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Hormonal components of altered developmental pathways in the annual killifish, *Austrofundulus limnaeus*

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ABSTRACT

The annual killifish, *Austrofundulus limnaeus*, typically enters embryonic diapause at two distinct points of development, termed diapause II and III. This study explores the role of maternal and embryonic steroid hormones, including 17-β-estradiol (E2), androstenedione (A4) and testosterone (T), in regulating the developmental decision to enter or escape diapause II. Steroid hormone levels were measured in tissues isolated from adult female killifish during the normal lifespan of this species and in individuals of the same age that were producing either high or low proportions of escape embryos. Levels of steroid hormones were also measured during early development and in fertilized eggs that were predicted to be on either an escape or diapausing developmental trajectory. Decreases in maternal E2 levels associated with age are correlated with decreasing escape embryo production. Maternal production of escape embryos is correlated with increased ratios of E2 to T in adult ovary tissue. Interestingly, neither hormone is significantly different in fish producing embryos on different developmental pathways when examined independently. Levels of steroid hormones in fertilized eggs are not correlated with entry or escape from diapause II, though levels of A4 tend to be higher in escape embryos. Escape embryos exhibit faster hormone metabolism and earlier hormone synthesis than embryos that will enter diapause II. Incubation of embryos in exogenous E2 is associated with a 7-fold increase in escape embryo production, and significantly elevated A4 levels. These data suggest that steroid hormones may be critical factors involved in determining developmental pathways in embryos of *A. limnaeus*.

1. Introduction

1.1. Diapause in annual killifish

Annual killifish persist in inhospitable environments and survive conditions that few other fish could endure [74]. The ephemeral ponds inhabited by the South American annual killifish, *Austrofundulus limnaeus*, exhibit large diurnal fluctuations in temperature, pH, and dissolved oxygen content [57]. Upon desiccation of the pools, adult and juvenile fish perish [47]. 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 quickly grow to sexual maturity. They are known to spawn continuously as adults for the short duration (weeks to several months) that the ponds remain inundated.

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 [74]. Each of these three stages confers increased resistance to environmental insult. Not all embryos will enter each stage of diapause, and the length of time spent in diapause is highly variable. Thus, in various combinations, the three possible occurrences 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 local population extinction [76]. Thus, survival of this species is very likely dependent on the production of a variety of developmental phenotypes, each of which may be better suited for survival in a different environmental scenario.

Diapause I is a developmental arrest interjected between epiboly and embryogenesis, a time that is typically associated with gastrulation in most species of fish [74–76]. In annual killifish, the embryonic blastomeres go through stages of complete dispersion and subsequent reaggregation prior to formation of the embryonic axis.

Abbreviations: dpf, days post-fertilization; L:D, light:dark; hpf, hours post-fertilization; E2, 17-β-estradiol; A4, androstenedione; P4, progesterone; T, testosterone; CORT, corticosterone; WS, Wourms' stage; DMSO, dimethyl sulfoxide; PBS, phosphate buffered saline; ANOVA, analysis of variance; KW, Kruskal–Wallis; JH, juvenile hormone; MAPK, mitogen activated protein kinase.

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Diapause I can occur early in this process in an embryo composed of randomly dispersed blastomeres [75]. In embryos of *A. limnaeus*, entry into diapause I is extremely rare or absent, although the embryos still go through the dispersion and reaggregation stages of development consistent with the annual killifish life history [75,76].

Diapause III is a state of arrested development that occurs directly prior to hatching, when the embryos are fully developed [76]. This developmental arrest is thought to be obligate in most embryos of *A. limnaeus*, although small subpopulations (≈10%) bypass developmental arrest at diapause III and hatch immediately when the embryos are reared in a laboratory environment [76]. Diapause III is characterized by a significant metabolic depression [54], although metabolic capacity continues to increase as diapause III embryos age [9].

Diapause II is the most extensively studied stage of diapause in annual killifish. Diapause II embryos have 38 pairs of somites, a beating heart, optic cups, and other basic elements of the central nervous system [54,74,76]. This stage very likely possesses the highest tolerance to environmental stress [60], and survival through extended drought is likely only possible in diapause II [58]. The physiologies of anoxia tolerance [14,16,59], dehydration tolerance [58], and salinity tolerance [37] have all been studied in diapause II embryos. A severe metabolic depression during diapause II is supported by a major reduction in global rates of protein synthesis [55]. Thus, diapausing embryos have a limited ability to synthesize new proteins. Perhaps to partially compensate for this and to help support survival of environmental stress, a typically heat-inducible form of a 70 kDa heat shock protein is constitutively expressed during early development and reaches peak expression during diapause II [56]. While arrest of development occurs at approximately 24 days post fertilization (dpf) when embryos are incubated at 25 °C, both metabolism and heart rate begin to decline about 12 days prior to cessation of development [54]. Thus, it appears that the molecular events that signal for entry into diapause, and perhaps survival during diapause II, likely occur many days prior to the observed cessation of morphological development.

1.2. Alternate developmental trajectories

In many situations, developmental arrest in diapause is manifested as a form of phenotypic plasticity [7,12,13,20,23,38]. This is also the case for embryos of *A. limnaeus* that have recently been shown to exhibit plasticity in developmental pathways controlled by both environmental and maternal cues [60]. A small proportion of *A. limnaeus* embryos incubated at 25 °C will not enter diapause II, but instead develop through an alternate developmental pathway directly to diapause III [60]. Wourms termed these "escape embryos" because they escape from arrest of development in diapause II [76]. Escape embryos can be distinguished from embryos that will enter diapause II by differences in the timing (heterokairy) of morphological and physiological characters [60]. The morphological differences are first apparent when the embryos reach stages possessing about 18–20 pairs of somites. Escape embryos exhibit a number of developmental characteristics that do not form until several days after embryos resume development following diapause II. For example, escape embryos are covered on the dorsal aspect of the head and trunk with melanocytes, 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 [60]. The two developmental trajectories appear to result in qualitatively similar larvae when they hatch, although a quantitative investigation has not yet been undertaken. However, escape embryos reared at 25 °C possess lower anaerobic and aerobic capacity at the termination of development than do embryos that have entered diapause II. 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 [9].

1.3. Regulation of diapause induction

Despite a population-level pattern for the reduced production of escape embryos as females age, there is substantial temporal variation in the production of escape embryos, with some females producing exclusively escape or diapausing embryos, while others produce a mixture of the two types [60]. Thus, there appears to be significant maternal control over induction of diapause II that may include some form of maternal programming or provisioning. The process by which this maternal control is affected, and also the possible maternal environmental variables influencing escape embryo production are currently unknown.

The cellular and organismal signaling pathways governing the induction of embryonic diapause in annual killifish are unknown. However, some evidence suggests that water-borne pheromones and hormones may play a role. In the East African annual killifish *Nothobranchius guentheri*, a hydrophilic compound excreted by adult fish (of any species) was found to induce diapause I and II [29,34]. Another study found that homogenates of ovary, and to a lesser magnitude testis and whole embryos, could alter developmental progression. A polar, hydrophilic agent of unknown size was shown to prolong dispersion–reaggregation in *Nothobranchius korthause* without affecting the incidence of diapause II [34,35]. In addition, the nonpolar and hydrophobic purification fractions from adult ovary also contain a bioactive substance that significantly reduced the proportion of embryos that enter diapause II [34]. Incubating embryos in a number of exogenous steroid hormones such as testosterone (T), estrogen (E), and progesterone (P4) caused a decrease in the proportion of embryos entering diapause II, although only 17-β-estradiol (E2) had an effect at physiologically relevant concentrations, as low as 0.001 μg/ml [34]. In addition, only E2 and T affected escape embryo production without causing significant developmental anomalies [34]. These data strongly suggest a role for steroid hormonal regulation of diapause in annual killifish embryos, and provide a possible mechanism for changes in developmental progression with season or population density; the presence of a high density of adult fish, or a reduction in the pond volume associated with the end of the rainy season could result in increased concentrations of these water-borne signaling molecules.

Major life history transitions are often controlled or influenced by hormones; these compounds are structurally conserved and powerful effector molecules that act to coordinate organism-wide responses [26]. Although the role of steroid hormones has been suggested in the regulation of embryonic diapause in African annual killifish, very little is known about the role that hormones may play in South American annual killifish, a lineage that is thought to have evolved diapause independently [28,46]. Here we investigate the role of maternal and embryonic steroid hormones on the regulation of diapause in *A. limnaeus*.

2. Materials and methods

2.1. Husbandry of adults and collection of embryos

Experimental animals were reared in the Portland State University (PSU) aquatic vertebrate facility, as previously described [53]. Briefly, breeding pairs of *A. limnaeus* were housed in 3.8 L aquaria attached to a shared sump and filtration system that provided biological, chemical, and mechanical filtration (21 aquaria per sump).