β -estradiol (E8875-1g) and cortisol (hydrocortisone; H4001-1g) were purchased from Sigma-Aldrich. Hormones were suspended in absolute ethanol, and added to embryo medium containing 1% dimethyl sulfoxide (DMSO). DMSO was used as a permeabilizing agent, to improve hormone movement into the embryos. Concentrations of 1, 5, 10, and 20 μ g/ml of hormone were prepared. Ethanol was supplemented to control as well as hormone treated embryos to an equal total (0.133%). Embryos were raised at 25°C, bleached at day 4 as previously described, then transferred in groups of 20 embryos per well to 6-well plates containing hormone medium at 6 dpf. The hormone medium was changed every other day, and the embryos scored as entering diapause II or as escape embryos at 18 dpf, 12 days following the commencement of hormone treatment.

Steroid hormone levels were quantified at 6, 10, 14, and 18 days following the initiation of exogenous hormone treatments. At the time of harvest, the embryos were transferred into clean culture plates, rinsed 3 times with nanopure water, soaked in nanopure water for 10 min to remove hormones from the perivitelline space, and then rinsed again 3 times. Embryos were pipetted onto a mesh screen, blotted dry, and flash frozen in liquid nitrogen. Hormones were extracted as previously described. Dechorionated embryos were treated and rinsed as previously described, then

deposited onto mesh screens inserted into 1 ml syringes. The syringe-screen assembly was placed into 15 ml falcon tubes, the plunger inserted to crush the embryos, and the assembly subjected to centrifugation at 0°C (Sorvall, Superspeed RC2-B) for 10 min (3000 x g). The dechorionated embryo fraction was flash frozen in liquid nitrogen and then extracted in ice cold 100% ethanol as previously described.

Steroid Hormones During Normal Development

Levels of 17 β -estradiol (E2) and androstenedione (A4) were quantified during early development in embryos of *A. limnaeus* reared at both 25 and 30°C. Embryos were sampled at the following developmental stages at both temperatures: full epiboly, 5-10 somite, 14-16 somite, and 25-30 somite. Additionally, embryos were sampled directly after fertilization (newly fertilized), and both during diapause and four dpf in embryos incubated at 25°C. At full epiboly the blastomeres have expanded to completely encircle the yolk sack as amoeboid cells, but remain relatively large and have not yet undergone multiple rounds of mitosis (stage 19; Wourms, 1972a); diapause I may be initiated directly following this stage. 5-10 somite embryos have a fully developed embryonic axis without a functional heart (stage 29-30; Wourms, 1972a). At this developmental stage, escape embryo production sharply increases from background levels under 30°C incubation (Podrabsky et al., 2010). 14-16 somite embryos have a beating heart and a widened cephalic region (stage 30-31; Wourms, 1972a). This is the final stage preceding

morphological divergence between embryos incubated at 25 or 30°C, though the majority of embryos incubated at 30°C until this stage will follow the escape trajectory (Podrabsky et al., 2010). By the 25-30 somite stage embryos at the different incubation temperatures have diverged sharply, both morphologically and physiologically, with embryos incubated at 25°C showing comparable morphological development only four days post-diapause II at approximately 45 somites. Due to the effects of temperature, embryos reared at 25 and 30°C reached these developmental stages at different times. Newly fertilized embryos were harvested within 4 hours of being spawned, and fertilization (formation of the perivitelline space) validated under a dissecting scope. Diapause II embryos were harvested approximately 4-5 days following initiation of diapause and had heart rates lower than 10 beats per minute. Embryos incubated at 30°C were transferred from 25 to 30°C at approximately 18 hours post fertilization, and cared for by identical protocols as previously detailed. Embryonic development was monitored using bright phase microscopy (Leica DMIRB inverted microscope).

Extraction of RNA

Adult fish were anesthetized in ice water for several minutes, followed by cervical dislocation. Adult female tissues were quickly harvested, placed into 14 ml polypropylene tubes, and flash frozen in liquid nitrogen. Embryos were collected onto a mesh screen using a wide-mouth transfer pipette, blotted dry with kimwipes,

weighed, and then flash frozen in liquid nitrogen. Embryonic development and escape embryo proportions were monitored as previously described. Samples were taken from newly fertilized embryos (within four hours of spawning), and at 14-16 somite stages in embryos reared at 25 and 30°C. Liver and ovary tissues were sampled from spawning females. Frozen embryos and adult tissues were stored at -80°C until immediately prior to processing.

RNA was extracted from frozen tissue and embryo samples by homogenization in 10 volumes (by weight) for tissues, and 20 volumes for embryos, of Trizol reagent (Invitrogen, Cat#15596-018) using an IKA rotary homogenizer (IKA Works, Ultra-Turrax S10) in 14 ml polypropylene tubes. The samples were homogenized in Trizol until they had an even consistency, then placed at room temperature for at least 15 min, allowing nucleoprotein complexes to dissolve. Samples were subjected to centrifugation at 10,000 x *g* (8-10°C) in a refrigerated centrifuge (Sorvall, Superspeed RC2-B) for 30 min to pellet insoluble material. The supernatant was decanted into a fresh tube, 0.2 volumes of 100% chloroform added per initial ml of Trizol used, then the sample gently vortexed until the phases had completely mixed. The homogenate was then subjected to centrifugation at 10,000 x *g* (8-10°C) for 20 min to separate the aqueous and organic phases. The aqueous phase (which contains the RNA) was carefully removed and placed into a fresh 14 ml tube on ice. The RNA was precipitated using a high salt precipitation which consisted of adding 0.25 volumes of high salt precipitation solution (0.8 M sodium citrate and 1.2 M sodium chloride) and 0.25 volumes 100% isopropanol to the supernatant for each

ml of Trizol originally used for the homogenization. The samples were placed at -20°C overnight to allow the RNA to precipitate, then centrifuged at 10,000 x g (8-10°C) for 30 min to pellet the RNA. The supernatant was removed without disturbing the RNA pellet. The pellet was washed twice with 2 ml 60% ethanol followed each time by centrifugation at 10,000 x g for 20 min. The ethanol was removed and the pellet was allowed to air dry for 5 to 10 min until clear. The isolated total RNA was covered in nuclease free water (20-100 µl) and heated to 55°C for 5 min to aid in solubilization. Following resuspension, the RNA samples were kept either frozen or on ice at all times. The amount of total RNA was quantified by UV absorbance at 260 nm using the extinction coefficient of 40 µg/OD₂₆₀. RNA purity was assessed by determining the ratio of absorbance at 260 and 280 nm (Range: 1.43-2). RNA quality was validated by visualization on 1% agarose gels stained with ethidium bromide (1X TBE) (100 V, run time=15 min). RNA samples requiring concentration were precipitated at -20°C overnight with 0.1 volume 3 M sodium acetate and 2.5 volumes 100% ethanol. After precipitation, RNA was pelleted by centrifugation at 12,000 x g for 20 min and the samples washed twice with 2 ml 80% ethanol followed by centrifugation at 12,000 x g for 20 min (Sorvall, Superspeed RC2-B). The ethanol was then removed, the RNA pellets allowed to air-dry, and the samples resuspended by the previously listed method. Aliquots of the RNA sample were frozen in separate tubes to avoid multiple freeze thaws cycle for each sample.

Amplification of Total RNA

Embryonic RNA samples were amplified using the MessageAmp 2 aRNA amplification kit according to the manufacturer's instructions (Applied Biosystems, Cat#AM1751), based on previously published techniques (Van Gelder et al., 1990). Full reactions were run on all day 0 embryo samples (1 µg input total RNA), and 1/2 reactions performed on 14-16 somite embryo samples (0.5 µg input total RNA, all steps 1/2 total volume of following protocol). The average ratio of absorbance measured at 260 versus 280 nm for each RNA sample used was 1.7 (Range: 1.4-2.0). All reagents used were provided in the amplification kit unless otherwise noted.

Single-stranded cDNA (ss cDNA) was prepared from 0.5-1.0 µg of total RNA in a final volume of 20 µl (11 µl RNA + nuclease free water, 1 µl T7 Oligo(dT) primer, 2 µl 10X first strand buffer, 4 µl dNTP mix, 1 µl RNase inhibitor, 1 µl Arrayscript reverse transcriptase). The reactions were incubated at 16°C for 2 hours in a thermocycler (Applied Biosystems, Gene Amp 2700) with the thermocycler lid open. After 2 hours the tubes were removed, placed on ice, then the contents added to 250 µl of cDNA binding buffer. The diluted ss cDNA was applied to a DNA filter cartridge and centrifuged at 10,000 x g for 1 min to bind the DNA to the column material. The column was washed once with 500 µl wash buffer and centrifuged again twice, each time at 10,000 x g for 1 min. The filter cartridges were then transferred to clean tubes and the cDNA eluted from the columns with 18 µl of nuclease free water (preheated to 55°C) by centrifugation at 10,000 x g for 1 min. The

eluted cDNA was then amplified in a reaction mixture containing 18 μ l of ss cDNA and 24 μ l of IVT master mix (IVT master mix; 4 μ l T7 ATP, 4 μ l T7 CTP, 4 μ l T7 GTP, 4 μ l T7 UTP, 4 μ l T7 10X Reaction buffer, 4 μ l T7 enzyme mix) incubated at 37°C for 14 hours in a thermocycler (Applied Biosystems, Gene Amp 2700). After the incubation, aRNA samples were brought to a final volume of 100 μ l with nuclease free water, transferred to new nuclease free tubes, and mixed with 350 μ l of aRNA binding buffer. The aRNA was mixed with 250 μ l of absolute ethanol and applied onto an aRNA filter cartridge. The samples were centrifuged at 10,000 x g for 1 min to allow the aRNA to bind to the column. The column was then washed with 650 μ l of wash buffer followed by 2 centrifugations at 10,000 x g for 1 min each to remove residual wash buffer. The aRNA was eluted in 200 μ l of nuclease free water (preheated to 55°C) by centrifugation at 10,000 x g for 1 min. RNA yield was quantified by UV absorbance at 260 nm as described above (Shimadzu Pharma Spec UV-1700). Average yield for newly fertilized embryo amplification samples was 86 μ g RNA with an average 260/280 nm ratio of 1.99 (Range: 1.7-2.15). Average yield for 14-16 somite embryos was 81.5 μ g RNA with an average 260/280 nm ratio of 2.08 (Range: 1.6-2.2).

Synthesis of Fluorescent Probes

Production of single-stranded amino-allyl cDNA

Fluorescently labeled probes were synthesized as previously described using Cy3 and Cy5 as the dyes (Podrabsky and Somero, 2004). Each experimental sample was competitively hybridized against a pooled reference RNA sample consisting of total RNA from liver and ovary tissues. 15 µg of total RNA (aRNA from embryos, total RNA for adult tissues) was used for the synthesis of all probes. Single-stranded cDNA was synthesized in a reaction mixture consisting of 8 µl of RNA + nuclease free water, combined with primers (1 µl of 5 µg/µl oligo dT₁₅VN, 1 µl of 2.8 µg/µl random hexamer; Integrated DNA Technologies Inc.) 4 µl of 5X reverse transcriptase buffer, 1 µl 20X dNTP/aa-dUTP (20X: 10 mM dATP, 10 mM dCTP, 10 mM dGTP, 5 mM dTTP, 5 mM aa--dUTP), 2 µl 100 mM DTT, 2 µl reverse transcriptase (40 units), and 1 µl nuclease free water for a total volume of 20 µl. The RNA and primers were first mixed and heated at 70°C for 10 min and then placed directly on ice. The rest of the components were then added and the samples incubated at 42°C for 90 min in a thermocycler (Applied Biosystems, GeneAmp 2700). After incubation the RNA was degraded by adding 6.67 µl 1 M NaOH, and 6.67 µl 0.5 M EDTA and heating the samples to 65°C for 15 min. The pH of the solution was neutralized by the addition of 17 µl of 1 M Tris (pH=7.5). The ss cDNA was purified using a Qiagen PCR purification kit according to the manufacturer's instructions with the exception that 80% ethanol was used instead of the wash buffer provided (QIAquick PCR purification kit, Qiagen, 28104). Single-stranded cDNA was eluted by two applications of 30 µl of nuclease free water (pH>7) warmed to 37°C followed by centrifugation at 10,000 x g for 1 min following each application. The eluted ss

cDNA was dried in a speedvac concentrator (ThermoSavant, SpeedVac Plus SC210A).

Fluorescent Labeling of Probes with Cy Dyes

The amino-allyl labeled cDNA pellet was resuspended in 5 μ l 0.1 M NaHCO_3 , then combined with 5 μ l of Cy3 (Amersham Biosciences, Q13104) or Cy5 mono-reactive dye (Amersham Biosciences, Q15104) dissolved in DMSO (80 μ l per tube dye), and incubated in the dark at room temperature for 1 hour. The reaction was stopped by a 15 min incubation (at room temperature) following the addition of 4.5 μ l of 4 M hydroxylamine. Each experimental sample was then combined with a reference sample labeled with the opposite colored probe. The probe mixtures were purified using a Qiaquick PCR purification kit according to the manufacturer's instructions (QIAquick PCR purification kit, Qiagen, 28104). The labeled ss cDNA probes were eluted from the column with two subsequent applications of 30 μ l of buffer EB warmed to 37°C followed by centrifugation at 10,000 $\times g$ for 1 min. The labeled probes were brought to a final volume of 80 μ l and a final concentration of 3X SSC, 0.75 mg/ml tRNA (Sigma, R8508-1ML) and 25 mM HEPES (pH=7), and 0.25% SDS. Immediately prior to use the probes were heated for 2 min at 100°C in a heat block and allowed to cool at room temperature for 1-2 min. The contents were collected into the bottom of the tube by a brief centrifugation (1.5 $\times g$ for 10 sec).

Hybridization of Fluorescent Probes

Microarray hybridizations were conducted on cDNA microarray slides, fabricated largely as previously published (Podrabsky and Somero, 2004). The arrays were printed using a GeneMachines Omnigrid Accent robotic array printer using a 32 pin configuration, with 150 μm spot spacing, and over 16,000 spots total. Microarrays were pre-processed according to standard protocols (Podrabsky and Somero, 2004). Immediately prior to use, the microarray slides were prepared for probe application by immersion in prehybridization blocking solution for at least 1 hour (blocking solution; 5X SSC, 1% Bovine serum albumin, 0.1% SDS), then washed twice in nanopure water (2 min each), and dried by centrifugation at 500 x *g* for 9 min (Eppendorf, 5810R). Probes were applied to the microarray under a LifterSlip cover slip (Erie Scientific Co., 25x541-2-4901) placed over the array. The slides were quickly transferred to an array hybridization chamber (Genetix, x2530) that was preheated and humidified at 65°C. Probes were allowed to hybridize overnight (12-14 hours) at 65°C in a hybridization oven (VWR International, model 5420). Following hybridization, the coverslip was removed after submersion of the slides into a wash buffer containing 0.6X SSC and 0.03% SDS. The slides were washed briefly in a second container of this wash solution followed by a brief wash in 0.06X SSC and finally in nanopure water. At each washing step the slides were gently plunged up and

down 5-10 times. Slides were dried by centrifugation at 500 x *g* for 9 min at 25°C (Eppendorf, 5810R).

Fluorescence Quantification and Data Analysis

The microarray slides were scanned on an Axon Genepix 4000B microarray scanner, using GenePix Pro 5.1 software, at both 635 and 532 nm wavelengths. Laser power was maintained at 100%, and normalized counts of the intensity distribution balanced using the PMT gain. Array spots were visually checked, and aberrant spots flagged and disqualified from analysis. Fluorescent intensity was quantified and used to calculate the median ratio of Cy3 to Cy5 for each spot. The background-corrected ratio of the median intensity for each spot was imported into GeneSpring GX 10.0 software. The overall ratios for each dye were normalized globally and changes in gene expression analyzed using an unpaired T-Test with asymptotic p-value computation (Benjamini-Hochberg multiple testing corrections, $p < 0.05$). Clones more than 2 fold differentially regulated, or with elevated significance values in relation to the other clones, were included in the results. It should be noted that the analysis presented here is of a fraction of the total genes identified as being differentially regulated in different tissues and at different developmental time points.

DNA sequencing

The *E. coli* clones (Invitrogen, DH10B) containing the plasmids of interest (Clontech, p λ TriplEx2) were removed from a -80°C freezer and thawed at 4-6°C (for cDNA library preparation see Podrabsky and Somero, 2004). The clones of interest were transferred from the cDNA library using 10 μ l pipet tips, into 200 μ l of LB broth containing 10% glycerol and Ampicillin (50 mg L⁻¹) in 96-well Costar round bottom plates (Fisher, 07-200-105). The plates were wrapped in plastic wrap, placed into tupperware with moist paper towels, and grown statically at 37°C for 48 hours. After the first growth period, the clones were transferred to 200 μ l of fresh LB/glycerol medium using a 96 pin replicator tool (V&P Scientific Inc., 250520) and allowed to grow for 12 hr at 37°C. The plates were sealed with aluminum adhesive seals (VWR, 100845-646) and frozen at -80°C. The 96-well plates were packed on dry ice in styrofoam coolers, and shipped overnight to Beckman-Coulter Genomics (Danvers, MA). The samples were sequenced unidirectionally with the 5'TriplEx2 primer (5 'CTCCGAGATCTGGACGAGC 3'), with an average of 678 high quality base pairs per read. The putative identity of each clones was assessed using the BlastX multiple alignment tool (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?PAGE=Translations&PROGRAM=blastx>) against the GenBank non-redundant protein database.

Graphs and statistical analyses

Statistical analyses were performed using Prism 4.0 software (Graph Pad). Correlation, analysis of variance (ANOVA), Kruskal-Wallis, Mann Whitney, and *t*-tests were used where appropriate. Tukey's multiple comparison test or Dunn's multiple comparison test were used *post hoc* to determine significantly different groups. P-values <0.05 were considered significant. Fractions of escape embryos were transformed prior to statistical analysis by taking the arc sine of the square root of the fraction ($\text{ArcSin}\sqrt{X}$).

Results

Estrogen levels are higher in younger compared to older females

Investigation of 17 β -estradiol levels in females of different age classes indicated higher levels in ovarian tissue and plasma in younger females (Fig. 1A,B). This pattern is similar to the pattern observed for production of escape embryos in females of different age classes, where the youngest females produce the highest proportion of escape embryos. The production of escape embryos decreases through maternal life, with the lowest level of escape production in the oldest females. The youngest age class (90-105 days old) had mean 17 β -estradiol levels in the ovary of $12,382 \text{ pg/g} \pm 1,452$ and $11,636 \text{ pg/ml} \pm 1,739$ in plasma. The mid-age class had intermediate 17 β -estradiol values (while not significantly different from older females) of $6,323 \text{ pg/g} \pm 1,490$ in ovarian tissue, and $5,250 \text{ pg/ml} \pm 1,541$ in plasma. The oldest age class had the lowest 17 β -estradiol levels, with $5,088 \text{ pg/g} \pm 873$ in ovary, and $3,204 \text{ pg/ml} \pm 744$ in plasma. The results indicate a decline of 59% in ovarian 17 β -estradiol concentrations from young to old females, and a decline of 73% in plasma levels.

Hormone panels in ovaries of young females

No significant differences in ovarian hormone levels were discovered between females producing different ratios of escape embryos (Fig. 2). However, there was a trend towards higher androstenedione values in females producing less than 8%

escape embryos compared to those producing a high proportion of escape embryos (2,952 $\mu\text{g/g} \pm 628$ vs 1,073 $\mu\text{g/g} \pm 603$, $p=0.0854$, $t=1.923$, $df=10$). The mean 17 β -estradiol level in young females was 11,551 $\mu\text{g/g} \pm 1299$, levels of progesterone averaged 497 $\mu\text{g/g} \pm 84$, androstenedione levels were 2314 $\mu\text{g/g} \pm 524$, and testosterone levels were 814 $\mu\text{g/g} \pm 217$. Interestingly, ratios of 17 β -estradiol to testosterone and progesterone exhibit a high degree of correlation with the proportion of escape embryos produced (Fig.3). Increased 17 β -estradiol/testosterone and 17 β -estradiol/progesterone ratios are associated with higher proportions of escape embryos (Pearson $r=0.768$, $p=0.0035$; Pearson $r=0.463$, $p=0.1491$, respectively). It is important to note that in both cases the relationship is influenced by possible outliers. When these points are removed, the association between 17 β -estradiol/testosterone is weakened although remains significant (Pearson $r=0.645$, $p=0.0323$), and the correlation between 17 β -estradiol/progesterone ratios and the production of escape embryos becomes significant (Pearson $r=0.872$, $p=0.0005$).

Embryonic hormone levels

There were no significant differences in hormone levels of newly fertilized embryos sampled from clutches with high (>85%) and low (0%) proportions of escape embryos produced (Fig. 4A,B). Therefore, hormone levels are presented as combined values for both escape and diapause groups. The mean hormone concentrations were: 0.9 $\mu\text{g/embryo} \pm 0.1$ 17 β -estradiol, 1.3 $\mu\text{g/embryo} \pm 0.2$ corticosterone, 0.8 $\mu\text{g/embryo} \pm 0.2$ progesterone, 0.4 $\mu\text{g/embryo} \pm 0.1$ androstenedione,

and $0.3 \text{ pg/embryo} \pm 0.08$ testosterone. As a function of tissue mass, the mean values are $379 \text{ pg/g} \pm 25$, $542 \text{ pg/g} \pm 92$, $311 \text{ pg/g} \pm 82$, $177 \text{ pg/g} \pm 63$, and $119 \text{ pg/g} \pm 35$ respectively.

17β -estradiol levels fall through early development, to a significantly lower level in 30°C embryos at the 5-10 somite stage (Fig. 5A: Two way ANOVA, $p < 0.05$, $t = 2.925$). Androstenedione values follow the same pattern as 17β -estradiol, although the drop is not significant (Fig. 5B). Hormone levels increase again following the 5 - 10 somite stage. This is the developmental period where 30°C treatment results in a steep increase in the production of escape embryos.

Embryonic hormone treatments

Exogenous treatment with 17β -estradiol and cortisol resulted in significantly increased numbers of escape embryos (Fig. 6AB). Incubation in 17β -estradiol caused the majority of embryos to follow the escape trajectory at concentrations above $5 \text{ } \mu\text{g/ml}$ (Kruskal-Wallis test $p = < 0.0001$, KW statistic = 40.64) (Fig. 6A). The magnitude of this effect did not significantly change by increasing the treatment concentration from 5 to 10 or $20 \text{ } \mu\text{g/ml}$ (escape percentages respectively: 64 ± 7 , 75 ± 7 , and 72 ± 11), suggesting a plateau effect (Fig. 6B). Cortisol treatment also significantly increased the percentage of embryos following the escape trajectory at concentrations above $5 \text{ } \mu\text{g/ml}$ (One way ANOVA $p < 0.0001$, $F = 26.24$). However, increasing cortisol concentrations above this concentration had an additive effect on the number of escape embryos, unlike the plateau effect seen with 17β -estradiol treatment.

17 β -estradiol treatment at levels above 1 $\mu\text{g/ml}$ led to a saturation effect in embryos. Higher concentrations in treatment levels were not significantly different at most points during treatment (Fig. 7). Dechorionated control embryos had estradiol levels that were not statistically different than hormone levels in 25°C post-diapause II embryos (Fig. 8). Estradiol levels were slightly higher than in 30°C 25-30 somite embryos, the stage developmentally closest to the dechorionated embryos. However, dechorionated embryos were sampled at 18 dpf whereas 25-30 somite embryos were sampled at 12-14 dpf. 17 β -estradiol levels in estrogen-treated embryos were significantly higher than in controls (t-test $p=0.0005$, $t=10.48$, $df=4$).

Androstenedione levels in estrogen treated embryos were also significantly higher than in controls ($p=0.0018$, $t=7.339$, $df=4$), indicating possible reverse aromatase activity. Removing the chorion dramatically changed the amount of estradiol detected in hormone treated embryos. Measured 17 β -estradiol levels decreased by more than 99% after chorion removal, from an average of 38,532 μg to 129 μg per embryo.

Differential Expression of Genes Associated with Escape Embryos

A total of 146 genes were identified as differentially expressed in maternal tissues isolated from females producing escape embryos and embryos on an escape trajectory (see appendix for sequences). Sequences identified as differentially expressed are presented in figure 9 organized as a function of tissue. Of the total, 44 genes were identified as differentially expressed in escape embryos of *A. limnaeus* (Fig. 10 & Table 1,2). Three differentially expressed genes were shared between the

14-16 somite escape embryos and newly fertilized escape embryos: Guanine nucleotide exchange factor MSS4, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase, and variations of histone H2A.V. Guanine nucleotide exchange factor MSS4 is downregulated in newly fertilized escape embryos and upregulated in 14-16 somite escape embryos. Six-phosphofructo-2-kinase/fructose-2,6-biphosphatase is upregulated in newly fertilized escape embryos and downregulated in escape embryos at the 14-16 somite stage. Histone H2A. V is downregulated in escape embryos compared to embryos on a diapause trajectory in both newly fertilized and 14-16 somite embryos. Additionally, two different isoforms of histone acetyltransferase 1, have been identified in 14-16 somite embryos, one highly expressed in escape embryos and the other in embryos bound for diapause II.

In newly-fertilized embryos, a total of 55 genes were identified as being differentially expressed (Table 1,2). Of these genes, 33 (60%) were upregulated in escape embryos and 22 (40%) in embryos that would enter diapause II. Forty-eight percent of these differentially expressed sequences have significant homology with sequences in the GenBank non-redundant protein database as determined by the Blastx search algorithm.

Twenty-two differentially expressed sequences were identified in 14-16 somite embryos on the two developmental trajectories (Table 3). Eleven of these (50%) were identified as upregulated in embryos on the escape trajectory and 11 (50%) were associated with embryos on the diapause II trajectory. A large proportion

($\approx 70\%$) of these sequences have significant homology with sequences in the GenBank non-redundant protein database.

Differential Gene Expression in Maternal Tissues

Sixty-nine sequences were identified as differentially expressed in ovary and liver tissues isolated from female fish producing either high or low proportions of escape embryos (Table 4 & 5). Of these sequences only one, the membrane-associated progesterone receptor component 1 (ACQ58387.1, *Anoplopoma fimbria*) shared a consistent expression pattern in both ovary and liver tissues. This gene was ≈ 7 -fold upregulated in both tissues of females producing escape embryos. Liver tissue had two differentially regulated sequences in common with newly fertilized embryos, one has no significant homology to known proteins (ALD37M09) and the other is an integrin-linked protein kinase (NP_956865.1, *Danio rerio*). These sequences were downregulated in both embryos and tissues associated with an escape embryo trajectory. Beta-tubulin was also differentially expressed in both liver and 14-16 somite embryos. Upregulation of this sequence was associated with escape embryo production in liver tissue (ALD09H02), but it was downregulated in 14-16 somite embryos on the escape trajectory (ALD15L11).

Twenty-three sequences were identified as differentially expressed in maternal ovary with respect to the production of escape or diapausing embryos. Thirteen ($\approx 57\%$) of these sequences were upregulated in females producing escape embryos and 10 ($\approx 43\%$) were associated with females producing diapausing embryos (Table

4). Less than 40% of the differentially expressed sequences identified from ovary tissue had significant homology with sequences in the GenBank non-redundant protein database.

Maternal liver tissue exhibited 46 differentially expressed sequences in females producing escape embryos relative to those producing diapausing embryos. Twenty-six ($\approx 57\%$) of these were upregulated in females producing escape embryos and 20 ($\approx 43\%$) were downregulated (Table 5). Interestingly, 3 genes were highly expressed in liver tissue isolated from females producing diapausing embryos: one is 40-fold upregulated and has no significant homology to known protein sequences (ALD37P02), the second is a Clasp2 protein that is 25-fold upregulated, and the third is a Chromobox homolog 3 that is induced 24-fold. Of the sequences identified as differentially expressed in liver tissue, $\approx 60\%$ had significant homology with sequences in the GenBank non-redundant protein database.

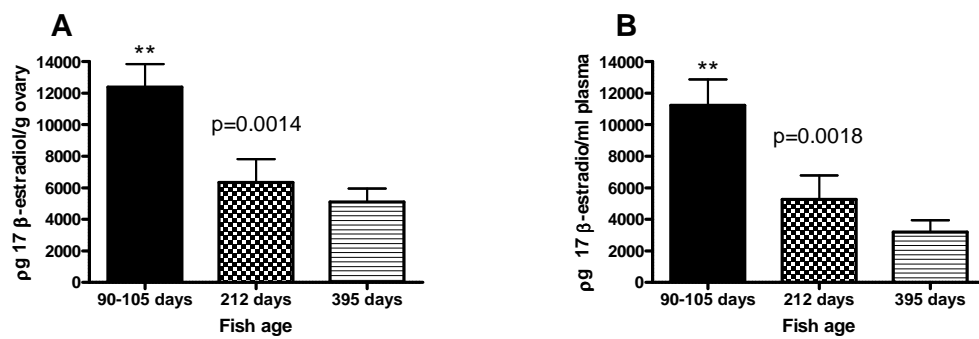


Figure 1. Levels of 17 β -estradiol in (A) ovarian tissue and (B) plasma of female *Austrofundulus limnaeus* as a function of age. Young females have higher ovarian levels of 17 β -estradiol than older females (ANOVA, $p=0.0014$, $F=8.54$, Tukey's multiple comparison test: 90-105 ($n=13$) vs 212 ($n=8$) $p<0.05$, 90-105 vs 395 ($n=8$) $p<0.01$). Plasma 17 β -estradiol levels are also higher in younger than older females (Kruskal-wallis test, $p=0.0018$, KW statistic=12.69, Dunn's multiple comparison test: 90-105 ($n=12$) vs 212 ($n=10$) $p<0.05$, 90-105 vs 395 ($n=8$) $p<0.01$). Bars represent mean \pm s.e.m.

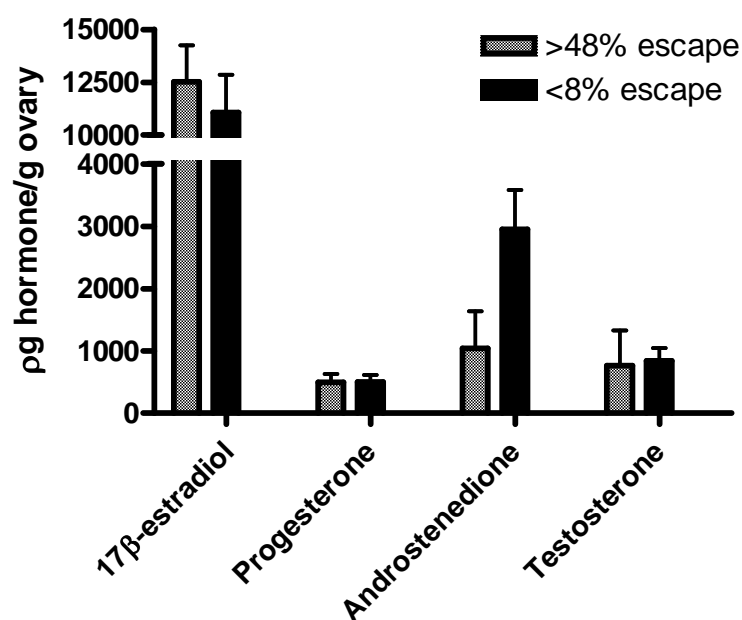


Figure 2. Hormone levels in ovarian tissues isolated from young female *A. limnaeus* (90-105 days). Black columns are hormone values for fishes producing less than 8% escape embryos (n=8) and shaded columns (n=4) represent values for females that produced over 48% escape embryos. The proportion of escape embryos produced was determined over the entire lifetime spawning for that individual fish (total number of spawns ranges from 3-7). There are no statistically significant differences in hormone levels in ovarian tissue isolated from the two groups. Bars represent mean \pm s.e.m.

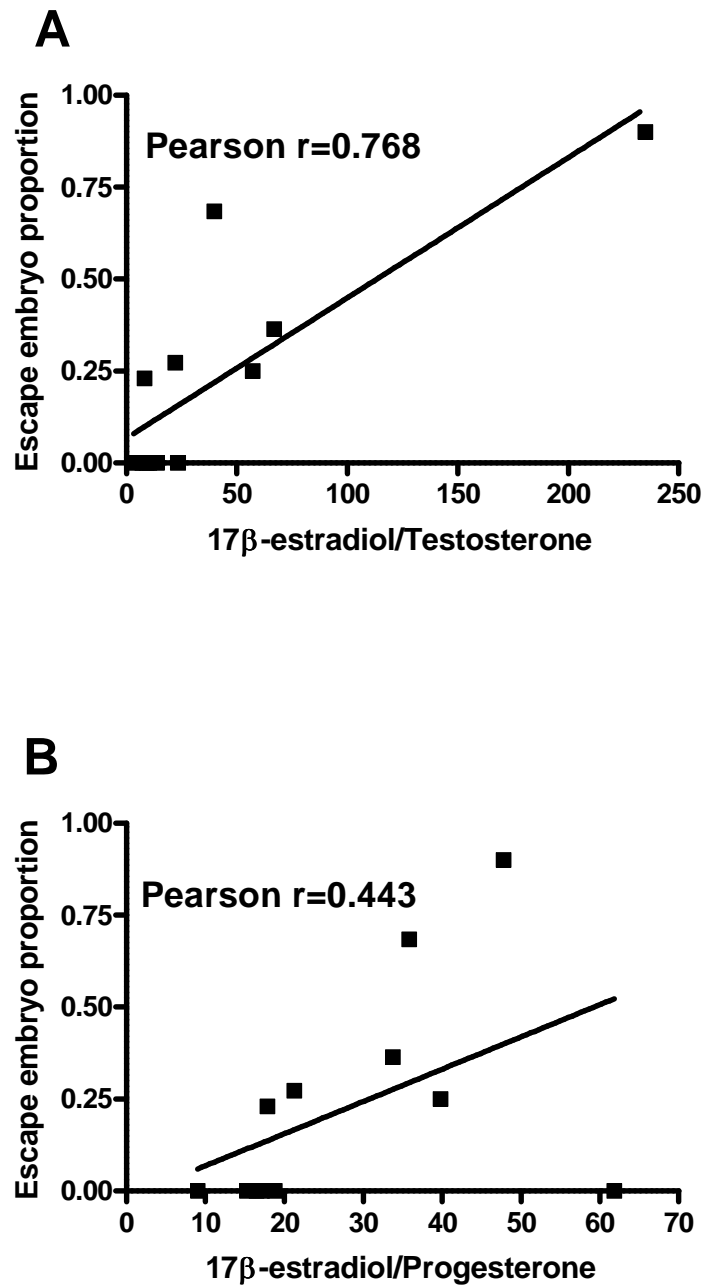


Figure 3. The relationship between the ratio of 17 β -estradiol to (A) testosterone, and (B) progesterone as a function of lifetime escape embryo production in young females (90-105 days). There is a significant correlation between the 17 β -estradiol/testosterone ratio and lifetime production of escape embryos ($p=0.0035$, Pearson $r=0.768$).

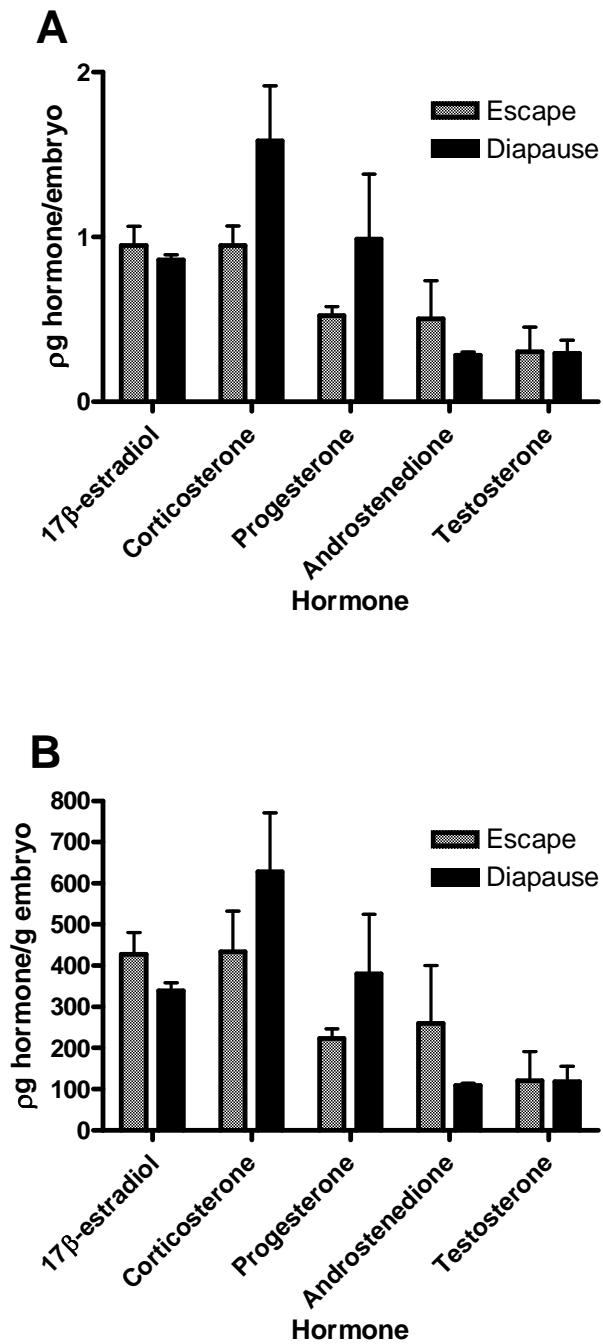


Figure 4. Hormone concentrations in newly fertilized embryos presented (A) per embryo and (B) per g embryo tissue. There are no significant differences between embryos presumed to be on either developmental trajectory. Bars represent mean \pm s.e.m. (n=4-5)

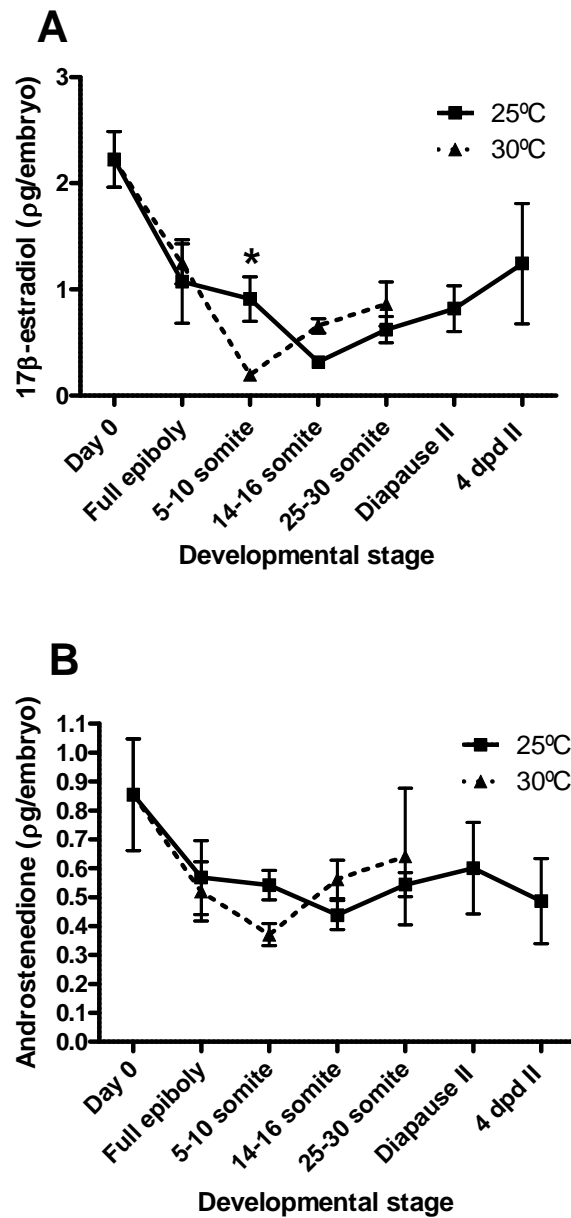


Figure 5. Hormone levels through early development. **(A)** 17 β -estradiol levels fall through early development, to a significantly lower level in 30°C embryos at the 5-10 somite stage (Two way ANOVA, $p < 0.05$, $t = 2.925$). **(B)** Androstenedione values follow the same pattern as 17 β -estradiol, although the drop is not significant. Hormone levels increase again following the 5-10 somite stage. This is the developmental period where 30°C treatment results in a steep increase in the production of escape embryos. Bars represent mean \pm s.e.m.

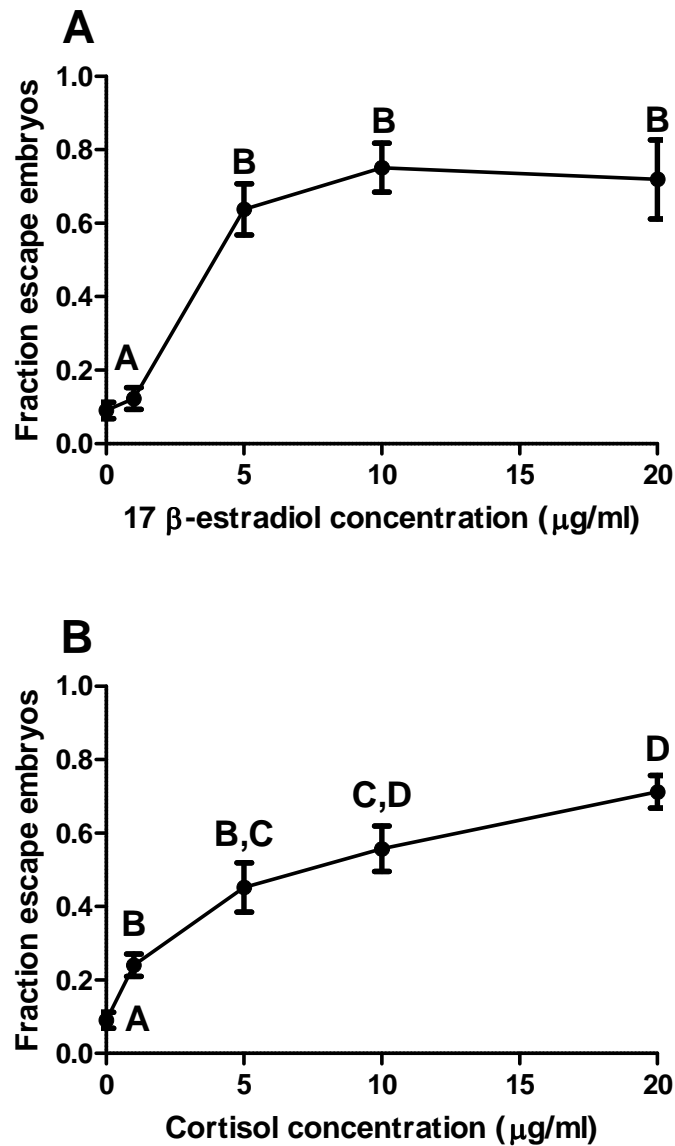


Figure 6. Effect of incubation in exogenous (A) 17 β -estradiol and (B) cortisol on the proportion of embryos that escape diapause II. Exposure to levels of 17 β -estradiol greater than 1 $\mu\text{g/ml}$ results in the majority of embryos following an escape embryo trajectory (Kruskal-Wallis test $p < 0.0001$, KW Statistic=40.64, $n = 12$). Cortisol treatment induces an increase in the proportion of escape embryos in a more dose-response typical fashion (ANOVA $p < 0.0001$, $F = 27.39$, $n = 12$). Different numbers above columns represent statistically significant differences. Bars represent mean \pm s.e.m.

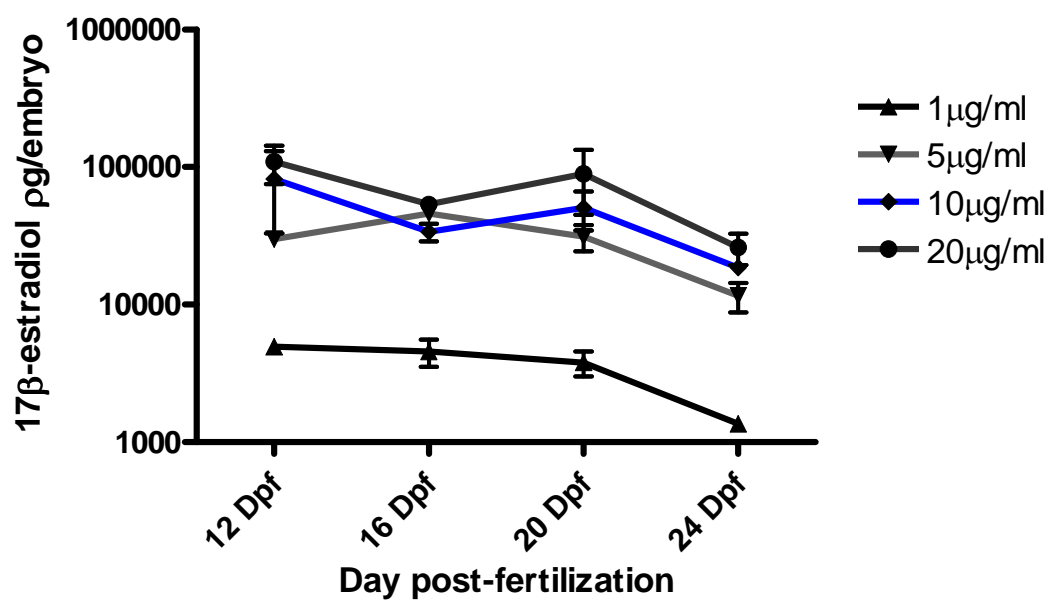


Figure 7. Hormone levels through embryonic hormone treatment; treatment began 6 dpf. Treatment above 1 $\mu\text{g/ml}$ resulted in hormone levels not significantly different at most time points. Bars represent mean \pm s.e.m.

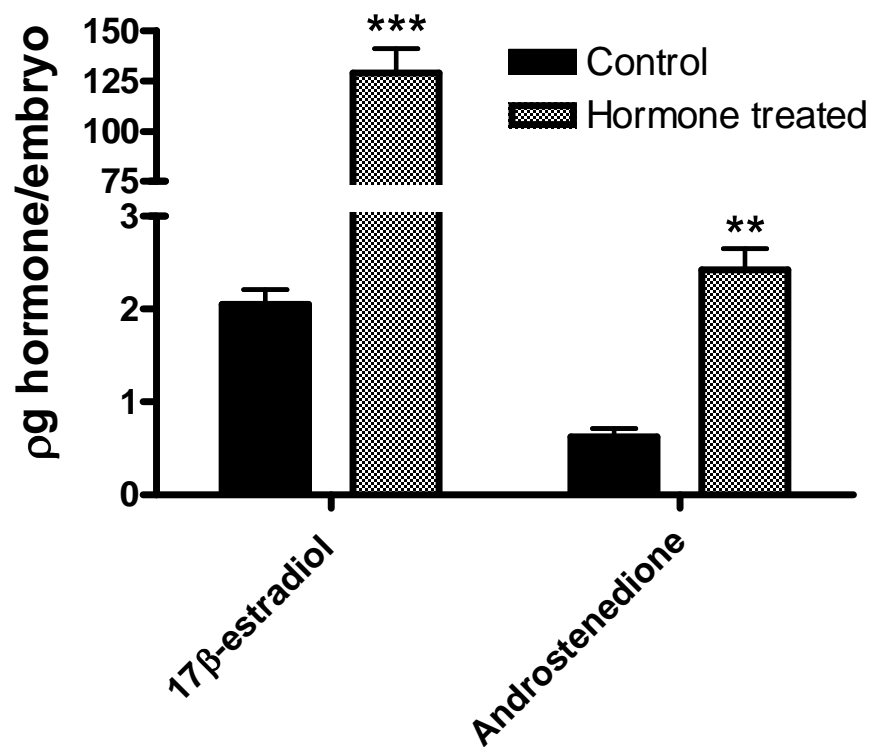


Figure 8. Hormone levels in 17 β -estradiol treated and control dechorionated embryos. Embryos sampled at 18 dpf, after 12 days hormone treatment 17 β -estradiol levels are significantly higher in treated embryos ($p=0.0005$, $t=10.48$, $df=4$). Androstenedione levels in estradiol-treated embryos are significantly higher than in control embryos ($p=0.0018$, $t=7.339$, $df=4$). Bars represent mean \pm s.e.m.

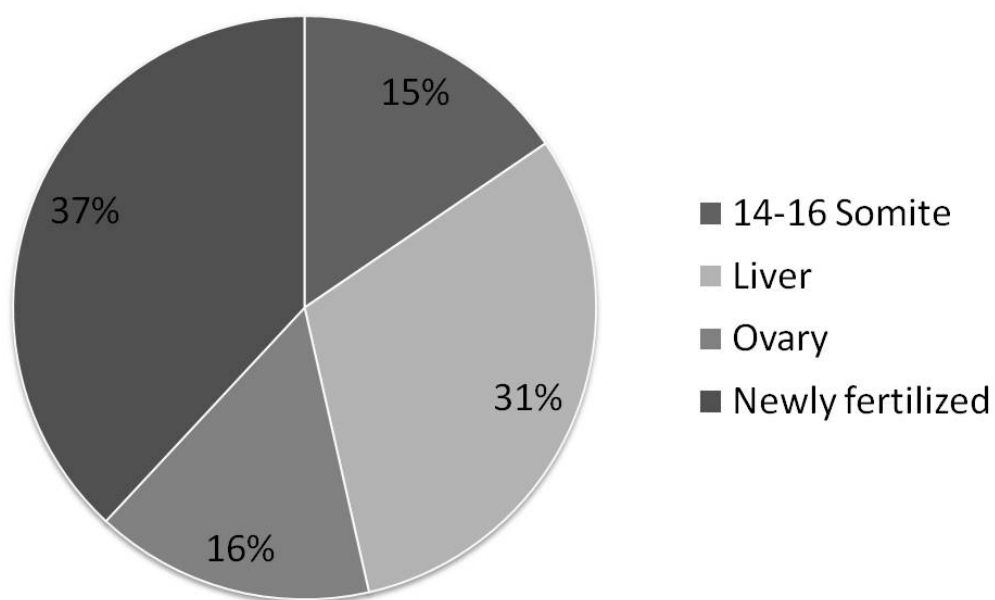


Figure 9. The distribution of sequences found to be differentially expressed in escape embryos and tissues isolated from females producing escape embryos by tissue. Some genes are shared between tissues, therefore total \neq 100%.

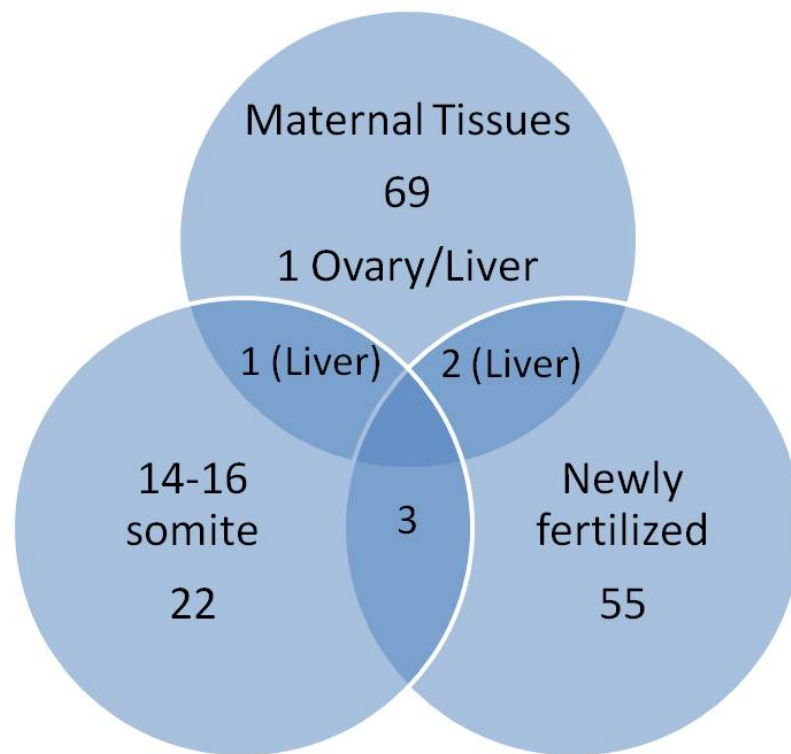


Figure 10. The number of differentially regulated genes identified in each microarray experiment to identify sequences unique to production of escape embryos. Maternal tissues include sequences identified in both ovary and liver tissues. Numbers in overlapping sections represent the number of differentially regulated genes common to both samples.

Gene ID	Corrected p	p-value	Fold Δ	Identity	Ref SEQ	E-value
ALD23E05	0.0494	0.0113	4.6	Guanine nucleotide-binding protein G(z) subunit alpha	NP_001156038.1	7E-112
ALD39F08	0.0494	0.0293	4.4	No significant homology		
ALD14B16	0.0494	0.0262	4.0	Warm temperature acclimation-related 65 kDa protein	ABL75414.1	4E-107
ALD41M22	0.0494	0.0455	4.0	No significant homology		
ALD05H19	0.0494	0.0402	3.9	No significant homology		
ALD40N06	0.0494	0.0066	3.8	No significant homology		
ALD11N08	0.0494	0.0110	3.8	No significant homology		
ALD37O10	0.0494	0.0363	3.7	Hypothetical protein Ssol98_08391	ZP_06388631.1	8E-16
ALD16C23	0.0494	0.0087	3.7	Retinol-binding protein-like protein	ACO40444.1	3E-09
ALD18B05	0.0494	0.0161	3.5	No significant homology		
ALD01F14	0.0494	0.0301	3.4	Myoglobin	BAG84239.1	5E-57
ALD12F21	0.0494	0.0313	3.4	No significant homology		
ALD06P19	0.0494	0.0036	3.2	Claudin 32a	AAT64086.1	1E-08
ALD03A14	0.0494	0.0345	3.1	NADH dehydrogenase subunit 1	YP_001491318.1	4E-58
ALD06P03	0.0494	0.0315	3.0	No significant homology		
ALD05N23	0.0494	0.0020	2.8	No significant homology		
ALD04P15	0.0494	0.0104	2.8	PREDICTED: 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase	XP_001919792.2	2E-72
ALD04H19	0.0494	0.0271	2.7	PREDICTED: deoxyguanosine kinase-like	XP_002662248.1	5E-12
ALD41H23	0.0494	0.0100	2.5	No significant homology		
ALD37E03	0.0494	0.0343	2.5	No significant homology		
ALD22E16	0.0494	0.0122	2.4	Ribosomal protein S25	BAF45913.1	2E-105
ALD04J15	0.0494	0.0107	2.4	No significant homology		
ALD22M16	0.0494	0.0444	2.4	Glutathione S-transferase theta-class	ABF70330.1	1E-89
ALD07O15	0.0494	0.0403	2.3	No significant homology		
ALD35J06	0.0494	0.0222	2.3	No significant homology		
ALD38F08	0.0494	0.0049	2.3	Deoxyhypusine synthase	NP_998387	1E-81
ALD25M22	0.0494	0.0206	2.3	No significant homology		
ALD08I08	0.0494	0.0478	2.2	No significant homology		
ALD38A07	0.0494	0.0468	2.1	PREDICTED: inhibitor of apoptosis protein-like isoform 1	XP_002666015.1	6E-94
ALD35F16	0.0494	0.0399	2.1	No significant homology		
ALD19J15	0.0494	0.0289	2.0	Inactive hydroxysteroid dehydrogenase-like protein 1	A5WWC6.1	8E-38
ALD33B24	0.0494	0.0307	2.0	Transcription factor ZBP-89	NP_001073649.1	9E-69
ALD18F02	0.0494	0.0109	2.0	No significant homology		

Table 1. Differentially regulated genes in newly fertilized embryos, fold changes are relative to embryos on escape developmental trajectory. Only sequences significantly ($p < 0.05$) differentially regulated are presented.

Gene ID	Corrected p	p-value	Fold Δ	Identity	Ref SEQ	E-value
ALD27K10	0.0494	0.0283	-3.8	No significant homology		
ALD38K20	0.0494	0.0247	-2.6	No significant homology		
ALD33F13	0.0494	0.0138	-2.6	Hypothetical protein LOC449817	NP_001005990.1	2E-90
ALD19D01	0.0494	0.0021	-2.4	Poly (ADP-ribose) polymerase family, member 12b	NP_001108558.1	4E-59
ALD35A12	0.0494	0.0438	-2.4	No significant homology		
ALD35I18	0.0494	0.0341	-2.4	Eukaryotic translation initiation factor 3 subunit 6	ACO08955.1	8E-128
ALD14G11	0.0494	0.0131	-2.3	Serine hydroxymethyltransferase [Danio rerio]	AAX45073.1	1E-118
ALD42B03	0.0494	0.0153	-2.3	Heat shock cognate 71	ADK88904.1	6.00E-106
ALD14K02	0.0494	0.0394	-2.3	No significant homology		
ALD38D03	0.0494	0.0116	-2.3	No significant homology		
ALD33F04	0.0494	0.0169	-2.3	PREDICTED: histone H2A.V-like	XP_002924084.1	1E-54
ALD24L13	0.0494	0.0127	-2.2	Guanine nucleotide exchange factor MSS4	ACQ58514.1	3E-37
ALD26J13	0.0494	0.0284	-2.2	No significant homology		
ALD28M07	0.0494	0.0208	-2.1	Cyclin-dependent kinase 2-interacting protein	NP_001134627.1	2E-11
ALD35G19	0.0494	0.0253	-2.1	No significant homology		
ALD08K10	0.0494	0.0021	-2.1	No significant homology		
ALD09K02	0.0494	0.0127	-2.1	No significant homology		
ALD26F21	0.0494	0.0359	-2.1	Integrin-linked protein kinase	NP_956865.1	2E-73
ALD33D20	0.0494	0.0478	-2.0	Coiled-coil domain-containing protein 111 homolog	NP_001032455.1	1E-65
ALD37M09	0.0494	0.0108	-2.0	No significant homology		
ALD35I20	0.0494	0.0063	-2.0	No significant homology		
ALD02J22	0.0494	0.0272	-2.0	PREDICTED: cyclin G1-like isoform 2	XP_002664422.1	3E-87

Table 2. Differentially regulated genes in newly fertilized embryos, fold changes are relative to embryos on escape developmental trajectory. Only sequences significantly ($p < 0.05$) differentially regulated are presented.

Gene ID	Corrected p	p-value	Fold Δ	Identity	Ref SEQ	E-value
ALD23D03	0.0484	0.0354	5.1	CXADR	NP_001124253.1	1E-72
ALD37I12	0.0478	0.0313	4.5	No significant homology		
ALD16D08	0.0467	0.0238	4.5	skin mucus lectin	BAI49518.1	2.00E-58
ALD32D03	0.0478	0.0328	4.1	Phenylalanyl-tRNA synthetase alpha chain [Salmo salar].	NP_001133621.1	1.00E-96
ALD05E02	0.0406	0.0024	3.0	No significant homology		
ALD36C23	0.0365	0.0001	2.6	No significant homology		
ALD40F05	0.0419	0.0045	2.2	No significant homology		
ALD28M24	0.0406	0.0016	2.2	Phosphate cytidylyltransferase 1, choline, beta b	NP_001032451.1	7E-94
ALD33H14	0.0406	0.0022	2.1	Histone acetyltransferase type B catalytic subunit	NP_001004572.1	3E-08
ALD39L22	0.0367	0.0003	2.1	Carnosine dipeptidase 1 (metallopeptidase M20 family)	NP_001107394.1	1E-74
ALD24L13	0.0406	0.0037	2.1	Guanine nucleotide exchange factor MSS4	ACQ58514.1	3E-37
ALD04P15	0.0464	0.0223	-8.7	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase	XP_001919792.2	2E-72
ALD34H19	0.0486	0.0400	-6.0	Histone H2A.V	XP_002924084.1	1E-54
ALD32F04	0.0484	0.0362	-5.9	Histone acetyltransferase type B catalytic subunit	NP_001004572.1	4E-110
ALD33D11	0.0495	0.0472	-5.6	No significant homology		
ALD15H03	0.0469	0.0258	-5.6	Single-stranded DNA binding protein, mitochondrial precursor	ACQ59073.1	1.00E-61
ALD15L11	0.0406	0.0018	-5.3	Tubulin, beta 2C	AAH05547.1	1E-123
ALD28D03	0.0495	0.0472	-4.6	No significant homology		
ALD29I17	0.0478	0.0310	-4.5	No significant homology		
ALD34N22	0.0406	0.0025	-4.3	Flavin containing monooxygenase 5	NP_001133267.1	2E-112
ALD19B23	0.0372	0.0008	-4.2	Alpha-1-antitrypsin	ACJ05607.1	2E-80
ALD23I15	0.0406	0.0021	-3.5	Dimethylaniline monooxygenase 5	NP_001133267.1	5E-178

Table 3. Differentially regulated genes in 14-16 somite embryos, fold changes are relative to embryos on escape developmental trajectory. Only sequences significantly ($p < 0.05$) differentially regulated are presented.

Name	Corrected p	p-value	Fold Δ	Identity	Ref SEQ	E-value
ALD26D16	0.0200	0.0002	7.1	No significant homology		
ALD38D17	0.0493	0.0307	7.1	Membrane-associated progesterone receptor component 1	ACQ58387.1	3E-30
ALD24N21	0.0493	0.0419	5.4	No significant homology		
ALD38C17	0.0493	0.0259	5.2	Brain protein 44	ACQ58713.1	3E-21
ALD28F11	0.0493	0.0389	4.9	No significant homology		
ALD28L24	0.0493	0.0376	4.8	No significant homology		
ALD35J11	0.0493	0.0117	4.5	No significant homology		
ALD31H06	0.0500	0.0476	4.3	No significant homology		
ALD30H07	0.0493	0.0083	4.2	Activating signal cointegrator 1 complex subunit 1	ACQ58089.1	8E-76
ALD29C17	0.0493	0.0335	4.2	No significant homology		
ALD38P21	0.0493	0.0071	4.1	No significant homology		
ALD01M11	0.0446	0.0021	3.5	No significant homology		
ALD08H16	0.0446	0.0017	2.3	Masparidin	ACO10004.1	1E-125
ALD11C23	0.0493	0.0093	-8.9	No significant homology		
ALD20F24	0.0493	0.0276	-7.2	14 kDa apolipoprotein	ACM41842.1	6E-73
ALD09K12	0.0493	0.0310	-6.9	No significant homology		
ALD01B24	0.0493	0.0103	-5.0	Fructose-bisphosphate aldolase B	ACO09921.1	3.00E-100
ALD31K17	0.0493	0.0313	-4.8	No significant homology		
ALD27G15	0.0500	0.0478	-4.7	Hypothetical protein	XP_001619336.1	0.00002
ALD37K01	0.0493	0.0313	-4.5	Brain protein 44	ACQ58713.1	5E-61
ALD37G21	0.0497	0.0440	-4.5	No significant homology		
ALD28M08	0.0493	0.0236	-4.2	PREDICTED: SET translocation-like	XP_002709599.1	8E-77
ALD35M22	0.0446	0.0026	-2.2	No significant homology		

Table 4. Differentially regulated genes in ovary of adult fish. Fold changes are relative to fish producing high proportions of escape embryos. Only sequences significantly ($p < 0.05$) differentially regulated are presented.

Name	Corrected p	p-value	Fold Δ	Identity	Ref SEQ	E-value
ALD41H13	0.0137	0.0000	13.0	No significant homology		
ALD21G08	0.0497	0.0251	10.9	Leucine-rich repeat flightless-interacting protein 2	NP_955773.1	1E-134
ALD39G18	0.0497	0.0220	10.7	No significant homology		
ALD41B02	0.0497	0.0194	10.5	No significant homology		
ALD14E08	0.0497	0.0272	8.9	Collagen type XI alpha-2	ADG36302.1	3E-09
ALD41D24	0.0408	0.0014	8.0	PREDICTED: neurexophilin-2-like	XP_002930275.1	7E-75
ALD20J24	0.0246	0.0002	7.6	No significant homology		
ALD40E16	0.0497	0.0032	7.3	No significant homology		
ALD41D11	0.0497	0.0442	6.7	Regulator of G-protein signalling 4	NP_954968.1	9E-56
ALD38E15	0.0497	0.0165	6.1	Membrane-associated progesterone receptor component 1	ACQ58387.1	1E-75
ALD39G15	0.0408	0.0016	5.8	No significant homology		
ALD21F15	0.0497	0.0302	5.2	Novel protein similar to vertebrate WAS protein family, member 1	CAN88174.1	2E-153
ALD28B18	0.0440	0.0020	5.0	No significant homology		
ALD32G09	0.0497	0.0083	4.8	No significant homology		
ALD14E16	0.0388	0.0007	4.6	No significant homology		
ALD28B21	0.0497	0.0241	4.2	Endothelin-converting enzyme 2	NP_001134861.1	2E-45
ALD28A20	0.0497	0.0199	4.1	Leucine rich repeat containing 4	NP_001093494.1	8E-114
ALD09H02	0.0414	0.0018	2.6	PREDICTED: tubulin beta-4 chain-like isoform 2	XP_002801082.1	5E-12
ALD26C03	0.0408	0.0012	2.2	Aminopeptidase N [Felis catus]	AAD09272.1	0.012
ALD29M01	0.0455	0.0025	1.9	No significant homology		
ALD36D24	0.0455	0.0026	1.8	No significant homology		
ALD33L21	0.0455	0.0022	1.7	Putative reverse transcriptase	AAK58879.1	7.00E-47
ALD03D04	0.0388	0.0008	1.6	No significant homology		
ALD33D21	0.0455	0.0026	1.5	PREDICTED: acid phosphatase 1, soluble isoform 1	XP_002723233.1	2E-13
ALD15D21	0.0408	0.0016	1.4	No significant homology		
ALD04D03	0.0408	0.0015	1.3	Hycin	NP_001026154.1	5E-69
ALD37P02	0.0497	0.0151	-39.9	No significant homology		
ALD37P01	0.0497	0.0117	-24.9	Clasp2 protein	AAH76002.1	1E-13
ALD37G10	0.0246	0.0001	-23.5	Chromobox homolog 3	ACQ58448.1	6.00E-71
ALD28L22	0.0374	0.0006	-10.7	RNA-binding protein 5	NP_001093608.2	5E-37
ALD35M13	0.0497	0.0219	-7.7	Integrin-linked protein kinase	NP_956865.1	4E-76
ALD37M10	0.0497	0.0475	-7.5	No significant homology		
ALD35H01	0.0497	0.0092	-6.9	Mediator of RNA polymerase II transcription subunit 28	NP_001007771.1	2E-54
ALD37M09	0.0497	0.0161	-5.7	No significant homology		
ALD40K12	0.0497	0.0088	-5.5	L-lactate dehydrogenase A chain	Q92055.3	3E-130
ALD40D17	0.0497	0.0052	-4.9	NFYA, nuclear transcription factor Y, alpha	CAN87934.1	4E-21
ALD41M14	0.0497	0.0307	-4.8	Sugar phosphate exchanger 2	NP_001133723.1	2E-32
ALD23L08	0.0497	0.0042	-4.6	Unnamed protein product	CAF94605.1	0.00002
ALD41J01	0.0497	0.0410	-4.3	No significant homology		
ALD35M04	0.0497	0.0102	-4.3	N-acetyltransferase NAT13	ACI67677.1	1E-77
ALD37K15	0.0408	0.0013	-3.7	PREDICTED: similar to multiple ankyrin repeats single KH domain protein	XP_414473.2	2E-24
ALD28D17	0.0455	0.0024	-2.9	No significant homology		
ALD18J24	0.0414	0.0017	-2.6	Integral membrane protein 2C	ACI33738.1	2E-88
ALD03J18	0.0246	0.0003	-2.3	No significant homology		
ALD14P11	0.0455	0.0025	-1.8	Hippocalcin-like protein 1	NP_001167347.1	6E-62
ALD09P18	0.0468	0.0028	-1.4	Alkylated DNA repair protein alkB homolog 7	NP_001133859.1	3E-65

Table 5. Differentially regulated genes in liver of adult fish. Fold changes are relative to fish producing high proportions of escape embryos. Only sequences significantly ($p < 0.05$) differentially regulated are presented.

Discussion

Steroid hormones are powerful modulators of organismal form and function. Entire suites of phenotypic traits, such as sexual dimorphism, can be coordinately expressed through the action of these signaling molecules. This study focuses primarily on the role of estrogens on the regulation of diapause in embryos of the annual killifish *A. limnaeus*. Estrogens play a major role in regulation of reproduction and metabolism in vertebrates and they are known to have numerous environmentally protective actions. Thus, I hypothesize that estrogen signaling may play a major role in the regulation of metabolic dormancy associated with diapause in embryos of *A. limnaeus*.

The effect of estrogens can be mediated through two major routes, genomic and non-genomic. Genomic actions depend on hormones binding to specific receptors initiating a unique gene expression program. Non-genomic actions do not necessarily result in the production of new gene products, but rather cause post-translational alterations in cellular physiology. Non-genomic actions are characterized by rapid initiation (too rapid to be mediated through transcription or translation). In fact, these effects may be realized in cell types lacking the machinery for protein synthesis, and are unchanged by inhibition of nuclear hormone receptors or the machinery of translation (Falkenstein et al., 2000; Revelli et al., 1998). Interestingly, the effects are not blocked by hormone adhesion to elements too large to pass through the cell

membrane, and thus are likely mediated by membrane-bound receptors. Non-genomic actions of estrogens are specific, and similar but non-identical hormone molecules can exert different magnitudes of response (Persky et al., 2000; Falkenstein et al., 2000; Revelli et al., 1998). These non-canonical actions of estrogens have been shown to initiate physiological changes that can protect heart muscle and skeletal elements from oxidative damage (Persky et al., 2000), and estrogen has also been shown to protect against ischemic damage (Dubal et al., 1998). Evidence presented below suggests estrogen may be acting via both genomic and non-genomic routes to regulate the fate of *A. limnaeus* to either enter or escape diapause II.

Maternal Effects on Developmental Trajectory

Overall, the steroid hormone levels reported here for female *A. limnaeus* correspond well to published values for other killifish species (*Fundulus heteroclitus* plasma; $\approx 10,000$ - $30,000$ $\mu\text{g/ml}$ 17 β -estradiol; *Kryptolebias marmoratus* plasma hermaphrodite $\approx 3,000$; male $\approx 7,000$ $\mu\text{g/ml}$ 17 β -estradiol), however estrogen levels are elevated in comparison to other reported values for teleosts (Minamimoto et al., 2005; Cochran et al., 1988; Webb et al., 2002; Wang et al., 2008; Sisneros et al., 2004; Bradford and Taylor, 1987; Rahman et al., 2000; Hobby and Pankhurst, 1997; Feist et al., 1990; Bayunova et al., 2003; Barannikova et al., 2003). 17 β -estradiol is higher in female *A. limnaeus* plasma (range 292-26,532 $\mu\text{g/ml}$) when compared to values reported for other non-killifish teleost species (*Porichthys notatus* undetectable-13,888

pg/ml plasma; *Acipenser guel denstaedti* Spring: 113.3 ± 22.50 , Winter: 249.9 ± 48.66 pg/ml plasma). This may be a function of annual killifish continually spawning, whereas other reported values are for fish spawning seasonally or on a more intermittent basis (Cochran et al., 1988; Wang et al., 2008; Sisneros et al., 2004; Bradford and Taylor, 1987). Conversely, testosterone levels are much lower than estrogen in female *A. limnaeus* ($814 \text{ pg/g} \pm 217$, range 65-2,460 pg/g ovary tissue), and also lower than testosterone values reported for some other species (*Fundulus heteroclitus* 620 ± 330 - $8,090 \pm 2,750$ pg/ml plasma; *Porichthys notatus* undetectable-7,960 pg/ml plasma; *Acipenser guel denstaedti* Spring: $19,100 \pm 6,700$, Winter: $18,700 \pm 3,320$ pg/ml plasma), although these values are reported in plasma (Cochran et al., 1988; Hobby and Pankhurst, 1996; Bayunova et al., 2003). The elevated estrogen levels and relatively low testosterone levels observed may be explained by higher aromatase activity in *A. limnaeus* ovary tissue, but no data is currently available on aromatase activity in this species.

Levels of 17 β -estradiol are known to decrease in female *A. limnaeus* as they age (Fig. 1), and this trend is correlated with a decrease in the production of escape embryos in older females (Podrabsky et al., 2010). This observation led me to hypothesize that differences in the maternal levels of 17 β -estradiol may affect the developmental trajectory of embryos. However, I found no direct correlation between estrogen levels in maternal ovary tissue and the production of escape embryos (Fig. 2). While overall levels of estrogen are not correlated with escape embryo production, the ratio of estrogen to testosterone is significantly correlated with the production of

escape embryos (Fig. 3, $r = 0.768$) and suggests that steroid hormone metabolism may indeed still be linked with the production of escape embryos. This correlation suggests a role for cytochrome P450 aromatases in determining developmental pathway; greater action by aromatases would result in this higher ratios and thus higher production of escape embryos. In teleosts, two aromatase proteins with similar catalytic activity are responsible for the production of estrogens through the aromatization of androgens (Callard et al., 2002; Piferrer and Blazquez, 2005). Interestingly, the membrane associated progesterone receptor component 1 (PGRMC1), that binds and positively regulates cytochrome P450 proteins (Hughes et al., 2007), is upregulated in ovary and liver of fish producing escape embryos (Table 4 & 5). PGRMC1 is known to be expressed in the cytoplasm but has also been shown to localize with cellular membranes in ovary tissue. The protein transduces the antiapoptotic action of progesterone in the ovary (Peluso, 2006; Peluso et al., 2008), although it does not directly bind progesterone (Min et al., 2005). The protein is thought to be involved in steroidogenesis (Zhang et al., 2008) and PGRMC1 interacts with multiple cytochrome P450's involved in progesterone and estrogen synthesis (Rohe et al., 2009). Inactivation of PGRMC1 can decrease the actions of cytochrome P450's, blocking cholesterol synthesis (Hughes et al., 2007), possibly through its interactions with a regulator of cholesterol homeostasis, Insig-1 (Suchanek et al., 2005). This action results in an increase in toxic sterol intermediates, and also interferes with pharmaceutical metabolism (Hughes et al., 2007). Thus, the

upregulation of PGRMC1 could change the hormonal environment of the ovary in females producing escape embryos through alterations in ovarian steroid metabolism.

Other evidence suggests a role for altered ovarian hormone metabolism with the production of escape embryos. The transcript for a 14kDa apolipoprotein involved in cholesterol transport and binding is downregulated in ovary tissue isolated from females producing escape embryos (Table 4). The activity of the closely related apolipoprotein A1 is enhanced by estradiol, and decreased by androgens (Hargrove et al., 1999), providing another link between hormonal levels and altered steroid metabolism. In chicken granulosa cells (Morley et al., 1992), human oocytes (Tesarik and mendoza, 1995), and rat endometrial tissue (Pietras and Szego, 1975; Pietras and Szego, 1977), treatment with 17 β -estradiol resulted in rapid uptake of Ca^{2+} (Revelli et al., 1998), which has been shown to inhibit aromatase activity (Balthazart et al., 2001; Balthazart et al., 2003). Importantly, this estrogen mediated increase in Ca^{2+} has been demonstrated on unfertilized oocytes (Tesarik and Mendoza, 1995), providing clear evidence that maternal hormone concentrations can immediately affect the intracellular milieu of developing oocytes. Aromatase expression has been demonstrated in embryos of *Danio rerio*, at different levels during development (Lassiter et al., 2002), and thus embryos of *A. limnaeus* very likely have the capacity to respond to steroid hormones through alterations in cytochrome P450 activity. Future studies should focus on the role of aromatase activity and steroid hormone metabolism in maternal tissues and their relationship to the production of escape embryos.

17 β -estradiol has also been shown to exert affects on other growth related molecular pathways via non-genomic pathways. The mitogen activated protein kinase (MAPK) pathway was induced by treatment with conjugated membrane impermeable 17 β -estradiol, through the phosphorylation of ERK-1 and ERK-2 (Watters et al., 1997). This pathway governs the duration of diapause in the silkworm (Fujiwara et al., 2006; Iwata et al., 2005) and the termination of embryonic diapause in a fleshfly (Fujiwara and Denlinger, 2007). Through these non-genomic actions, higher maternal estrogen levels could constitute the basis of profound effects on developing embryos, through modification of transcript deposition and phosphorylation state of maternally derived proteins.

In any hormone system, signaling can only be accomplished by the presence of active hormone and an active cellular receptor. The hormonal environment of unfertilized eggs has profound influences on the quality of the developing fish (Brooks et al., 1997; McCormick, 1998). Thus, the effects of estrogen on the regulation of diapause in *A. limnaeus* could be mediated through the expression of estrogen receptor variants. In teleosts, three different estrogen receptors have been identified, showing differences in tissue expression and differences in estrogenic stimulation based on tissue (Bardet et al., 2002; Greytak and Callard, 2007; Hawkins et al., 2000; Menuet et al., 2001; Callard et al., 2001; Menuet et al., 2002). Estrogen receptor activity can also be rapidly modified through phosphorylation (Balthazart et al., 2001). Nothing is currently known about estrogen receptor expression, activity, or regulation in relation to production of escape embryos in *A. limnaeus*. This an

obvious area for future research to focus based on the interesting effects of estrogen on the production of escape embryos, but the lack of a clear relationship between levels of estrogen and escape embryo production.

The upregulation of the transcript for a nuclear-receptor coactivator, activating signal cointegrator-1 (ASC-1), in ovary tissue from females producing escape embryos suggests an intriguing link between nutritional environment, steroid hormones, and the production of escape embryos. This protein acts as a transcriptional coactivator of nuclear receptors, including estrogen receptors and the steroid receptor coactivator, SRC-1 (Kim et al., 1999; Lee et al., 2001). ASC-1 binds target nuclear receptors or transcription factors, and additionally binds the RNA polymerase 2 complex, modifying transcriptional dynamics (Kim et al., 1999). In addition to enhancing the effects of steroid hormones, this family of coactivators also possesses histone acetyltransferase activity and is thus likely involved in mediating chromatin remodeling directed by steroid hormones (Xu and O'Malley, 2002). Rapid changes are found in the subcellular localization of ASC-1 under different nutritional states. ASC-1 is normally located in the nucleus, however after 24 hours of serum deprivation it is largely translocated to the cytoplasm. This change in localization is reversed when SRC-1 or its other coactivators are present, or under serum supplementation (Kim et al., 1999; Lee et al., 2001). ASC-1 could act as a link between nutritional status, endogenous hormonal cues, and changes in the ovary that alter the developmental trajectory of embryos produced. Annual killifish embryos can also respond to the presence of adults by entering developmental arrest at diapause I

or II (Inglima et al., 1981; Levels, 1986; Levels, 1988). It is tempting to suggest that this signal or a similar one, could function to alter the developmental trajectory of embryos as a function of nutritional status.

The effect of maternal nutritional status on the production of escape embryos has not been investigated, but this is a very promising and ecologically relevant cue; poor nutritional status of the female would likely cue for an unsuitable environment for hatching and growth of offspring. Nutritional status is known to affect entrance into developmental dormancy in a number of other systems. *C. elegans* larvae enter the dauer developmental arrest in response to dauer hormone, produced by nearby juveniles and adults under poor nutritional conditions (Butcher et al., 2007; Tatar et al., 2003; Gerisch and Antebi, 2004; Patel et al., 2008). Dormancy in *Daphnia* has also been shown to respond to maternal nutritional status. A poor maternal nutritional environment can result in a switch from parthenogenetic to bisexual reproduction and the production of ephippia, two dormant offspring attached to the back of the mother (LaMontagne and McCauley, 2001; Alekseev and Lampert, 2001; Alekseev and Lampert, 2004). This effect can be transmitted through multiple generations, and is also modified by environmental cues such as photoperiod and predator abundance (Alekseev and Lampert, 2001; Alekseev and Lampert, 2004). There are many similarities between dormancy in these invertebrate systems and in regulation of diapause in annual killifish embryos. The results of the present study suggest that maternal nutritional status may be one way that the production of diapausing embryos

is regulated. Future studies on the role of maternal nutritional status and the putative role of ASC-1 in mediating this signal will likely shed light on this possibility.

The potential role of epigenetic changes in maternal determination of developmental pathway, suggested through the interaction of ASC-1 with SRC-1, is strengthened by differential expression of other sequences identified. The SET-translocation like gene is upregulated in ovary from females producing diapausing embryos. SET is involved in gene silencing through the inhibition of demethylation. Specifically, it binds to targets of histone acetyltransferases (Jung et al., 2002). Chromobox homologue 3 (Cbx3), also known as heterochromatin protein 1 γ , is 25-fold upregulated in liver of females producing diapausing embryos compared to those producing escape embryos. The most common function of this protein is the production of heterochromatin through histone binding, typically resulting in gene silencing (Lomber et al., 2006). Additionally, members of the Cbx family are known to interact with non-histone proteins involved in transcriptional regulation, DNA repair, and chromatin modification (Lomber et al., 2006). These factors could induce epigenetic changes, resulting in relatively rapid changes in proportions of embryos on alternate developmental pathways, as seen in *A. limnaeus*.

Embryonic Hormonal Environment and Developmental Trajectory

Exposure to high levels of both estrogen and cortisol can cause embryos of *A. limnaeus* to shift from a diapausing to an escape developmental trajectory (Fig. 6).

Quantification of estrogen levels in embryos treated with exogenous 17 β -estradiol indicate similar levels of hormone accumulated in embryos that is independent of hormone concentrations exceeding 1 $\mu\text{g/ml}$ concentration (Fig. 7). The levels of hormone delivered to whole embryos in this manner suggests a massive dosage of hormones. However, when estrogen levels were measured in embryos first exposed to 5 $\mu\text{g/ml}$ estrogen and then dechorionated prior to quantification of hormone levels, the concentration of hormones in the cellular and yolk compartments of the embryos are several orders of magnitude lower (Fig. 8). Thus, greater than 99% of the hormone is absorbed to the chorion, and is not likely active within the embryo, which results in exogenous exposures that are about 100 times those found in control embryos (Fig. 8). Importantly, 17 β -estradiol treatment in embryos also resulted in significantly elevated levels of androstenedione (Fig. 8), a precursor of both testosterone and estrogen biosynthesis. This provides circumstantial evidence of aromatase activity modifying embryonic hormone levels with high levels of estrogen. This effect could result from aromatase activity in reverse, converting exogenous estrogens into androgens, or by high estrogen levels inhibiting aromatase as has been reported in another system (Shimizu et al., 2003), that could result in an increase in biosynthetic precursors such as androstenedione from normal metabolism.

Traditionally, lipophilic hormone levels in embryos were thought to be regulated by hormone levels in the maternal environment, with passive transfer from maternal plasma and the cytosol of theca and granulosa cells into developing offspring (Schwabl, 1993; Moore and Johnston, 2008). This passive model of

hormone transfer would result in higher levels of hormones in the yolk than maternal plasma, because of the lipophilic status of steroid hormones and the lipid nature of yolk (Moore and Johnston, 2008). However, newly fertilized embryos of *A. limnaeus* exhibit hormone levels that are considerably lower than those in the ovarian tissue from which they are derived (Fig.2, 4B). This suggests active regulation of hormone partitioning into embryos. Some mechanisms for regulation of hormone transfer have been proposed including an enzymatic barrier (Moore and Johnston, 2008) and a barrier to diffusion (Licht et al., 1998). Localized steroid production could also create an ovarian hormone gradient, although this is unlikely in light of our data showing the large differences between ovary and embryo hormone levels. At present the mechanism resulting in this difference between newly fertilized embryos and the ovary environment is unclear.

Embryos developing along the two different developmental trajectories appear to have different rates of estrogen metabolism (Fig. 5). Embryos incubated at 30°C (which will escape diapause II) metabolize both estrogen and androstenedione at a faster rate than embryos developing at 25°C that will enter diapause II. These differential rates of degradation result in significantly lower concentrations of these hormones in the 5-10 somite embryo. This stage also appears to be the developmental time that embryonic estrogen synthesis is initiated in escape embryos, while in embryos entering diapause it is not initiated until the 14-16 somite stage. There has been surprisingly little research on embryonic hormone production in teleosts. Some earlier studies have indicated little capacity for hormone synthesis in teleost embryos

prior to full yolk absorption (Brooks et al., 1997). Other results indicate an initial decline in steroid hormone levels, followed by a moderate increase (De Jesus et al., 1992). In Coho salmon embryos, steroid hormones decreased through development until hatching (Feist et al., 1990). Estradiol levels also decreased through development in snapping turtle embryos (Elf et al., 2002). Our results indicate that in *A. limnaeus*, hormone synthesis is initiated during the early stages of somitogenesis, and at different developmental stages for the two developmental trajectories. This period overlaps with the developmental window when incubation at 30°C results in a developmental switch from a diapausing to an escape embryo trajectory (Podrabsky et al., 2010). Thus, differences in hormone metabolism are correlated with developmental trajectory in this species, although a causative link has yet to be identified.

There is no direct relationship between hormone concentrations in newly fertilized embryos that will follow a diapausing or escape embryo trajectory (Fig. 4). The fact that high levels of estrogen can affect developmental trajectory, and the evidence presented above for altered gene expression in maternal tissues suggests that the effects of hormones on developmental trajectory could be mediated through alteration of hormone receptor expression or activity. Altered activity of steroid receptors in embryos could easily result in different sensitivity to the same total quantity of hormones. Increasing the hormone levels during oogenesis may alter hormone receptor levels or program for a different expression program for these receptors during early development. Steroid hormone receptor mRNA's are known to

be deposited into zebrafish embryos (Pikulkaew et al., 2009). Although our microarray studies did not identify differential levels of estrogen receptors in embryos, a transcript for a hydroxysteroid dehydrogenase (HSD) enzyme is upregulated in embryos destined to become escape embryos compared to those on a diapausing developmental trajectory (Table 1). HSD's have generated much interest due to their roles in hormone metabolism and disease (Moeller and Adamski, 2006; Tomlinson et al., 2004). In *C. elegans*, HSD1 null mutants initiate the dauer diapause stage inappropriately, and are hypersensitive to dauer hormone (Patel et al., 2008). The HSD identified in this study has the highest homology to a catalytically inactive form, HSDL1. This protein has a point mutation in the active center, that renders it enzymatically inactive in humans and zebrafish, and that is conserved in many other vertebrates (Meier et al., 2009). However, the widely conserved status of this protein, together with identified protein interactions in yeast, suggest a regulatory role for HSDL1 (Meier et al., 2009).

As in adult ovary tissue, epigenetic changes seem to play a role in the switch between escape and diapause developmental pathways within the embryo. One of the genes that is differentially expressed in newly fertilized escape embryos, and 14-16 somite stage escape embryos induced by incubation at 30°C is histone H2Av. This transcript is downregulated at both developmental stages in association with the escape embryo trajectory. Histone H2Av is a member of the H2Az variant histone family; members of this family are involved in transcriptional activation and repression (Dhillon and Kamakaka, 2000). In *Drosophila*, H2Av is involved in

heterochromatin formation and gene silencing (Swaminathan et al., 2005), and in yeast seems to flank euchromatin regions, inhibiting the spread of heterochromatin (Kobor et al., 2004). The downregulation of transcripts for this gene may imply structural remodeling to facilitate changes in gene expression that are linked with induction of the diapausing trajectory.

Newly fertilized embryos exhibit differences in transcript levels that are consistent with the later morphological and physiological differences observed in the diapausing and escape embryo trajectories. The sequence for 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-2 (PFKFB-2) is upregulated in newly fertilized embryos on an escape trajectory, and downregulated in escape embryos at the 14-16 somite stage. This enzyme has both phosphorylation and kinase activities, and directly regulates levels of fructose-2,6 bisphosphate, a potent activator of phosphofructokinase (PFK-1) and inhibitor of fructose-1,2 biphosphatase (Minchenko et al., 2003). PFK-1 is the key enzyme regulating glucose flux through glycolytic pathways, therefore higher levels of PFKB-2 would likely result in a high glycolytic/gluconeogenic ratio (Kurland and Pilkis, 1995). Expression of this protein has also been shown to be highly responsive to hypoxia and hypoxia-mimics, where hypoxia increases expression in liver and testis (Minchenko et al., 2003). At the 14-16 somite stage, this sequence is 8-fold downregulated in embryos on escape versus diapause trajectories which is consistent with a high glycolytic capacity in embryos that will enter embryonic diapause. This fits well with the observed morphology and physiology of these developmental trajectories (Chennault and Podrabsky, 2010).

Conversely, the PFKB-2 transcript is almost 3-fold more abundant in newly fertilized embryos on the escape trajectory than embryos on the diapause trajectory. The basis of this transcriptional difference is unclear, however it is tempting to venture that this could be a form of metabolic bet-hedging in escape embryos, where rapid metabolism and development are pre-determined. The sequence for hemoglobin is also upregulated in embryos that will follow the escape trajectory. Development of circulating and respiratory pigment islands are some of the most readily apparent morphological differences between escape and diapausing embryos (Podrabsky et al., 2010). Heat shock cognate 71 is upregulated in newly fertilized embryos that will enter embryonic diapause; expression of an inducible 70 kDa heat shock protein was found to be elevated in embryos of *A. limnaeus* during diapause II in an earlier study (Podrabsky and Somero, 2007). Two sequences for genes involved in cell cycle were also downregulated in newly fertilized embryos that will enter embryonic diapause; Cyclin-dependent kinase 2-interacting protein and cyclin G1- like isoform 2. Downregulation of these proteins would be consistent with the slow progression though the cell cycle that is observed in embryos developing along the diapausing trajectory.

Hormone levels of 17 β -estradiol decline as female *A. limnaeus* age, following the same pattern as escape embryo production. There is a significant correlation between estrogen and testosterone ratios and the production of escape embryos. This relationship suggests differences in aromatase activity, as well as possible receptor dynamics. There are concurrent transcriptional differences in tissues of female fish

producing embryos on altered developmental trajectories, in many processes that are hormonally responsive. Genes involved in epigenetics have been identified in the production of embryos on altered developmental pathways, fitting the often rapid temporal change seen in the type of embryos produced. These epigenetic changes are likely responsive to the hormonal environment, further being affected by the nutritional and environmental status of the fish. This is also seen in qualitative and quantitative differences in maternally packaged transcripts of embryos on different developmental pathways. While there is maternal control over embryonic developmental pathway, the immediate environment of the embryo during development also determines ultimate trajectory. Incubation at 30°C, that initiates the escape phenotype, also results in a more rapid metabolism of hormones in escape embryos, and the initiation of embryonic hormone synthesis at an earlier developmental stage (5-10 somites vs 14-16 somites). This initiation of hormone synthesis coincides temporally with the critical period that temperature treatment induces the majority of embryos to escape. At this same point we also see transcriptional differences in genes involved in metabolism, epigenetics, and processes associated with the morphological and physiological development of escape embryos. Further, hormone treatment induces the majority of embryos to escape embryonic diapause. Taken together, these experimental results strongly suggest that hormonal dynamics regulate developmental pathways, both during oocyte development and through embryonic development in *A. limnaeus*. Future studies in receptor dynamics and aromatase activity will likely further clarify the

components of this remarkable developmental program, and may elucidate basic mechanisms of embryonic development not apparent in a system of continuous embryonic development.

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Appendix A

Table 1 sequences

ALD23E05

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ALD39F08

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ALD14B16

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ALD41M22

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ALD05H19

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ALD40N06

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ALD11N08

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[illegible][illegible]

ALD01F14

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ALD12F21

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ALD06P19

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ALD03A14

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ALD06P03

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ALD05N23

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ALD04P15

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ALD04H19

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ALD41H23

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ALD37E03

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ALD19J15

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ALD33B24

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ALD18F02

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Appendix B

Table 2 sequences

ALD27K10

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ALD38K20

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ALD33F13

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ALD19D01

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Appendix C

Table 3 sequences

ALD23D03

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Appendix D

Table 4 sequences

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ALD31K17

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ALD27G15

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ALD37G21

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ALD35M22

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Appendix E

Table 5 sequences

ALD41H13

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ALD21G08

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ALD39G18

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ALD41B02

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ALD14E08

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ALD41D24

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ALD20J24

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ALD41D11

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ALD38E15

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ALD39G15

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ALD14E16

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ALD28B21

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ALD28A20

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ALD09H02

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ALD26C03

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ALD29M01

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