Terrestrial Deposition of Embryos as a Strategy to Reduce Predation and Enhance Development in the Mangrove Rivulus, *Kryptolebias marmoratus*

by

Michael W. Wells

A Thesis

Presented to

The University of Guelph

In partial fulfillment of requirements

for the degree of

Master of Science

In

Integrative Biology

Guelph, Ontario, Canada

ABSTRACT

TERRESTRIAL DEPOSITION OF EMBRYOS AS A STRATEGY TO REDUCE PREDATION AND ENHANCE DEVELOPMENT IN THE MANGROVE RIVULUS, *KRYPTOLEBIAS MARMORATUS*

Michael W. Wells
University of Guelph, 2014

Advisor:
Dr. Patricia A. Wright

Strategies used by amphibious fishes to survive air-exposure have been studied, but how biotic and abiotic factors affect terrestrial development has received little attention. I hypothesized that rivulus deposit embryos in terrestrial environments to avoid cannibalism and to access higher oxygen levels. Adults recognized kinship of single embryos, cannibalizing unrelated embryos, but not their own. In air, cannibalism was not observed. Rivulus released ~2-fold more embryos in air than water. Air-reared embryos had accelerated development, greater yolk reserves, lower oxygen uptake, but similar morphology relative to water-reared embryos. These results suggest that exposure to an oxygen-rich aerial environment is energetically less costly than development in water. Overall, I conclude that kin recognition and terrestrial development increased embryonic survival; furthermore terrestrial incubation accelerated development possibly through higher oxygen supplies. This research sheds light on the biotic and abiotic factors that influenced early development of ancestral vertebrates out of water.

ACKNOWLEDGEMENTS

I would first like to thank my advisor, Dr. Patricia Wright, for her guidance and support during my time in graduate school. Pat advised me early in my 4th year at the University of Guelph during an undergraduate thesis course and has been an incredible supervisor ever since I began independent research. I would also like to thank my committee members, Dr. Fred Laberge and Dr. Andreas Heyland for their advice and assistance during my Master's program. Dr. Alice Gibb from the University of Northern Arizona, and Dr. Ryan Earley from the University of Alabama were very helpful with data analysis and provided valuable feedback on my thesis. Thanks to Dr. Andrew McAdam for statistical advice.

I would like to thank Bob Frank, Matt Cornish and Mike Davies for aiding in the maintenance of the colony of mangrove rivulus and for construction of and advice on experimental setups. My research would not have been possible if it was not for the hard work of the many work study students and volunteers who were involved in maintaining, feeding and cleaning the colony of mangrove rivulus.

I'm grateful and consider myself lucky to have been surrounded by so many helpful lab members, including Cayleih, Kristin, Quentin and Alex. Andy Turko was especially helpful throughout my entire research career since I began in 2010 as both a mentor and a great friend. I'd like to thank my fellow students and friends from other labs / departments who have helped and supported me through my Master's degree, especially Bryce, Anne, Cameron, Kristina, and Cory. My family and girlfriend Kaitlyn were/are always supportive, and I appreciate them more than anything. Finally, thanks to OGS for funding.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

GENERAL INTRODUCTION

Evolution of Terrestriality

Terrestrial invasion by aquatic vertebrates was a major evolutionary transition that resulted in the current diversity of life on land. Over the past century, several fossils of the earliest terrestrial vertebrates have been reported (Clack 1988; Coates and Clack 1991; Clack 2002a; Downs et al. 2008; reviewed by Long and Gordon 2004). It is hypothesized that our aquatic ancestors went through several amphibious stages before becoming fully terrestrial (Daeschler et al. 2006; Shubin et al. 2006). It would be valuable to understand the biotic and abiotic factors that prevailed at the time of this major transition in evolutionary history (Long and Gordon 2004). The study of extant amphibious teleost species and the mechanisms they use to survive on land has become widely used to shed light on the ultimate factors that drove aquatic vertebrates to leave the water (Graham and Lee 2004; Long and Gordon 2004).

The study of amphibious fishes, along with fossil evidence, has generated several hypotheses which describe the various environmental conditions that prevailed when aquatic animals moved to land. One hypothesis suggests that vertebrates moved onto land from marine intertidal environments (Little 1990; Long and Gordon 2004). However, Graham and Lee (2004) explained that the favourable aquatic conditions in a marine intertidal habitat were not strong enough selective pressures to drive life out of water. An alternative explanation is that vertebrates invaded land from freshwater, swamp environments in response to chronic hypoxia (Graham and Lee 2004; Clack 2012). A third hypothesis is that aquatic vertebrates left water in search of food, selecting for animals that were better able to cope with extended periods of terrestriality (Clack 2002b; Graham and Lee 2004; Niedźwiedzki et al. 2010).

The majority of research on amphibious fishes has focused on the adult life stage (e.g Jew et al. 2013; Kawano and Blob 2013), while the highly sensitive developmental period has received little attention. The amniotic egg is resistant to several challenges of terrestrial environments and is considered to be one of the major innovations that allowed vertebrates to permanently leave aquatic environments (Packard and Seymour 1997; Stewart 1997). However, early vertebrates that left the water were anamniotes and therefore did not possess this derived egg (Skulan 2000). Therefore, an investigation of the costs and/or benefits of terrestrial versus aquatic development in anamniotic eggs could help provide valuable information on the selective pressures that resulted in the invasion of land. My research focused on the biotic and abiotic advantages and possible disadvantages of terrestrial embryo deposition in the amphibious mangrove rivulus (*Kryptolebias marmoratus*).

Terrestrial Embryonic Development in Teleosts

Arguably, the most beneficial aspect of development in air is the difference in environmental dissolved oxygen compared to water. The oxygen capacitance of air is 30-fold higher relative to water and the diffusion coefficient is 8000-fold larger (Liss 1973; Dejours 1988). This is of significance because teleost embryos are considered to be oxygen conformers in early development (e.g. Matschak et al. 1997; Barrionuevo and Burggren 1999; Miller et al. 2008). As such, terrestrial development can provide embryos access to a richer, more quickly replenishing supply of oxygen, which can strongly impact growth and development (Strathmann and Hess 1999). Furthermore, aquatic hypoxia (Diaz and Rosenberg 2008) delays development in fishes (e.g. Alderdice et al. 1958; Garside 1959, 1966; Silver et al. 1963; Miller et al. 2011; Bianchini and Wright 2013). Waterborne chemicals (Carls et al. 1999; Incardona et al. 2004;

Johnson et al. 2007) are another major problems in many aquatic environments and development out of water can avoid these unfavorable conditions.

However, the largest challenge of terrestrial development is desiccation, as prolonged dehydration is lethal to embryos (Macro 2001; Touchon and Warkentin 2010). To avoid desiccation, embryos developing out of water need to remain in a moist environment (Mitchell 2002; Martin and Carter 2013). The efficient elimination of nitrogenous wastes in terrestrial embryos is also an issue, as the abundance of water required for ammonia excretion is not available (Gibson 1982) and species must find alternatives, such as converting ammonia into urea (e.g Wright et al. 1995; Chadwick and Wright 1999; Barimo et al. 2004). Hatch time regulation is another problem that presents a challenge for embryos on land, as hatching when water is unavailable may be lethal (Geiser and Seymour 1989; Martin and Carter 2013). If embryos do hatch out of water, locomotion on land is also another potential problem, as newly hatched embryos unable to make it to water may die from dehydration or starvation (Gibson 1982).

A small number of extant teleost species are able to overcome the above constraints and take advantage of development out of water. Several fish species incubate their embryos above the water line, for example in the high intertidal zone of beaches, buried in wet sand, hidden in sea grasses or inside shells (Middaugh 1981; Taylor 1999; Martin and Swiderski 2001; McDowall and Charteris 2006). The California grunion (*Leuresthes tenuis*) swim just beyond the upper limit of the tide and deposit their embryos into the moist sand before returning to water (Walker 1949). The embryos develop in the substrate, where they remain in a humid environment, safe from predation, until they are triggered to hatch by the agitation of the next spring tide (Walker 1949; Speer-Blank and Martin 2004). The common galaxias (*Galaxias*

maculatus) can develop out of water in moist sea grasses (McDowall and Charteris 2006). Intertidal sculpins (*Clinocottus acuticeps*) survive air exposure under the cover of rockweeds (Marliave 1981). Several embryos that develop out of water also use environmental hatching triggers, such as aquatic hypoxia in *F. heteroclitus* (DiMichele and Taylor 1980) or wave agitation in *L. tenuis* (Griem and Martin 2000) as a signal that the external environmental is favorable for hatching, i.e. water is present. Some killifish embryos are able to survive extended periods of time out of water by using different mechanisms to reduce water loss (Podrabsky et al. 2010). The mummichog (*F. heteroclitus*) can survive dehydration associated with aerial exposure by decreasing the expression of aquaporins in the skin (Tinguad-Sequeira et al. 2009). Embryos of the annual killifish (*Austrofundulus limnaeus*) handle desiccation by reducing water loss during air exposure by increasing the number of proteins elements (intermolecular β-sheets) in the chorion to reduce water permeability (Podrabsky et al. 2001). Research on these few species that use terrestrial incubation is focused on developmental rate and strategies to avoid desiccation without mention of the various biotic pressures that can affect survival.

Aquatic predation

Aquatic predation of embryos (by heterospecifics or conspecifics) is another major threatto development in water. Teleosts use several unique strategies to reduce embryo predation, such as unique modes of reproduction that protect their young from consumption (Gibson 1982; Graham and Lee 2004). Parental care, e.g. nest guarding (DeMartini 1987 in *Oxylebius pictus*) or mouth brooding (Iles and Holden 1969 in *Tilapia galilaeus*) is employed by several teleosts to reduce predation on embryos. Filial cannibalism (cannibalism of own offspring) is a strategy used to regain energy during nest guarding or if clutches are too small to energetically justify parental investment (Forsgren et al. 1996; Manica 2002; Mehlis et al. 2009). Conspecific

predation of unrelated embryos occurs in several species (Smith and Reay 1991; Pfennig et al. 1993) to gain energy from the high protein content of eggs (Heming and Buddington 1988) and eliminate possible competitors (Pfennig et al. 1994). However, most species display no parental care (Gittleman 1981) but can protect embryos by hiding them in unique locations (e.g. *Adinia xenica* Cunningham and Balon 1985). Species that display no parental care and do not remain with the embryos need to ensure that they can recognize kin, as cannibalism of related individuals can entail a large inclusive fitness cost (Hamilton 1964a,b).

The ability of adult fishes to recognize kinship of embryos has received little attention, besides a few studies (e.g. Frommen et al. 2007; Mehlis et al. 2010). The ability to recognize kin entails several benefits, such as a reduction in both inbreeding and filial cannibalism (Smith and Raey 1991; Pfennig et al. 1993; Pfennig et al. 1994; Arnold 2000). Consuming kin can also increase the transmission of pathogens as relatives may possess similar immune systems (Rice 1983; Shykoff and Schmid-Hempel 1991). The main determinants of relatedness in animals are the major histocompatibility complex (MHC) genes (Penn 2002), first discovered in mice (Yamaksi et al. 1976), and also found in several fish species (e.g. Kaastrup et al. 1989; Klein et al. 1993; Sato et al. 2002; Ward and Hart 2003; Ellison et al. 2012a). Fish are able to indirectly detect relatedness through olfaction of waterborne cues influenced by MHC genes. The ability to recognize non-kin can result in favourable heterocannibalism, as seen in several fish species that will consume unrelated embryos and juveniles (Smith and Reay 1991; Pfennig et al. 1993). MHC genes are also involved in mate selection, e.g. Female three-spined sticklebacks (*Gasterosteus aculeatus*) smell male waterborne odours influenced by MHC and will actively select adults with greater MHC diversity (Reusch et al. 2001). It has been suggested that females prefer males with a higher diversity of MHC genes because they have stronger immune systems (Hamilton et al.

1990; Brown 1997; Reusch et al. 2001). Kin recognition via MHC gene detection could allow adults to recognize their own or related embryos to reduce consumption of related individuals.

The release of embryos in a terrestrial environment could also be an effective strategy to reduce aquatic predation as the majority of fishes do not leave the water (Nelson 2006). Only a limited number of amphibious fishes swallow while on land as suction feeding is mechanistically impossible out of water and as such, the terrestrial release of embryos drastically reduces fish predation (Sayer and Davenport 1991; Van Wassenbergh 2013). Although the release of embryos out of water can eliminate aquatic predation, it may also introduce terrestrial predation (Middaugh 1981). However, embryos may be hidden in complex terrestrial environments, such as under leaf litter, which could reduce terrestrial predation (Middaugh 1981; Gibson 1982). Terrestrial deposition could therefore function as a strategy to reduce predation and to exploit higher oxygen levels, enhancing the development of embryos.

Studies on how teleost fish embryos are able to survive life out of water rarely combine abiotic and biotic pressures. A species that is capable of developing in both aquatic and terrestrial environments is required to examine the consequences of aerial development. The mangrove rivulus is as an ideal species to study the abiotic factors because of their ability to develop in both environments (M. Wells, personal observation). While most species that develop terrestrially have primarily aquatic adult lives (e.g. California grunion or the common galaxias) and species that are amphibious as adults (e.g. lungfish) have aquatic development (Sayer and Davenport 1991; Martin 1999; Taylor 1999) the rivulus is unique because it is amphibious at all stages of development. Therefore, the mangrove rivulus can be used to study the biotic pressures of early development as cannibalism of embryos could occur in both environments.

Study Organism

The mangrove rivulus (formerly killifish), *Kryptolebias* (formerly *Rivulus*) *marmoratus* (Poey 1880) is a small cyprinodont that is endemic to the Neotropical mangrove forests of the West Atlantic (Taylor et al. 1995). The mangrove rivulus lives in stagnant pools, mosquito ditches, and within crab burrows of the land crabs *Cardisoma guanhumi* (Taylor et al. 2004) or *Ucides cordatus* (Davis et al. 1990). The aquatic conditions in crab burrows are unfavourable so few other fish species can be found in this environment (Kristensen 1970; Taylor 2012). Because they live in an environment hostile to other fishes, conspecific predation may be relatively more important on killifish embryos. Rivulus also frequently experience periods of environmental stress, such as drought, extreme hypoxia, fluctuations in salinity, and high levels of hydrogen sulphide (Abel et al. 1987; Taylor et al. 1995; Frick and Wright 2002; Ellison et al. 2012b). When rivulus emerse, they find refuge in damp leaf litter or even inside logs (Taylor et al. 2008), where they are thought to respire cutaneously (Grizzle and Thiyagarajah 1987; Ong et al. 2007).

K. marmoratus is one of only two known self-fertilizing hermaphroditic vertebrates, the other species is *K. hermaphroditicus* (Harrington 1961; Tatarenkov et al. 2009; Costa 2011). They possess a unique reproductive organ known as the ovotestis and are able to self-fertilize embryos internally, releasing up to 10 clones per week (Harrington 1961; Soto et al. 1992). The use of "clonal" homozygous lineages to study behaviour and physiology can eliminate confounding genetic effects (Turko et al. 2011; Earley et al. 2012).

Despite extensive field efforts, mangrove rivulus embryos have never been observed in the wild (Abel et al. 1987; Taylor et al. 1995). In the laboratory, however, adults are known to voluntarily deposit embryos when immersed and emersed, without obvious consequences for development and hatching (Taylor 1990; M. Wells, personal observation). To my knowledge,

this species is the only fish that can hatch out of water. Therefore, it is plausible that wild mangrove rivulus embryos may develop in aquatic and/or terrestrial environments.

Hypothesis

My overall hypothesis was that amphibious fish use terrestrial niches for the deposition of embryos as a strategy to reduce aquatic predation and exploit higher oxygen supplies. For my first chapter, I hypothesized that under high egg cannibalism, inclusive fitness theory predicts that selection will favour the evolution of embryo-kin recognition. If this is true, then I predicted that *K. marmoratus* will readily consume non-kin but avoid kin embryos in aquatic environments. My second hypothesis was that the risk of cannibalism is much lower on land than in water because of physical constraints on suction feeding. If so, then embryos will have higher survival on land than in water.

In my second chapter, I focused on the influence of environmental factors on embryonic development. Given that embryonic oxygen uptake is limited in aquatic environments, which in turn, limits development of embryos, I hypothesized that the terrestrial environment would provide a greater supply of oxygen for embryos. If so, I predicted that (1) adult *K. marmoratus* will release embryos terrestrially, (2) development will be enhanced in terrestrial environments and (3) embryos will show behaviours that enhance oxygen exchange in water to minimize boundary layers within the chorion.

CHAPTER 1

HOW TO AVOID EATING YOUR KIDS: KIN RECOGNITION AND TERRESTRIAL EMBRYO DEPOSITION AS STRATEGIES TO INCREASE EMBRYONIC SURVIVAL IN AN AMPHIBIOUS FISH

INTRODUCTION

Survival and reproduction are the two main aspects of individual fitness. Optimal fitness is obtained through a balance between high reproductive output and survivability. However, if an individual is able to help increase the reproductive output and/or survival of relatives (i.e. individuals that share a high percent of genes) this can also increase the fitness of this individual. Hamilton (1964a,b) was the first to describe this concept, known as inclusive fitness and explained the theory behind why individuals will help relatives over non-relatives. To maximize inclusive fitness, an individual should recognize and provide benefits to individuals that share genes (i.e. kin), without aiding unrelated individuals (i.e. non-kin) (Hamilton 1964b; Reeve 1989). Since the first description of inclusive fitness, several species have been found to discriminate between related and unrelated individuals (Brown and Brown 1996; Olsén et al. 1998; Pfennig et al. 1993; Pfennig et al. 1994; Stacey and Ligon 1991; Ward and Hart 2003). Individuals determine relatedness by comparing unknown conspecifics to themselves using various cues (Brown and Brown 1996), the most important being olfactory cues (Olsén et al. 1998).

Kin recognition of early developmental stages in fishes has received very little attention. For example, there are only a couple of studies on the direct recognition of groups of embryos by adult fishes (Frommen et al. 2007; Mehlis et al. 2010). Three-spined sticklebacks (*Gasterosteus aculeatus*) have been observed to cannibalize entire nests when artificially manipulated to contain above 50% non-kin (Mehlis et al. 2010). To my knowledge, no study has tested the ability of a fish to recognize kinship of a single embryo. This is likely because it is difficult to recognize kinship, as their small size results in a low concentration of waterborne odour

(Courtenay et al. 2001). There are various other strategies that can reduce predation of embryos, which can be a large problem for aquatic animals, other than kin recognition.

Fishes have evolved several different reproductive strategies to reduce embryo predation (Gibson 1982). Some species will remain with their embryos throughout development to protect and ensure their survival (Iles and Holden 1969; DeMartini 1987). Parental care is costly though, and as such, most species show no parental care (Gittleman 1981) and will spawn in hidden locations (e.g. Cunningham and Balon 1985). The deposition of embryos in a terrestrial environment is one possible strategy to reduce aquatic predation by conspecifics (Podrabsky et al. 2010). Embryos can be hidden in complex terrestrial environments (Middaugh 1981; Gibson 1982) and since most fishes are suction feeders, they are unable to feed out of water (Sayer and Davenport 1991; Gibb and Ferry-Graham 2005; Van Wassenbergh 2013). The potential use of terrestrial embryo deposition as a strategy to reduce conspecific predation has received little attention. The objectives of this study were to 1) examine kin recognition and cannibalism of embryos in aquatic environments and 2) test if the terrestrial deposition of embryos reduces predation by conspecifics.

The mangrove rivulus (formerly killifish), *Kryptolebias* (formerly *Rivulus*) *marmoratus* (Poey 1880) makes an excellent model to study kin recognition and terrestrial deposition of embryos for several reasons. The mangrove rivulus is a small, internal self-fertilizing hermaphroditic cyprinodonts endemic to the West Atlantic mangrove forests (Harrington 1961, Taylor 2012). It is one of two fish species to possess this unique reproductive strategy, where adult hermaphrodites will produce “clonal” embryos that are genetically identical to the parent (Harrington 1961; Costa 2011). However, *K. marmoratus* are androdioecious and a low percentage of males can sexually reproduce with hermaphrodites leading to heterozygous

individuals in the wild (Mackiewicz et al. 2006b). The genetic relatedness between embryo and parent is especially high for hermaphrodites, as the embryo is 100% related to the parent. This should favour the ability of rivulus to recognize kin, as consuming an embryo would entail a high inclusive fitness cost (Hamilton 1964b). It has recently been demonstrated that adult mangrove rivulus are less aggressive toward kin compared to more distantly related adults (Edenbrow and Croft 2012), but the ability of these fish to recognize embryonic kin has not been tested. Given the relatively small clutch sizes produced by mangrove rivulus (~1-12 embryos; M. Wells, personal observation) and their selfing reproductive strategy, the ability of these fish to recognize kin would be especially beneficial compared to other species. Therefore, my first hypothesis was that under high egg cannibalism, inclusive fitness theory predicts that selection will favour the evolution of embryo kin recognition. If this is true, then I predict that *K. marmoratus* will readily consume non-kin embryos but will avoid their own.

If adults are cannibalistic towards conspecific embryos, then it is possible that the terrestrial release of embryos could reduce predation. There is very little information on the location of *K. marmoratus* embryo deposition in the wild. However, in artificial terrariums adults will release embryos in both terrestrial and aquatic environments (Abel et al. 1987; Taylor 1990). As such, I hypothesized that the risk of cannibalism is much lower on land than in water because of physical constraints on suction feeding. If so, then embryos released in a terrestrial environment will have higher survival compared to water.

METHODS

Animal Care

Laboratory-reared mangrove rivulus were maintained at the Hagen Aqualab, University of Guelph, under constant environmental conditions (25 °C, 16 ‰, pH 8, 12 h light: 12 h dark photoperiod; Frick and Wright 2002). Fish were held individually in 100 mL plastic specimen containers filled with 50 mL of artificial brackish water (16‰) made with reverse osmosis water and sea salt (Instant Ocean®, Spectrum Brands Inc., Madison, WI, United States). A small (~0.5cm^2) hole in the lid allowed for air exchange. Fish containers were cleaned once weekly. Fish were fed newly hatched brine shrimp nauplii (San Francisco Bay Brand®, Newark, CA, United States) three times per week and frozen, chopped bloodworms (San Francisco Bay Brand®, Newark, CA, United States) once per month. Two isogenic strains of fish were used for all of the experiments. The 50.91 strain (Strain 1) originated from Twin Cayes, Belize and have been bred in captivity for over 30 generations and the SLC8E (Strain 2) originated from St. Lucie County, Florida, United States and have been bred in captivity for 20 generations (Tatarenkov et al. 2010). All experiments were carried out under the Animal Utilization Protocol 10R068 at the University of Guelph.

Experimental Protocol

Series I: Aquatic kin recognition

To test the ability of adult rivulus to discriminate between kin and non-kin embryos, individuals from both strains (n = 25 – 30) of similar mass (0.077 ± 0.001 g, mean ± s.e.m.) were monitored in the presence of an embryo from one of three randomly selected parental origins. Embryos originated from the parent (parental), from an adult of the same genetic strain (same

strain) or from an adult of an unrelated genetic strain (different strain). Adults were placed individually in 100 mL plastic specimen containers with 50 mL of brackish water (25°C, 16 ‰, pH 8) for 30 min prior to experimentation. Containers were suspended 30 cm above an upward facing webcam (Logitech Quickcam Pro®, Fremont, CA, United States) attached to a laptop for 24 h of video recording. A single embryo (24 hours post release) was added to the bottom of the chamber. Embryos were obtained within one day of release from the parent and were held in water for up to 24 h to ensure that fertilization had occurred, as indicated by pigmentation. To record at night with minimal disturbance, a red incandescent light (60 W) illuminated the containers from below (Turko et al. 2011). Adults were fed 24 h before the experiment.

Five different behaviours were recorded through video analysis; the latency to investigate the embryo, the number of investigations, contacts and bites, as well as embryo consumption. Latency to investigate was measured as the amount of time (s) required for the adult to initiate an investigation of the embryo. Analysis was restricted to the first hour of video recording, except for consumption of the embryo by the adult, which was measured as a binary response; consumption or no consumption over the entire 24 h period. Following the experiment, adults were weighed.

Series II: Effects of hunger level on embryo cannibalism in water

To test whether the hunger of adult fish influenced cannibalistic behaviour wo groups of adults (fasted and fed; each n = 18) were observed for 24 h in the presence of an embryo from an unrelated strain. This was chosen because preliminary analysis indicated that adults most often consumed non-kin embryos. The fasted group received no food for two weeks while the fed group received brine shrimp three times per week for two weeks prior to experimentation, include 24 hours before the experiment. Fish were transferred to new containers before

experimentation to remove any effect of food debris. After a 30 min acclimation period, an unrelated embryo was placed in the chamber. Fish behaviour, as described above, was quantified during the first hour and consumption of the embryo was assessed after 24 h. Fish were weighed at the end of the experiment.

Series III: Predation of embryos by adult rivulus in air

To quantify embryonic predation rates by adult rivulus in air, individuals from two strains (each n = 12) were monitored in the presence of an embryo. Since adults may potentially encounter embryos in different terrestrial environments under natural conditions, fish were tested in both semi- and fully terrestrial environments. For the semi-terrestrial environment, rivulus were placed in 100 mL containers with 50 mL of brackish water as described above, but with a small piece of filter paper stuck to the side by surface tension. This filter paper allowed an embryo (from one of the three parental origins) to be stuck to the side of the cup out of water. For the terrestrial environment, adults were placed on a piece of damp filter paper in contact with a moist reservoir (three cotton balls soaked with water (25°C, 16 ‰, pH 8; Ong et al. 2007) in 100 mL containers. Embryos were added on top of the filter paper. For both fully and semi-terrestrial conditions, video was taken from above the containers (as the cotton balls restricted the possibility of video capture from below) illuminated with a red incandescent light throughout the 24 h. Adults were fasted 24 h before the experiment. Consumption of the embryo by the adult was measured as a binary response; consumption or no consumption over the entire 24 h period. Following the experiment, adults were weighed.

Statistical Analysis

All statistical analyses were carried out using R 2.15.1 (R Core Team®, 2012) with $\alpha = 0.05$. To analyze the cannibalistic behaviours of adult rivulus in water, general and generalized

linear models were created. Generalized linear models were used for count and binary data that followed Poisson and binomial error distributions, respectively. General linear model was used to analyze latency data. The five response variables (latency to investigate, investigations, contacts, bites and consumption) were analyzed using three predictor variables:

1. Mass: the mass of adults in grams.
2. Strain: the genetic strain of the animal, either strain 1 or 2.
3. Treatment: Adults paired with embryos from one of three sources, parental, within strain or different strain

A log transformation was performed on the latency to investigate data to improve normality. To control for the size of the animal, the mass was included first in the model. The genetic strain of the animal was included second to account for any strain-specific differences in cannibalistic behaviours. The parental relationship between the adult and embryo was included last in the model. The significance of this model was analyzed using an ANOVA.

Three generalized linear models with the Poisson family were created for the number of investigations, contacts and bites at the embryo during the first hour using the predictors above. In the preliminary analysis of residual plots for the number of bites, several points were found to have high leverage (Cook's distance > 0.5). As such, a square root transformation was applied to the number of bites. As well, a generalized linear model with the binomial family was created using the three predictor variables mentioned above to analyze embryo consumption. An analysis of deviance was used to determine the significance of each model (reported as deviance, residual df, p-value) and the coefficients from the generalized linear models were reported to show the direction of the relationships and power of each predictor (reported as the coefficient, z-stat and

p-value). After preliminary trials, weight was found to have a strong influence on the ability of an animal to eat an embryo. To analyze the consumption data, animals that had a mass below 0.08 g (fish below this mass never consumed an embryo) were removed and the generalized linear model was tested using the heavier fish.

General linear and generalized linear models were used to determine the effect of hunger on the cannibalistic behaviours of adult rivulus. Simple linear models were created to assess the latency to investigate, the number of investigations, contacts and bites during the first hour. The three predictor variables in each model were adult mass, strain of the individual and hunger level. The significance of each model was analyzed using an ANOVA. The consumption data was binomial, and as such a generalized linear mode with the binomial family was created. The three predictors were included in the model in the same order and an analysis of deviance was used to assess the significance of the model.

RESULTS

Series I: Aquatic kin recognition

The latency to investigate the embryo was lower for animals that were with an unrelated strain (different strain) relative to adults paired with a genetically related embryo (parental and same strain) ($F = 6.19$, $df = 2, 125$, $p = 0.0027$; Figure 1.1). Strain 1 investigated embryos of a different strain more quickly than strain 2 ($F = 7.95$, $df = 1, 125$, $p = 0.0056$; Figure 1.1).

Genetic relatedness was found to be a strong predictor of adult cannibalistic behaviours in both strains of rivulus tested (all $p < 0.001$). The number of times an adult investigated the embryo was ~2-fold higher for unrelated individuals (different strain) relative to the related treatment groups (parental and same strain) (different strain: $\beta = 0.82 \pm 0.15$, $z = 5.66$, $p < 0.001$; Figure 1.2A). The two strains did not differ in the number of investigations ($\beta = -0.0327 \pm 0.16$, $z = -0.21$, $p = 0.83$; Figure 1.2A). The number of adult contacts with the embryo followed similar trends as the number of investigations. Animals that were unrelated (different strain) were found to come into contact with the embryo twice as often compared to embryos that were genetically related (parental and same strain) (different strain: $\beta = 0.74 \pm 0.18$, $z = 4.17$, $p < 0.001$; Figure 1.2B). There was no significant difference between the two genetic strains with respect to contacts per hour ($\beta = -0.22 \pm 0.15$, $z = -1.48$, $p = 0.14$; Figure 1.2B).

For both strains, the number of bites at the embryo increased when animals were unrelated to the embryo ($\beta = 0.93 \pm 0.28$, $z = 3.36$, $p < 0.001$; Figure 1.2C). As well, strain 2 adults were found to bite their own embryos significantly less than related embryos (same strain), but this was not observed in strain 1 ($\beta = 2.08 \pm 0.82$, $z = 2.55$, $p = 0.01$; Figure 1.2C).

Figure 1.1 The latency (s) of two adult *K. marmoratus* strains to investigate an embryo from one of three parental origins in aquatic conditions (Series I). Adults paired with an embryo from an unrelated strain (different strain) were significantly faster to investigate an embryo than adults paired with their own embryo (parental) or an embryo from the same strain (same strain). Asterisk indicates that strain 1 was significantly faster to investigate an embryo than strain 2. Data are presented as means ± S.E. (n = 20 – 32). Different uppercase letters indicate significant differences between treatment groups (parental, same strain and different strain) within strain 1. Different lowercase letters indicate significant differences between treatment groups within strain 2 respectively ($p < 0.05$).

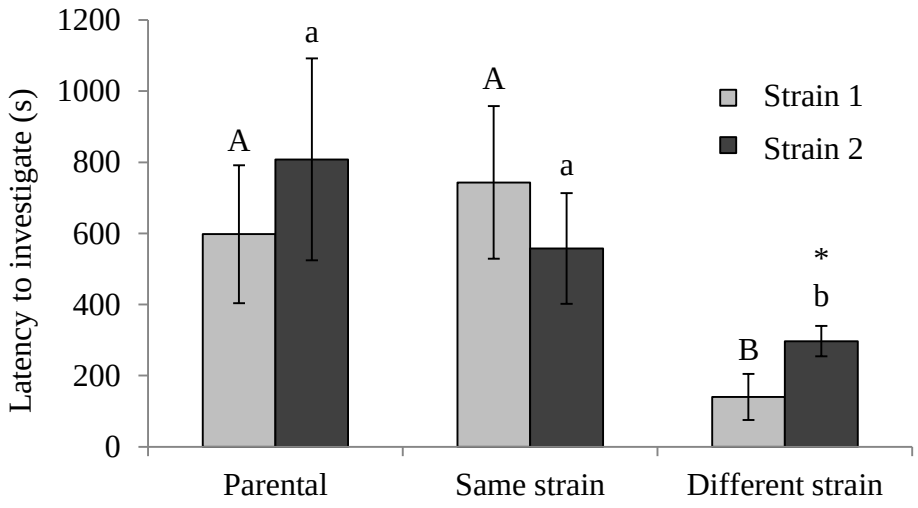
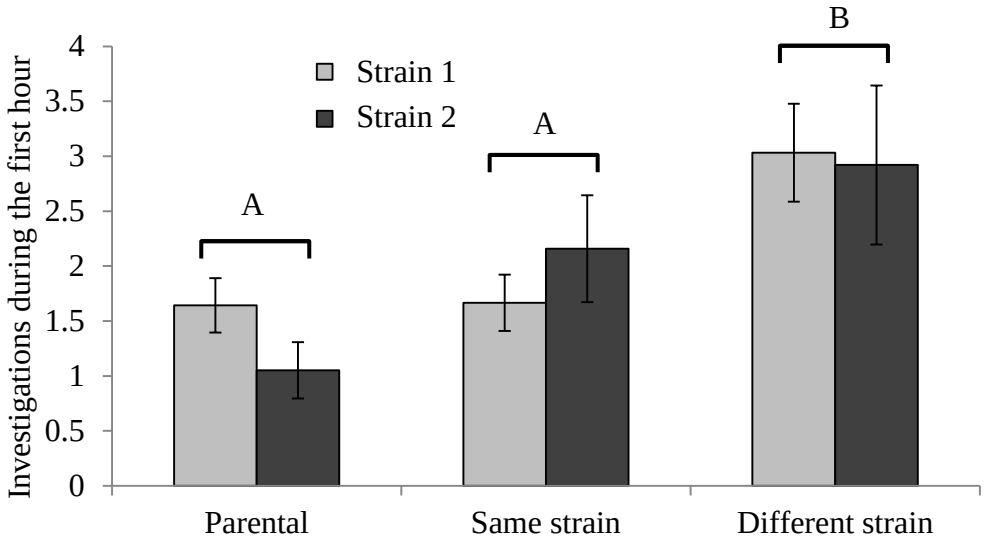
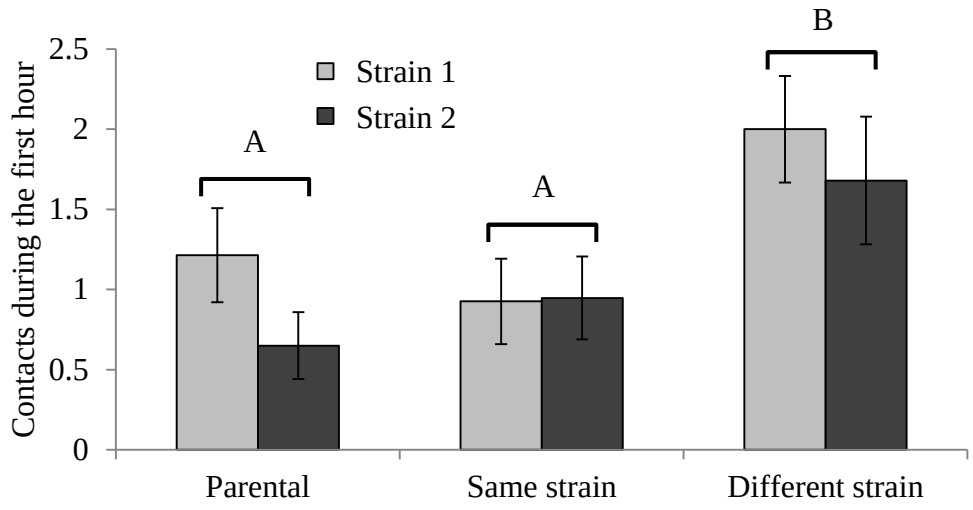

1200
1000
800
600
400
200
0
Latency to investigate (s)
A
a
A
a
B
*
b
Strain 1
Strain 2
Parental
Same strain
Different strain

Figure 1.2 The number of (A) investigations, (B) contacts, and (C) bites at an embryo from three different parental origins by two adult *K. marmoratus* strains during the first hour of exposure in aquatic conditions (Series I). Adults paired with an unrelated strain (different strain) investigated and came into contact with the embryo significantly more than adults paired with their own embryo (parental) or one from the same strain (same strain). The strains did not significantly differ in the number of investigations or anterior contacts of the embryo. Strain 1 was found to bite at parental embryos significantly more than strain 2 individuals. Strain 1 was also found to bite at unrelated embryos (different strain) more than related individuals (parental and same strain). Individuals from strain 2 were found to bite at related embryos (same strain) more than their own (parental), and would bite the most at unrelated embryos (different strain). Data are presented as means ± S.E. (n = 20 – 32). For the number of (A) investigations and (B) contacts, different uppercase letters indicate significant differences between treatment groups (parental, same strain and different strain). For the number of (C) bites, different uppercase letters indicate significant differences between treatment groups (parental, same strain and different strain) within strain 1. Different lowercase letters indicate significant differences between treatment groups within strain 2. Asterisks indicate differences between strains ($p < 0.05$).

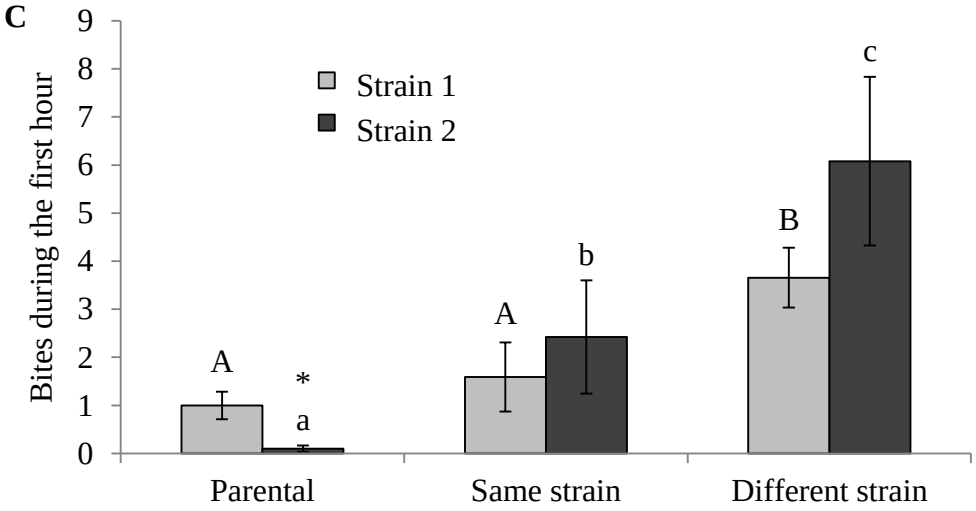
A


Investigations during the first hour
4
3.5
3
2.5
2
1.5
1
0.5
0
Strain 1
Strain 2
A
A
B
Parental
Same strain
Different strain

B


Contacts during the first hour
2.5
2
1.5
1
0.5
0
Strain 1
Strain 2
A
A
B
Parental
Same strain
Different strain

C

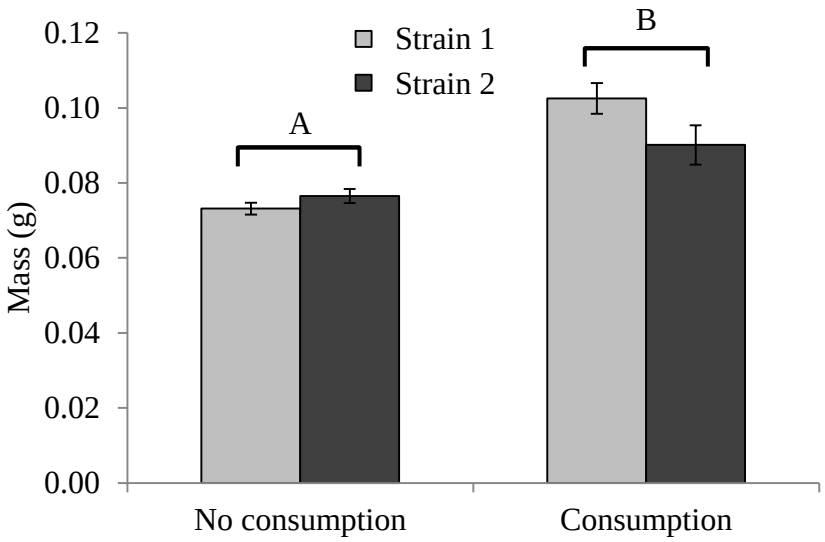

Bites during the first hour
9
8
7
6
5
4
3
2
1
0
Strain 1
Strain 2
A
*
a
A
b
B
c
Parental
Same strain
Different strain

The effect of genetic relatedness between adult and embryo on biting frequency differed between strains (Interaction deviance = 10.5, df = 145, $p = 0.005$; Figure 1.2C). The strains differed in the number of times they were observed to bite their own embryos (parental).

A correlation was found between whether a fish consumed an embryo and the mass of the fish ($\beta = 94.0 \pm 18.5$, $z = 5.08$, $p < 0.001$; Figure 1.3A). As the mass of the adult increased, the likelihood of consumption increased as well (Figure 1.3B). After smaller adults (<0.08 g) were removed, embryo relatedness was found to have a significant effect on consumption of the embryo ($p < 0.05$; Figure 1.4). There was a significant interaction between the strain of the fish and embryo relatedness; adults from strain 1 consumed same strain embryos ~60% of the time, while strain 2 never did ($p = 0.0033$; Figure 1.4).

Figure 1.3 (A) The mass (g) of adult *K. marmoratus* from two strains tested in predation trials and (B) the predicted rate of consumption depending on the mass of the adult in aquatic conditions (Series I). Adults that consumed an embryo (strain 1, n = 12; strain 2, n = 9) were significantly heavier than adults that did not consume embryos (strain 1, n = 72; strain 2, n = 58). Different letters indicate significant differences between cannibalistic and non-cannibalistic animals ($p < 0.05$). Data are represented as means ± S.E. Consumption of embryos is predicted to increase with larger animals when adults are paired with embryos from an unrelated strain ($\beta = 92.0 \pm 17.5$, $z = 5.27$, $p < 0.05$). The light grey box highlights individuals that had below a 10% chance to consume the embryo (mass < 0.08 g) while the dark grey box highlights individuals that were more likely to consume the embryo (mass > 0.08 g).

A


0.12
0.10
0.08
0.06
0.04
0.02
0.00
Mass (g)
Strain 1
Strain 2
A
B
No consumption
Consumption

B

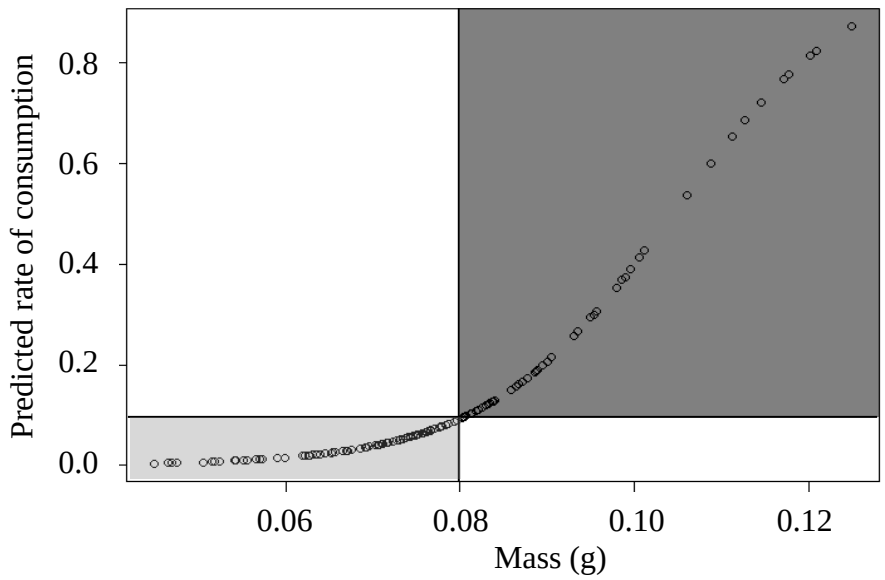
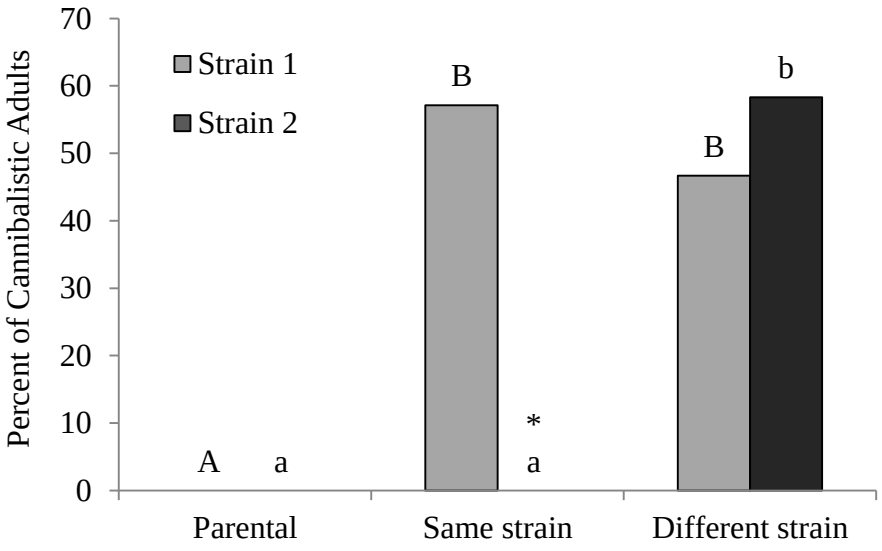

0.8
0.6
0.4
0.2
0.0
Predicted rate of consumption
0.06
0.08
0.10
0.12
Mass (g)

Figure 1.4 The percent of adult *K. marmoratus* greater than 0.08 g mass from two strains that consumed an embryo from one of three egg origins in aquatic conditions (Series I). Adults paired with an unrelated strain (different strain) were found to consume the embryo the most, while adults with their own embryo (parental) were never observed to consume an embryo. Strain 1 was found to consume the embryos that were genetically related (same strain) significantly more than strain 2. Data are presented as the proportion of cannibalistic adults ($n = 5 – 15$). Different uppercase letters indicate significant differences between treatment groups (parental, same strain and different strain) within strain 1. Different lowercase letters indicate significant differences between treatment groups within strain 2. Asterisks indicate differences between strains ($p < 0.05$).


Percent of Cannibalistic Adults
70
60
50
40
30
20
10
0
Strain 1
Strain 2
A
a
B
*
a
B
b
Parental
Same strain
Different strain

Series II: Effects of hunger level on embryo cannibalism in water

Hunger level did not strongly affect cannibalistic behaviours. The number of embryo investigations by adults was not different between fed and fasted fish ($F = 0.0007$, $df = 1, 30$, $p = 0.98$; Table 1.1). The number of contacts and bites at the embryo were similar to investigations, whereby hunger level had no effect on either behaviour (contacts $F = 0.500$, $df = 1, 30$, $p = 0.48$; bites $F = 0.038$, $df = 1, 30$, $p = 0.85$; Table 1.1). However, fasted fish investigated the embryo 7-fold faster relative to adults that had been fed consistently over a two week period ($F = 20.67$, $df = 1, 29$, $p < 0.001$; Table 1.1). An analysis of deviance demonstrated that the percent of adults that consumed the embryo was similar between fed and fasted fish (deviance = 5.08, $df = 52$, $p = 0.75$; Table 1.1).

Series III: Predation of embryos by adult rivulus in air

Adult rivulus exposed to a terrestrial environment were not observed to consume embryos (Table 1.2). When the embryo was positioned at the water's edge, 100% of adults tested were observed to jump at the embryo and knock it into the water. Once the embryo was immersed, adults consumed the embryo if it was unrelated to the adult (different strain).

Table 1.1 The number of observed *K. marmoratus* cannibalistic behaviours during the first hour of exposure, latency to investigate and percent of cannibalistic individuals for fed or fasted adults in water (Series II).

Feeding regime	**Fed**	**Fasted**
Investigations[1] / h	2.16 ± 0.44	3.18 ± 0.72
Contacts / h	0.67 ± 0.26	1.41 ± 0.39
Bites / h	2.56 ± 0.81	4.41 ± 0.98
Latency to investigate (s)	198.5 ± 51.8	29.0 ± 16.9*
Cannibalistic / 24 h	61.1 %	47.1 %

1. Data are represented as means ± S.E. or percentage (n = 18).

* indicates a significant difference between fed and fasted ($p < 0.05$)

Table 1.2 Contingency table of *K. marmoratus* cannibalistic behaviours in water, in air and when the embryo was just above the water's edge (Series III).

Environment	**Embryo Consumption**		**Percent Cannibalistic**
Water	Parental	(0/48)	0%
	Different Strain	(14/27)	52%
Air[1]	Parental	(0/10)	0%
	Different Strain	(0/10)	0%
Semi-Terrestrial[2]	Parental	(0/12)	0%
	Different Strain	(6/12)	50%

1. Adults did not investigate, come into contact with or bite at embryos

2. Embryos were consistently knocked into the water by adults

DISCUSSION

I have shown for the first time that a fish can recognize the genetic relatedness of an individual embryo. I hypothesized that under higher egg cannibalism, inclusive fitness theory predicts that selection will favour kin recognition. As predicted, mangrove rivulus discriminated between unrelated embryos and their own, readily consuming non-kin (more than ~50% of the time). The latency to investigate was greater for kin than non-kin embryos, suggesting that distant odour cues may also be important. As well, adults consistently came into contact with and bit at embryos regardless of relatedness, suggesting that physical contact with the embryo may also be required to discriminate between kin and non-kin. Adults of different genetic strains differed in their ability to differentiate between genetically related embryos and their own, which suggests that there may be different strategies used by each strain to recognize kin. As predicted, the terrestrial release of embryos offered protection against conspecific predation. Overall, my findings suggest that *K. marmoratus* may prey on unrelated embryos in the wild, but could use the terrestrial niche to reduce embryonic predation of their own offspring.

Kin recognition

My results suggest that waterborne odours may be partially involved in recognizing the kinship of *K. marmoratus* e2mbryos. Adults from both strains were much faster at investigating embryos from unrelated fish compared to their own embryos or ones related to them (Figure 1.1). This suggests waterborne odour cues may be involved as the introduction of an embryo to the water stimulated the adults to investigate faster when animals were genetically dissimilar. Waterborne odour cues are also used to identify kinship in several fish species (Quinn and Hara 1986; Brown et al. 1993; Olsén et al. 1998; Reusch et al. 2001). However, my data indicates that waterborne cues were not sufficient for complete kin recognition.

Adult rivulus investigated, came into contact, and bit at unrelated embryos significantly more times than related embryos, however, they still contacted and bit at parental and genetically related embryos (Figure 1.2B and 1.2C). Most animals are able to indirectly detect relatedness through olfaction of waterborne odours influenced by major histocompatibility complex (MHC) genes, comparing foreign odours to their own (e.g. African clawed frog *Xenopus laevis*; Villinger and Waldman 2008). Mate choice by female three-spined sticklebacks (*Gasterosteus aculeatus*) was originally thought to be only based on the MHC influenced waterborne odours of males (Reusch et al. 2001). However, further studies by Sommerfeld and colleagues (2008) found that androgens may also be involved in the ability of female three-spined sticklebacks to determine male reproductive condition. Coming into contact with and biting at embryos (Figure 1.2B and 1.2C) may provide the adult with a higher concentration of odour cues. The small size of individual embryos (mass = 2.07 ± 0.05 mg) likely results in a very low odour concentration (Courtenay et al. 2001). Adults that came into contact with or bit an embryo cannibalized non-kin embryos while avoiding their own (Figure 1.4). Pfennig and colleagues (1993) observed similar behaviours in spadefoot toad tadpoles (*Scaphiopus bombifrons*); cannibals would nip at both related and unrelated conspecifics, but would only consume non-kin. Therefore, contacting and biting at related embryos may have helped adults recognize kin by taste or by providing a greater concentration of odour cues than waterborne odours alone.

Genetic differences between strains may result in different strategies used by adults to recognize kin. Both strains of fish were held under identical rearing conditions, therefore environmental factors were consistent as well as constant. Adults from strain 1 were found to bite at parental embryos significantly more times than strain 2 (Figure 1.2C). Therefore, it is possible that strain 2 were able to recognize their own embryos using waterborne cues alone,

while strain 1 required additional information. As well, adults from strain 2 never consumed embryos that were related to them while strain 1 adults were observed to cannibalize genetically-related embryos more than 50% of the time (same strain; Figure 1.4). The inability of strain 1 but not strain 2 to differentiate between parental and same strain embryos further suggests different cues are involved in kin recognition between these two strains. Parental and same strain embryos were genetically identical, sharing the same MHC genes, suggesting that if MHC recognition was involved, then it was not the only cue. Moreover, adults from strain 1 were found to consume genetically related (same strain) and unrelated (different strain) individuals at similar rates (Figure 1.4), further suggesting that a non-MHC recognition system(s) must be involved.

The ability of rivulus to produce effectively "clonal" individuals allowed me to investigate the type of kin recognition strategy used by *K. marmoratus*. There are three general strategies used to recognize kin (1) phenotype matching, (2) kin template building and (3) spatial distribution. Phenotype matching involves comparing an unknown conspecific to the one's own phenotype, requiring no pre-exposure (e.g. comparing odours of unknown individuals to their own; Holmes and Sherman 1982; Reeve 1989; Brown et al. 1993). Kin template building requires individuals to spend time with kin in order to make a template for relatives, then will compare new individuals to this known template to determine kinship (Holmes and Sherman 1982; Olsén et al. 2002). Spatial distribution is when individuals identify kin based on proximity (e.g. juvenile birds in a nest are assumed to be kin; Holmes and Sherman 1982). In the present study, pre-hatch rivulus were reared in the presence of ~2 but up to ten genetically identical embryos, but then were held in isolation for their entire life after hatching. Therefore, it is possible that rivulus use phenotype matching to recognize kinship of embryos. Coho salmon (*Oncorhynchus kisutch*) discriminate using kin templates but cannot build a template until after

the embryonic period, as they are unable to recognize kin if held in isolation after hatching (Courtenay et al. 2001). Arctic charr (*S. alpinus*) can discriminate between water scented with kin and non-kin siblings (Olsén et al. 1998), but lose this ability if they are held in isolation from hatch (Winberg and Olsén 1992). As such, it is believed that Arctic charr (*S. alpinus*) require some pre-exposure to juvenile or adult siblings to make a kin template. Taken together, it is likely that strain 2 individuals were able to discriminate through phenotype matching as they avoided eating both their own (parental) embryos as well as the embryos produced by other adults of the same strain (same strain). Phenotype matching was also suggested by Edenbrow and Croft (2012) who found that previously isolated adult *K. marmoratus* were able to recognize adult kin, possibly through odour and/or visual cues.

My results also suggest that strain 1 adults may be using an alternative mechanism to recognize kinship of embryos. Adults from strain 1 never consumed their own embryos but would consume both genetically similar and dissimilar embryos. Adults would have only been previously exposed to their own embryos (parental) because of the laboratory conditions at which rivulus were maintained. As rivulus did not consume their own embryos (parental), but would consume embryos produced by other fish of the same genotype (same strain), it is possible that rivulus from strain 1 are using kin template building to recognize kin. Further studies would be required to determine the specific strategies used by *K. marmoratus* and if these strategies differ based on genotype.

There are several possible reasons why I found behavioural differences between strains. First, it is possible that the male to hermaphrodite ratios in the original wild populations resulted in the observed differences. Strain 1 is from Twin Cayes, Belize where populations with a high percentage of males have been found (20% males; 80% hermaphrodites), while strain 2 is from

Florida, USA, where there is a much lower percentage of males (1-2% males; 98-99% hermaphrodites) (Turner et al. 1992; Mackiewicz et al. 2006b; Tatarenkov et al. 2010). Strain 1 individuals thus originally inhabited an environment with mainly heterozygous individuals, as genetic outcrossing with males was frequent, while strain 2 was originally from an environment dominated by homozygous individuals (Mackiewicz et al. 2006b). As such, the importance of kin recognition may be greater in the environment inhabited by strain 2. If an individual from strain 2 encounters an embryo with an unknown origin, the inclusive fitness cost of consuming the embryo would be higher than when the individuals are not related to the adult. Therefore, it is very important that adults from strain 2 are able to differentiate between kin and non-kin embryos, as seen in this study (Figure 1.4). However, if an individual from strain 1 encounters an embryo, there is a lower probability that the embryo will be genetically identical to the adult, as outcrossing is more frequent, resulting in the majority of the population being heterozygous (Mackiewicz et al. 2006b). Strain 1 individuals consumed related and non-related embryos at the same rate (Figure 1.4). Therefore, different acceptance thresholds for cannibalism may have been selected for over evolutionary time (Reeve 1989). This creates stronger selection for kin recognition in the highly related population (strain 1) because the inclusive fitness cost of consuming kin is higher resulting in the observed differences in kin recognition.

A second possible explanation for the strain differences is the difference in the length of time that each strain has been held in captivity. Strain 1 fish have been held in captivity since 1992 at the University of Guelph under standard conditions for over 25 generations. Strain 1 adults were exposed to their own embryos for several days before separation. Strain 2 fish were originally held at a different lab (D. Bechler, Valdosta State University, Georgia), under somewhat different holding conditions from 1995 to 2009 before being transferred to the

University of Guelph in the same holding conditions as strain 1. At their previous location, strain 2 embryos were immediately separated from adults via needlepoint mesh at the bottom of containers (Bland et al. 2001; Mackiewicz et al. 2006a). Thus, strain 2 has only been kept under standard conditions at the University of Guelph for 5 generations. It is possible that differences in embryo exposure to adults and the number of generations under different holding conditions may have altered the strategies used for kin recognition. However, this possibility seems less likely because captive animals are held in isolation under the same environmental conditions, therefore reducing any effects of selection. Nonetheless, my provisional conclusion about a relationship between hermaphroditism and kin-recognition now needs to be tested using other strains with different proportions of hermaphroditism.

The ability to recognize kin in rivulus did not appear to be affected by mass, however the ability to consume the embryos was strongly influenced by the size of the adults (Figure 1.3). The observed correlation with weight is likely caused by an inability of smaller fish to consume embryos because they cannot physically swallow the embryo. A trend was observed, where smaller adults bit at the embryo more than larger adults, suggesting small fish would continue to attempt to eat the embryo, however unsuccessful. Therefore, during the analysis of the consumption of embryos by adults, only the larger animals (mass > 0.08 g) were analysed. This cut off point was selected as post-hoc analysis demonstrated that no individuals smaller than 0.08 g were observed to consume an embryo. Including these small fish in the data analysis did not change the overall conclusions regarding kin recognition or inter-strain differences (data not shown), but the inclusion of these small fish underestimated cannibalism rates by 30%. Further work is required to investigate the relationship between consumption of the embryo (prey) and mouth gape (Schmitt and Holbrook 1984; Nilsson and Brönmark 2000) in *K. marmoratus* to

determine the threshold mass necessary in order for juveniles to demonstrate cannibalistic behaviours.

These results suggest that differences in hunger levels affected fish foraging and/or exploration behaviour, but not the decision to cannibalize non-kin embryos. Although both strains of rivulus were kept on approximately the same feeding regime, the confounding effect of hunger on cannibalism was considered. However, when the cannibalistic behaviours of fasted and fed fish were compared, no differences were observed (Table 1.1). The latency to investigate an embryo was different between fed and fasted fish, which was expected, as fasted animals will actively search out food items.

Predation of embryos by adult rivulus in water or air

Adult rivulus did not consume embryos while exposed to a fully terrestrial environment. Most teleosts are suction feeders (Gibb and Ferry-Graham 2005), and therefore cannot swallow in the absence of water (Van Wassenbergh 2013). However, some species of Cyprinidontiformes, including the mangrove rivulus, are able to "grab" terrestrial prey while out of water (Hernandez et al. 2009; Pronko et al. 2013). Recently, Pronko and colleagues (2013) documented terrestrial excursions by rivulus to acquire food (pinhead crickets), but this was always followed by the return to water to consume the prey. No feeding behaviours were observed while out of water, suggesting that the rivulus may be unable to consume embryos while emersed. I found that when embryos were at the water's edge, adults jumped at the embryos and successfully knocked them into the water. Adults were non-discriminatory towards embryos out of water and would attempt to knock them down regardless of genetic relatedness. However, once the embryos had fallen into the water, adults would only consume unrelated embryos (M. Wells, unpublished data). This suggests that visual cues alone are inadequate to

determine kin relations, as observed in Arctic charr (e.g. Olsén et al. 1998). Visual identification of embryos by fish out of water may be further complicated by different refractive indexes of light in air and water (Dejours 1988, Sayer 2005).

Conclusions

Adult mangrove rivulus were able to accurately discriminate between parental and unrelated embryos in water. This is the first time that the ability to recognize the genetic relatedness of a single embryo by a fish has been described. I found evidence to suggest that adult rivulus are able to recognize kin through phenotype matching. The observed strain differences in ability to recognize kin may have originated from genetic variation in the populations of origin or laboratory holding conditions, but this merits further investigation.

My results support the prediction that if embryos were released in a terrestrial environment away from the water's edge, predation from conspecifics could be reduced. The complex basin of mangrove swamps or emergent logs could serve as an effective area where embryos could remain hidden away from predators (Taylor et al. 2008). Terrestrial and aquatic predators of rivulus that have been documented include the mangrove water snake (*Nerodia fasciata compressicauda*), mangrove fish species (e.g. *Cichlasoma uropthalmus* and *Belenesox belizanus*) and wading birds (e.g. Wood Stork, *Mycteria Americana* and Tricolored Heron, *Hydranassa tricolor*) (Taylor 2012). If embryos are deposited in aquatic rivulus habitat, e.g. crab burrows, then the genetic composition of adult rivulus within these burrows might be used to predict predation risk. To my knowledge, information on the genetic relatedness between *K.*

marmoratus within a single burrow is not published but is soon to be available (R. Earley, A. Turko, P. Wright, S. Currie, and S. Taylor, unpublished data).

CHAPTER 2

A TERRESTRIAL ENVIRONMENT ENHANCES EMBRYO DEPOSITION AND DEVELOPMENT IN THE AMPHIBIOUS *KRYPTOLEBIAS MARMORATUS*

INTRODUCTION

The environmental oxygen levels that animals experience have several implications for growth and development. External oxygen levels significantly impact ontogeny of fishes because embryos are considered to be oxygen conformers (e.g. Matschak et al. 1997; Barrionuevo and Burggren 1999; Miller et al. 2008). Hypoxia generally delays development in fishes (e.g. Alderdice et al. 1958; Garside 1959, 1966; Silver et al. 1963; Miller et al. 2011; Bianchini and Wright 2013), while the effects of hyperoxia are more variable and have received less attention. A few studies of hyperoxia reported increased growth rates and muscle development (Matschak et al. 1997, 1998), while others found decreased swim bladder size and a blunted hypoxia response (Pelster and Burggren 1996; Vulesevic and Perry 2006). Aquatic hyperoxia is rarely found in nature, but aerial exposure could be considered the functional equivalent (Martin and Swiderski 2001). This is due to the fact that the oxygen capacitance of air is about 30 fold higher relative to water (Liss 1973; Dejours 1988), which can provide embryos with a rich supply of oxygen.

Development out of water can provide embryos with more oxygen but also exposes them to a suite of environmental challenges, such as desiccation (Macro 2001; Touchon and Warkentin 2010). To survive in a terrestrial environment, embryos are deposited in moist environments (Middaugh 1981; Taylor 1999; Martin and Swiderski 2001; McDowall and Charteris 2006). For example, California grunion (*Leuresthes tenuis*) spawn in damp sand, which offers embryos physical protection and higher oxygen levels relative to water (Walker 1949; Martin et al. 2011). Mummichog (*Fundulus heteroclitus*) embryos are periodically exposed to an aerial environment during low tides where they hatch earlier relative to embryos in water (Taylor et al. 1977; Tingaud-Sequeira et al. 2009). The Japanese mudskipper, *Periophthalmus modestus*,

incubates embryos aerially in underground nests to avoid hypoxia (Ishimatsu et al. 2007). However, studies comparing the metabolic costs and benefits of development in fully terrestrial or aquatic environment are non-existent.

There is some evidence that oxygen regulation occurs in species that incubate their embryos terrestrially (e.g. African clawed frog *Xenopus laevis* (Hastings and Burggren 1995); tropical frog *Eleutherodactylus coqui* (Burggren et al. 1990)). It has been suggested that red-eyed treefrog, *Agalychnis callidryas* increase movements and metabolic rate in late-stage embryos to reduce boundary layers and mix perivitelline fluid, therefore increasing oxygen availability (Warkentin et al. 2005). As well, embryonic movement of rainbow trout (*Oncorhynchus mykiss*) embryos increased prior to hatching, possibly as a strategy to enhance mixing (Ninness et al. 2006). Thus, although there is evidence that embryos are oxygen conformers, there may be environmental situations where embryos regulate oxygen diffusion by increasing movement within the chorion. Amphibious fish that can rear embryos in air or water may provide important insight into the ability of embryonic fish to response to environmental oxygen levels and regulate oxygen uptake.

The amphibious mangrove rivulus (formerly killifish), *Kryptolebias* (formerly *Rivulus*) *marmoratus* (Poey 1880) is an excellent model species to address questions related to early development in aquatic and terrestrial environments. Rivulus live in mangrove swamps of the Neotropical mangrove forests of the West Atlantic (Taylor et al. 1995) where they experience a wide range of environmental stressors such as hypoxia, high levels of hydrogen sulphide and large fluctuations in temperature and salinity (Abel et al. 1987; Taylor et al. 1995; Ellison et al. 2012b). They possess a unique reproductive system, internal self-fertilization, where adult hermaphrodites produce isogenic strains (Harrington 1961). The unique reproductive system of

adults (Harrington 1961) makes them an excellent model for studying how the environment affects development, as genetic differences can be eliminated (Turko et al. 2011; Earley et al. 2012). Adults have been observed to release embryos in both aquatic and terrestrial habitats in the lab (M. Wells, personal observation) however there is no information on rivulus embryos in the wild (Taylor 2012). Therefore, it is possible that wild mangrove rivulus embryos develop in terrestrial environments.

I hypothesized that embryonic oxygen uptake is limited in aquatic environments. Given that oxygen availability limits development of embryos, if the terrestrial environment provides greater oxygen supplies for embryonic *K. marmoratus* I predict that (1) adult *K. marmoratus* will release embryos terrestrially, (2) embryonic development will be enhanced in terrestrial environments and (3) embryos will show behaviours that enhance oxygen exchange in water to minimize boundary layers within the chorion.

METHODS

Animal Care

Mangrove rivulus were maintained under constant conditions as described previously in Chapter 1. All experiments were carried out under the Animal Utilization Protocol 10R068 at the University of Guelph.

Experimental Protocol

Three series of experiments were performed:

Series I: Reproductive output of adult rivulus in water or air

Series II: Impact of rearing environment on developmental rate, growth and metabolism

Series III: Impact of rearing environment on embryo movement

Series I: Reproductive output of adult rivulus in water or air

To measure the fecundity of mangrove rivulus in air and water, reproductive adults (>5 months old, n = 58) from strain 1 were placed in individual 100 mL containers and monitored for 96 h. The exposure time of 96 h was selected to ensure that sufficient time was available for internal fertilization of eggs and embryo release (M. Wells, personal communication). To maximize reproductive output, the feeding regime was increased to chopped bloodworms once per week on top of the regular brine shrimp feedings for three weeks prior to experimentation. Fish were exposed to both air and water in a randomly determined order one week apart for pair-wise comparisons. For the water treatment, adults were placed individually in 100 mL plastic specimen containers with 50 mL of brackish water (25°C, 16 ‰, pH 8). In water, the addition of a small (5 cm diameter), inverted filter paper funnel (Whatman®, GE Healthcare Companies,

Little Chalfont, UK) elevated off the bottom of the plastic container allowed embryos to fall out of reach of the parent to prevent any possible cannibalism. In air, fish were placed on a piece of moist filter paper in contact with a moist reservoir (three cotton balls soaked with water (25°C, 16 ‰, pH 8, Ong et al. 2007)) in 100 mL containers. Consumption of embryos was not explicitly prevented during air exposure, but preliminary tests showed that rivulus did not feed while out of water (M. Wells, personal observation). Embryo production was measured daily. At the end of the 96 h period, adults were returned to standard conditions for one week and then retested in the alternative environment. Fecundity was measured as the number of embryos released per adult during each 24 h period.

Series II: Impact of rearing environment on developmental rate, growth and metabolism

To determine the consequences of an aerial environment on embryonic development and metabolism, individual embryos (within 24 h of release from adults held under constant conditions) were reared in simulated aquatic and terrestrial environments. It should be noted that approximately 20% of rivulus embryos are held within the parent for 24 – 96 h post internal fertilization (Harrington 1963, Swain and Lindsey 1986) and as such, if any signs of organogenesis were evident (i.e. pigmentation), the embryos were not used. Air- or water-exposed embryos were maintained in the same manner as described above for adults. Filter paper was placed on the bottom of the water container (same as air container) to control for any confounding effects the paper may have on development. After 15 and 30 days post release (dpr), embryos were imaged for morphology and developmental staging as described by Mourabit et al. (2011). Embryos were photographed under a dissecting microscope with a Venus 2.0 microscope camera (Am Scope®, Irvine, CA, United States). For 15 dpr, images were blindly analyzed for developmental staging only. At 30 dpr, all embryos were at hatching

competency and analyzed for developmental staging and morphology. Embryos were manually de-chorionated under a dissecting microscope with fine forceps, in order to standardize hatch time at 30 dpr. A separate group of embryos were reared for 30 dpr in aquatic and terrestrial conditions for mass measurements. Embryos were blotted dry and wet mass was recorded with the chorion (intact) and without the chorion (embryonic body and yolk sac; embryo wet) and dry mass (embryonic body and yolk sac; embryo dry) was determined after drying to constant mass (40˚C for 20 min).

To measure metabolic rate of embryos in air and water, a fiber-optic optode (PreSens Precision Sensing GmbH®, Regensburg, Germany) was used in both air and water. Oxygen consumption was measured in a respirometry chamber constructed out of a small 12x32 mm glass screw neck vial (Waters®, Milford, Massachusetts). The vial was placed inside a glass chamber with circulating water (25˚C ± 0.1). The temperature inside the glass chamber was maintained using a circulating water bath in a thermo-control room. A small stir bar (4 mm) was placed in the bottom to mix water or air throughout the experiment. A mesh stand held the embryo above the stir bar in water. For aerial oxygen consumption, the chamber was ¾ filled with wax to reduce the volume. An air-tight lid screwed onto the chamber with a small hole allowed insertion of the optode. Two blank trials were performed to determine if the chamber was sealed appropriately showed that there was no leakage of oxygen into or out of the chamber. The optode was calibrated for 100% DO saturation using brackish water (25 °C, 16 ‰, pH 8) aerated with atmospheric air and 0% DO saturation using a 2M solution of Na_2SO_3.

For oxygen consumption measurements in water, the chamber was filled with oxygenated water and one embryo reared in an aquatic environment. For oxygen consumption measurements in air, the chamber was filled with oxygenated air and five embryos reared in a terrestrial

environment were added. Metabolic rate in embryos was measured at 7, 15 and 30 dpr. Embryos were allowed to acclimate for 1 h in the vial before oxygen consumption was measured. Aerated water was slowly flushed through the chamber to ensure aquatic embryos remained in well-oxygenated water. For aerial embryos, the chamber was not sealed to allow gas exchange during the acclimation period (1 h). Oxygen consumption was measured for 1 h, with DO saturation always above 70%. The embryonic oxygen consumption was calculated using the rate of decrease in oxygen inside the closed chamber, mass of the embryo, volume of water inside the chamber and the duration of exposure. Oxygen consumption was presented as μmol O_2/g/h.

Series III: Impact of rearing environment on embryo movement

To test the second prediction, embryo movement in air versus water was quantified. To determine if the introduction to an aquatic environment affected the metabolic rate and movement of terrestrially-reared embryos, individual embryos (15 dpr, n = 8) were analyzed in both environments. Embryos were placed in a Petri dish on a piece of wet filter paper for air-reared embryos or filled with 10 ± 0.5 mL of brackish water (16‰, 25˚C, pH 8) for water-reared embryos. Embryos were video recorded through a dissecting scope for 1 h. At 30 dpr, air- and water-reared embryos were placed in a Petri dish filled with brackish water. Movement of the embryos was categorized as either large body movements (Tattersall and Spiegelaar 2008) or opercular movements. Values were calculated as movements per hour.

The aquatic metabolic rate of the rivulus embryos (strain 1; 30 dpr; n = 9) reared in water or air was determined using closed respirometry via dissolved oxygen (DO) consumption per wet gram of embryo per h. A custom built respirometry chamber was constructed out of a 1.7 mL microcentrifuge tube (graduated tube with flat cap, Fisherbrand®, Hampton, NH, United States). A stir bar (4 mm) was placed in the bottom of the tube to mix the water and a mesh stand was

placed above the stir bar to avoid agitation of the embryos. The chamber was maintained at 25˚C ± 0.5 in a glass container with circulating water attached to a water bath. The glass container was held above a stir plate with a micromanipulator to allow for movements of the entire setup. One hole (~1 mm diameter) was punctured in the tip of the tube to allow the insertion of the micro-oxygen electrode needle (OX -7245, Unisence®, Aarhus, Denmark). The electrode was unable to take aerial readings, and therefore embryos reared terrestrially were measured in water. The tube was sealed once the electrode was inserted using blue tack. A picoammeter (PA2000, Unisense®, Aarhus, Denmark) was used to measure DO saturation via the relative amplitude of the water. These measurements were recorded using the program LabChart 6 through PowerLab 4/30 (AD instruments Inc.®, Colorado Springs, CO, United States).

The micro-oxygen electrode was calibrated for 100% DO saturation using brackish water (25 °C, 16 ‰, pH 8) aerated with atmospheric air and 0% DO saturation using a 2M Na_2SO_3 solution. The electrode was calibrated before and after the experiment to account for the minute drift in readings. A blank run was carried out prior to all experimental runs to determine the relative rate of oxygen consumption of the electrode. Fully developed embryos were then placed inside the respirometry chamber. Embryos were pooled in groups of five and two for water and air reared animals, respectively. Embryos were acclimated to the chamber for 1 h prior to the experiment with aerated water slowly flowing through the chamber. Experiments lasted 1 h, with DO saturation always above 70% to ensure the metabolic rate was at normoxia as described by Rodela and Wright (2006). The oxygen consumption was calculated using the rate of decrease in oxygen inside the closed chamber minus the rate of oxygen consumption of the electrode (determined via blank run prior to the experimental trial), mass of the embryos, volume of water

inside the chamber, the duration of exposure and the temperature of the system. Oxygen consumption was presented as µmol O_2/g/h.

Embryonic Morphology and Statistical Analysis

To determine gross morphology of 30 dpr embryos, 8 landmarks were selected from a sagittal view of the hatched embryo. The landmarks were**:** (1) tip of the snout (2) ventral point where the head meets the yolk sac (3) most dorsal and posterior portion of the head (4) posterior and proximal point of operculum most ventral point of yolk sac (5) most dorsal point of the yolk sac (6) most ventral point of the yolk sac (7) where the posterior point of yolk sac meets tail (8) posterior end of the tail before the caudal fin. Landmarks were digitized using TPS Dig2 developed by Rohlf, F. J. (Department of Ecology and Evolution, State University of New York at Stony Brook, NY, United States). The total length of the embryo was determined by producing vectors using points 1-4-8. Body depth was determined using points 3-5. The yolk sac surface area was determined by creating two representative triangles on the images using points 2-5-7 and 2-6-7 and calculating the cumulative area.

Data was compiled and graphed using Excel® 2010 and statistical analyses were carried out on R 2.15.1 (R Core Team®, 2012) with $\alpha = 0.05$. Paired Student's t-tests were used to compare the reproductive output of adult rivulus in water and air at 24, 72 and 96 h.Two-sided Student's t-test were used to determine if air-exposure had an effect on the mass of the embryo (intact, embryo wet and embryo dry). The morphological measurements at 30 dpr were analyzed using two-sided Student's t-test.

Two-sided Student's t-test for independence were run to determine the effects of aerial rearing on embryonic oxygen consumption. The embryonic movement within the chorion was

analyzed using two-sided Student's t-test. Preliminary analysis of the ventilation residuals showed several high leverage points (Cook's distance > 0.5) and as such, a log transformation was applied to the number of ventilations per hour. A two-way ANOVA was used to determine the effects of development in air or water on metabolic rate at 7, 15 and 30 dpr.

RESULTS

Series I: Reproductive output of adult rivulus in water or air

Adult rivulus exposed to a terrestrial environment released more than 2-fold as many embryos after 96 h relative to when held in water ($t = 2.50$, $df = 57$, $p = 0.015$; Figure 2.1). Thirty eight percent of adults released embryos while in a terrestrial environment compared to 15% in water.

Series II: Impact of rearing environment on early development and metabolism

At 15 dpr, a greater number of aerially reared embryos had reached hatching competency (95% at Stage 32; Figure 2.2) relative to those reared in water (45% at Stage 32; Figure 2.2). However, at 30 dpr body morphology was similar except for yolk sac surface area in embryos reared in aquatic and terrestrial environments. Mass of rivulus embryos (intact, wet and dry embryo) was not affected by rearing environment (all $p > 0.05$; Figure 2.3A). The mean standard length and body depth of rivulus embryos was not significantly different between treatments (body depth $t = 1.49$, $df = 22.5$, $p = 0.149$; standard length $t = -0.59$, $df = 22.9$, $p = 0.56$; Figure 2.3B and 2.3C). In contrast, embryos reared out of water had a yolk sac surface area 2-fold higher relative to aquatic animals ($t = 2.21$, $df = 22.6$, $p = 0.037$; Figure 2.3D).

Figure 2.1 The cumulative numbers of embryos released by adult *K. marmoratus* exposed to a moist terrestrial or fully aquatic environment for 96 h. Individuals were randomly exposed to both treatments one week apart to allow for a pair-wise comparison (Series I). After 96 h, adults held out of water released significantly more embryos than individuals kept in water. Data are represented as means ± S.E. ($n = 58$). Asterisks indicate differences between treatments ($p < 0.05$).

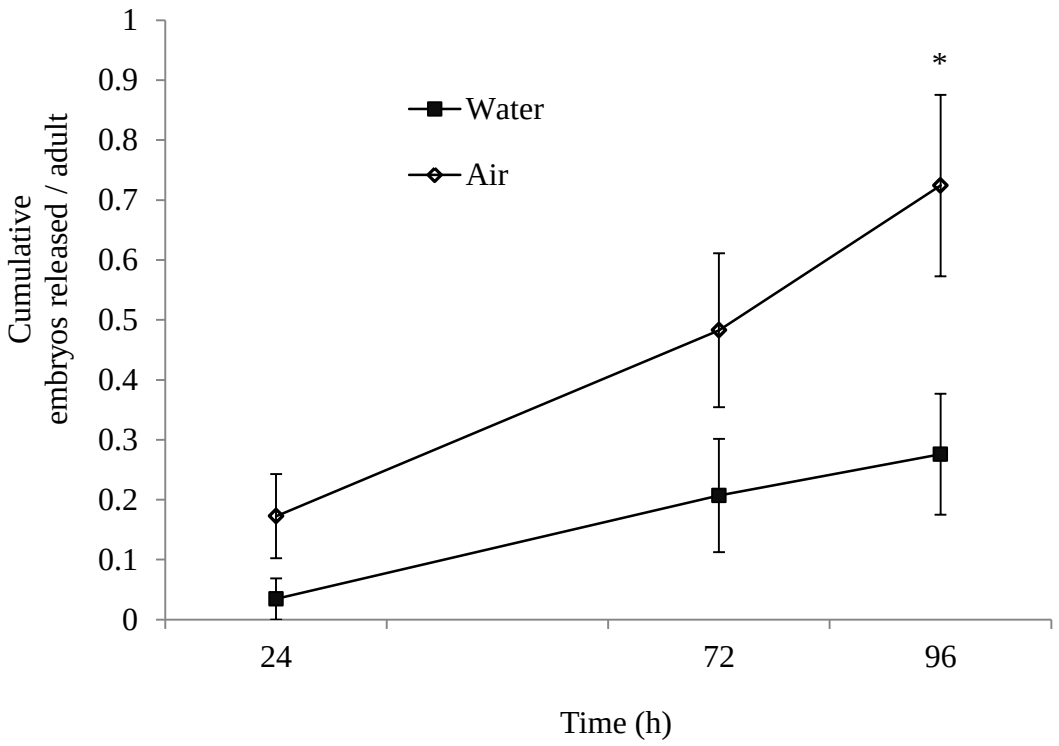
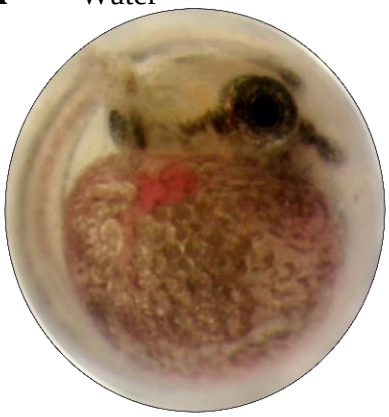

1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Cumulative embryos released / adult
Water
Air
*
24
72
96
Time (h)

Figure 2.2 Representative images of embryos developing in water at (A) 15 and (B) 30 days post release and developing in air at (C) 15 and (D) 30 days post release (Series II). At 15 dpr embryos reared in air had reached stage 32, hatching competency, while embryos reared in water were at an average of stage 31. However, at 30 dpr, water embryos were at the same stage as aerially reared embryos but had a visually distinct reduction in their yolk sac.

A Water

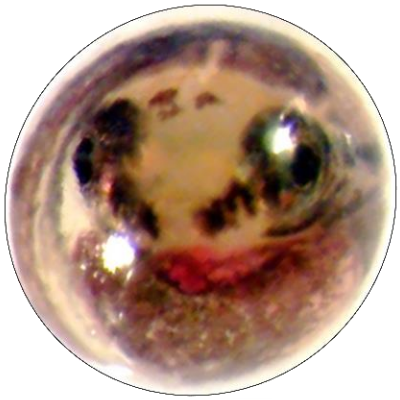


15 dpr, Stage 31

B Water

3 mm

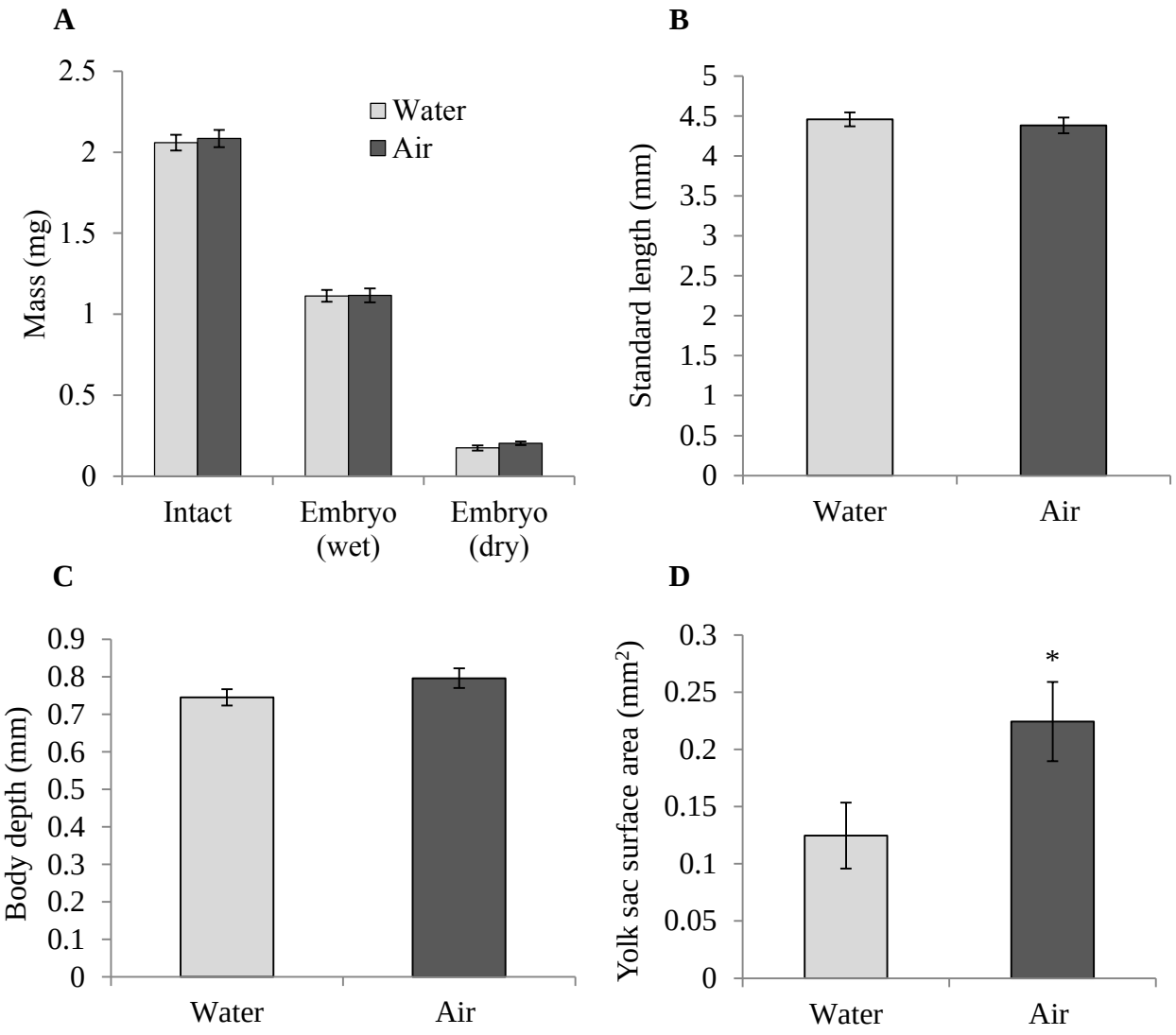
C Air



15 dpr, Stage 32

D Air

Figure 2.3 (A) The mass (mg), (B) standard length (mm), (C) body depth (mm) and (D) yolk sac surface area (mm^2) of *K. marmoratus* embryos reared in fully aquatic or moist terrestrial environments at 30 days post release (Series II). The mass is reported as intact embryos (with the chorion and yolk sac), after the chorion had been removed (embryo + yolk sac) and the dry mass of the embryo (embryo + yolk sac). There were no significant differences found between embryos reared in air or in water for all three measures of weight. There was no difference in standard length or in body depth between embryos reared in water or air. However, embryos reared in air had a significantly larger yolk sac surface area compared to embryos reared in water. Data are represented as means ± S.E. (mass n = 10; morphometrics n = 11). Asterisks indicate significantly differences between treatment groups ($p < 0.05$).


A
Mass (mg)
2.5
2
1.5
1
0.5
0
Water
Air
Intact
Embryo (wet)
Embryo (dry)
B
Standard length (mm)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Water
Air
C
Body depth (mm)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Water
Air
D
Yolk sac surface area (mm2)
0.3
0.25
0.2
0.15
0.1
0.05
0
*
Water
Air

The mean oxygen consumption rate of embryos reared in air was ~44% lower than embryos reared in water ($F = 40.8$, $df = 1, 34$, $p < 0.001$; Figure 2.4). Embryos reared in water followed a significantly different trend in oxygen consumption rate over developmental time compared to embryos reared aerially (Interaction $F = 3.29$, $df = 2, 34$, $p = 0.049$). The mean oxygen consumption rate of embryos reared in water significantly decreased at stage 30 relative to earlier stages ($F = 6.10$, $df = 2, 34$, $p = 0.0054$; Figure 2.4). However, oxygen consumption in embryos reared in air was consistent across embryogenesis (Figure 2.4).

Series III: Impact of rearing on embryo movement

Embryos reared in air for 15 dpr did not differ in ventilation rate or the number of times they rotated within the chorion compared embryos reared in water ($p > 0.05$; Figure 2.5). At 30 dpr, however, the number of ventilations decreased by about 5-fold in water reared embryos relative to 15 dpr. Air-reared embryos had a significantly higher ventilation rates when returned to water compared to individuals reared in water ($t = 3.75$, $df = 6.09$, $p = 0.0092$; Figure 2.6A). The rearing environment did not affect the number of times they rotated within the chorion ($t = 0.53$, $df = 9.79$, $p = 0.6$; Figure 2.6A). The aquatic oxygen consumption of embryos reared in a terrestrial environment but returned to water was 2-fold higher than embryos reared in water and tested in water ($t = 4.07$, $df = 7.84$, $p = 0.0037$; Figure 2.6B).

Figure 2.4 The oxygen consumption (µmol O_2/g/h) of *K. marmoratus* embryos reared in water or air for 7, 15 and 30 days post release and measured in their respective respiratory media (Series II). Embryos reared in water had a higher oxygen consumption rate compared to embryos reared out for water at 7 and 15 dpr. However, oxygen consumption was significantly lower at 30 dpr compared to 7 and 15 dpr for embryos reared in water, while embryos reared in air had similar oxygen consumption rates throughout development. Data are presented as means ± S.E. (n = 6 – 8). Different uppercase letters indicate significant differences between embryonic stages (7, 15, and 30 dpr) for embryos reared in water. Different lowercase letters indicate significant differences between embryonic stages for embryos reared in air. Asterisks indicate differences between embryonic rearing environments ($p < 0.05$).

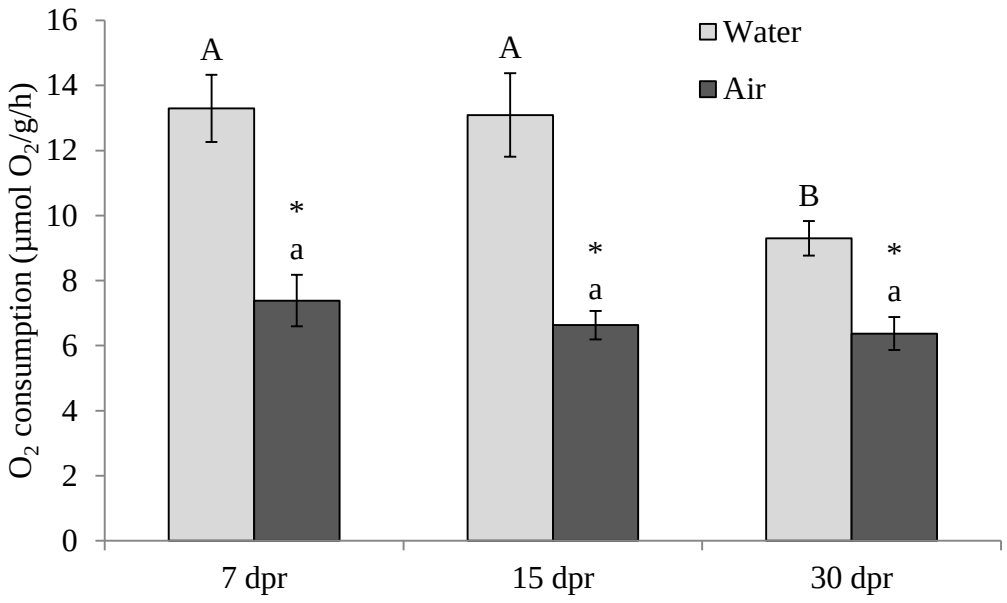

16
14
12
10
8
6
4
2
0
O2 consumption (µmol O2/g/h)
Water
Air
A
A
B
*
a
*
a
*
a
7 dpr
15 dpr
30 dpr

Figure 2.5 The number of ventilations and large body movements per hour of *K. marmoratus* embryos in air or water at 15dpr (Series III). Embryos were first reared in an aquatic or terrestrial environment and then moved into the environment they were reared in. Rearing environment had no effect on movements at 15 dpr. However, the environment in which embryos developed had no effect on the number of movements. Data are represented as means ± S.E. (n = 8).

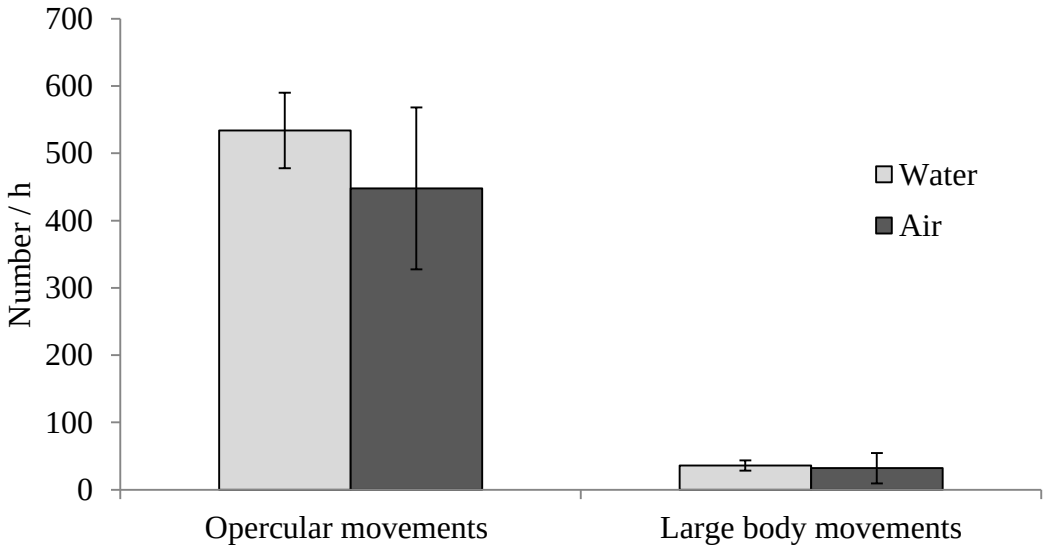
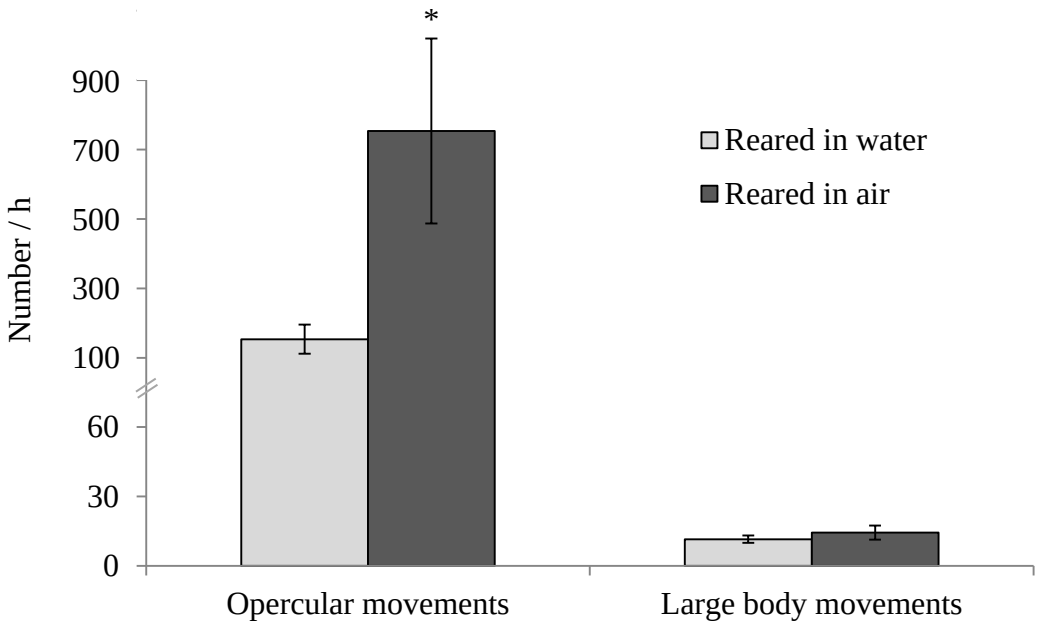
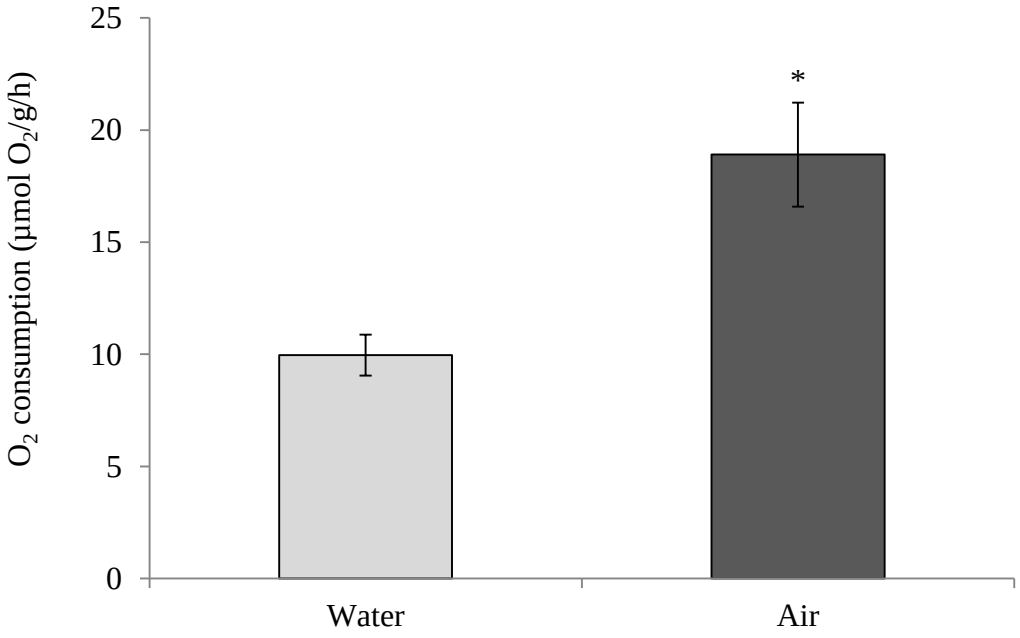

700
600
500
400
300
200
100
0
Number / h
Water
Air
Opercular movements
Large body movements

Figure 2.6 (A) The number of ventilations and large body movements per hour and (B) the oxygen consumption (µmol O_2/g/h) of *K. marmoratus* embryos previously reared in air or water for 30 days and returned to water for measurement (Series III). All individuals were from Strain 1. (A) Once returned to water, embryos reared in air were found to ventilate significantly more compared to embryos reared in water. (B) The oxygen consumption of embryos reared in a terrestrial environment was 2-fold higher than embryos reared in a terrestrial environment. The asterisk indicates a significant difference between treatments ($p < 0.05$). Data are presented as means ± S.E. ($n = 9$).

A


*
900
700
500
300
100
60
30
0
Number / h
Reared in water
Reared in air
Opercular movements
Large body movements

B


25
20
15
10
5
0
O_2 consumption (µmol O_2/g/h)
*
Water
Air

DISCUSSION

This is the first time the spawning rates of an amphibious fish have been measured in both terrestrial and aquatic environments. I found that adults released a higher number of embryos in air relative to water, suggesting a preference for terrestrial environments. Terrestrial incubation accelerated development to 15 dpr and decreased yolk utilization at 30 dpr compared to embryos in water. In previous research on amphibious fishes, metabolic rate of embryos in air or water has not been measured over several developmental time points. I found that the metabolic rate of *K. marmoratus* embryos in air was ~44% lower compared to those reared in water, suggesting differences in energetic costs depending on rearing conditions. These results support my first two predictions that rivulus can release embryos out of water and development was accelerated up to 15 dpr. However I found evidence for and against my third prediction, that embryos will manipulate their micro environment to maintain oxygen uptake. Embryos reared in water or air for 15 dpr did not differ in their movement rates, in contrast to my prediction. However, air-reared embryos increased movement and metabolic rate when acutely returned to water, which could indicate micro-environment manipulation to maintain oxygen uptake or a hatching response. Taken together, my results show that terrestrial incubation could be an effective strategy to avoid a variable and harsh aquatic mangrove environment.

Reproductive output of adult rivulus in water or air

Adult rivulus held out of water for 96 h released a significantly higher number of embryos than when the same animals were held in water (Figure 2.1). This finding supports my first prediction, that rivulus will release embryos out of water. Previous literature describes anecdotal observations of adult *K. marmoratus* that are able to release embryos in an artificial terrestrial terrarium (Abel et al. 1987; Taylor 1990). Most fish species are unable to spawn in

both environments, however rivulus are able to internally self-fertilize (Harrington 1961) and therefore reproduction out of water is not constrained. The greater number of embryos released in air suggests that although adults use both environments for the release of embryos, they may prefer a terrestrial environment for embryo deposition under certain circumstances. To show a true preference, however, fish would need to be tested in both environments simultaneously.

Adult rivulus released embryos at a consistent rate between each time point (Figure 2.1), which suggests they were not purging their system, i.e. removing any unwanted materials from their body that could hinder the ability to survive environmental challenges. Rivulus have been shown to adjust the allocation of resources depending on environmental conditions. Dunson and Dunson (1999) showed that shifts in resource allocation allow amphibious fish to devote more energy to survival in air. Therefore, it was possible that rivulus would purge any developing embryos when exposed to an aerial environment to reduce energy usage. If air exposure caused the rivulus to purge their system, then an early (24 h) spike in embryo production would have been expected, however this was not observed (Figure 2.1).

Impacts of rearing environment on early development and metabolism

The developmental rate and energetic costs were different between embryos reared in water and air, however, embryo morphology (e.g. standard length, body depth) was not affected by rearing environment. After 15 dpr, the majority of embryos reared in air had reached hatching competency (stage 32), while embryos reared in water were at an average stage of 31 (Figure 2.2A and 2.2C). The time gap between these stages was over 24 hour at 25ºC (Mourabit et al. 2011), which demonstrates accelerated development in an aerial environment. These results provide partial evidence for my second prediction that embryos develop at a faster rate in a terrestrial environment, however growth rate was unaffected. Although *F. heteroclitus* hatched

earlier when exposed to an aerial environment, they also showed increased growth rates (Tingaud-Sequeira et al. 2009). Therefore, terrestrial environments accelerate development of *K. marmoratus*, but unlike *F. heteroclitus*, growth does not appear to be affected.

My results show that embryos reached hatching competency at different rates depending on rearing environment. At 15 dpr, embryos reared in air, but not in water, had reached hatching competency. From 15 to 30 dpr, there were no further developmental changes in embryos reared in air. The natural hatching time of rivulus is extremely variable, between 20 to 90 days (Sakakura and Noakes 2000), which may explain why air-reared embryos had not hatched between 15 and 30 dpr, despite being hatching competent. Water-reared embryos had also reached hatching competency by 30 dpr but had significantly less of their yolk sac remaining relative to air-reared embryos (Figure 2.2B and 2.2D). The observed difference in yolk sac utilization between embryos reared in air or water suggests that terrestrial incubation in rivulus results in development to hatching competency using less energy. This may influence hatching time because embryos reared in water would running out of food quicker than embryos in air.

The metabolic rate of air-reared embryos was significantly lower than the metabolic rate of embryos reared in water at the three time points measured (Figure 2.4), in contrast to what I predicted. Decreased metabolic rates closer to hatch have been observed in air-reared embryos relative to water-reared embryos in the amphibian species *Pseudophryne bibroni* (Bradford and Seymour 1985). However, Bradford and Seymour (1985) did not observe significant differences in oxygen consumption at earlier developmental stages in *P. bibroni*. It is possible that because embryos reared in aquatic environments are exposed to lower oxygen concentrations relative to air, they might invest more energy into their respiratory systems. This would result in an additional energetic cost to development, which would explain the reduced yolk reserves,

delayed development and increased metabolic rate in water-reared embryos. African cichlids (*Pseudocrenilabrus multicolor victoriae*) alter gill morphology to maximize surface area when broods of juveniles are exposed to chronic hypoxia in early development (Chapman et al. 2008). Although embryonic fish gills are used primarily for ion regulation (Rombough 1999; Fu et al. 2010), if rivulus embryos are exposed to an external environment low in oxygen (e.g. aquatic normoxia) relative to air, they may use more energy to optimize respiratory systems (e.g. developing larger gills) in anticipation of future low oxygen environments. This could be tested by examining the metabolic rate, critical oxygen tension or gill morphology of larval fish that were previously reared in either water or air.

In the present study, the rate of oxygen consumption by air-reared embryos was consistent across all three time points measured. However, the metabolic rate of embryos reared in water was significantly different between time points; oxygen consumption was highest at 7 and 15 dpr and dropped significantly by 30 dpr (Figure 2.4). In annual killifish (*Austrofundulus limnaeus*), embryonic development can be halted pre-hatch, known as diapause III, which is characterised as a metabolic depression (Wourms, 1972). During diapause III there is a dramatic decrease (~66%) in metabolic rate in the annual fish *Nothobranchius korthausae* (Levels et al. 1986). At 30 dpr, rivulus embryos reared in water decreased oxygen consumption by ~30% relative to 15 dpr (Figure 2.4). It is unlikely that the drop in metabolic rate was caused by an embryonic diapause because in the present study the drop in metabolic rate was modest relative to the depression observed in other fish (Levels et al. 1986). Mourabit et al. (2011) suggested that metabolic arrest occurs in late stage *K. marmoratus* embryos, however no data was presented to support this. There was no metabolic depression in embryos reared in air. Likewise,

in air-exposed California grunion embryos, metabolic rate is maintained for two weeks after reaching hatching competency (Darken et al. 1998).

Alternatively, the decreased metabolic rate at 30 dpr may be related to insufficient yolk reserves (Figure 2.3D). Wood (1932) suggested that the decreased metabolic rate of brown trout (*Salmo trutta*) near the end of development was linked with the amount of yolk reserves. Rivulus embryos held in laboratory conditions frequently do not hatch and remain within the chorion for months unless they receive a hatching trigger or the chorion is removed manually (M. Wells, personal observation). Darken and colleagues (1998) measured the yolk reserves depletion in *L. tenuis* embryos during delayed incubation. When yolk reserves were exhausted, they observed mortalities from starvation if embryos did not receive the proper environmental hatching cues. As such, it is possible that the decline in metabolic rate is caused by a depletion of energy reserves.

A third possibility is that metabolic rate in water-reared embryos declines as a result of changes in embryo surface area to volume ratios. As development proceeds, the mass of anamniotic embryos increases without large changes in surface area which results in increased absolute metabolic rate (e.g. Rombough 1986, 1994; Moses et al. 2008; Mueller et al. 2011), but a decreased mass-specific metabolic rate (e.g Von Bertalanffy 1957; Alderdice et al. 1958; Burggren et al. 1990). In my study, I reported mass-specific metabolic rate (Figure 2.4) and observed a decrease in metabolic rate between 15 dpr and 30 dpr. However, the changes in mass between 15 dpr and 30 dpr are minimal (less than ~2%) and should therefore not result in changes in mass-specific metabolic rate. In rivulus, the majority of growth between stage 31 (15 dpr embryos) and stage 32 (30 dpr embryos) occurs in the fins (Mourabit et al. 2011), which would hypothetically increase the surface area of the embryo and not greatly increase the mass.

According to Von Bertalanffy (1957), as surface area increases, so should the absolute metabolic rate. This was not observed in my study. Therefore, it is likely that the observed decrease in metabolic rate in water-reared embryos at 30 dpr were not from surface area: volume changes. Given the three possibilities discussed above, it is probable that the lower metabolic rate in water-reared embryos relates to insufficient fuel reserves.

Impact of rearing environment on embryo movement

I predicted that embryos will manipulate their micro-environment to maintain oxygen delivery. I found partial evidence for this prediction. Embryos in an aquatic environment were not observed to increase movement relative to air-reared embryos at 15 dpr (Figure 2.5). However, when air-reared embryos were acutely returned to water at 30 dpr, they increased movement by 7-fold (Figure 2.6A). The metabolic rate of air-reared embryos returned to water was also 2-fold higher compared to embryos reared in water (Figure 2.6B). Therefore, the increase in metabolic rate in air-reared embryos may be partly or wholly due to the energetic costs associated with opercular movements. Previous work on adult rainbow trout (*Oncorhynchus mykiss*) found that switching from active ventilation to ram ventilation decreased oxygen consumption by ~10% (Steffensen 1985). Increased opercular movements in air-reared *K. marmoratus* embryos after the return to water likely explain the increase in metabolic rate. Increased opercular movements in encapsulated embryos may help to disrupt of boundary layers to facilitate oxygen transport. As development proceeds in rainbow trout, boundary layers within and outside the chorion increase in size if ontogeny occurs in stagnant aquatic environments (Dhiyebi et al. 2013).

An alternative explanation for the observed differences in metabolic rate and movements in air-reared embryos returned to water could be the initial stages of a hatching response, as

described by DiMichele and Taylor (1981). Hypoxia is a known hatching trigger in some species of killifish and amphibians (Dimichele and Taylor 1980; Bradford and Seymour 1988). Because of differences in oxygen capacitance, well-oxygenated water is hypoxic relative to air, which may have caused the response in rivulus embryos as they have been observed to hatch with different external cues (e.g. hypoxia, aquatic agitation, M. Wells, unpublished data). When exposed to an environmental hatching trigger, California grunion will increase oxygen consumption and the number of movements within the chorion (Speer-Blank and Martin 2004; Martin et al. 2011). Rivulus embryos are able to hatch relatively quickly (within ~30 min) when exposed to aquatic or aerial hypoxia (M. Wells, unpublished data). Therefore, it is possible that the observed increase in oxygen consumption and embryonic movements were caused by the initiation of a hatching response.

The results of this study suggest that *K. marmoratus* embryos do not appear to conform to external oxygen levels early in development but are regulating oxygen later in ontogeny. Developmental trajectories were similar between rearing environment as embryos were morphologically similar. Embryos did not increase growth or metabolic rates in air, but developmental rate was accelerated out of water. However, as the rate of oxygen consumption did not mirror the external oxygen concentration, it does not appear that embryos are conforming early in development (Randall et al. 2001). Ontogeny in water resulted in complete consumption of the yolk sac, suggesting that development in water is more costly, which is further supported by the higher metabolic rate of embryos in water. Because embryos developing in water are in an environment with significantly less oxygen, they may need to devote additional energy to maintaining oxygen uptake suggesting that they are oxygen regulators. When air-reared embryos were returned to water, they significantly increased both metabolic rate and embryonic

movement. If embryos are oxygen conformers throughout development, then the introduction to a relatively hypoxic environment (compared to air) would cause a decrease in oxygen consumption. This has been observed in some oxygen conforming amphibian embryos that are unable to maintain oxygen consumption at mild levels of hypoxia (Hastings and Burggren 1995). The increased metabolic rate and number of opercular movements per hour in air-reared embryos returned to water indicates that they are responding to the new environment, i.e. they are oxygen regulators. Thus, although there is no evidence for oxygen conforming in embryos up to 15 dpr, I found strong evidence for oxygen regulating in late stage embryos. Measuring the critical oxygen tension of embryos at 15 dpr and 30 dpr would provide valuable information on their ability to regulate oxygen.

Conclusion

Rivulus were observed to deposit eggs in both aquatic and terrestrial artificial environments. Aerial incubation increased developmental rates while apparently reducing the energetic costs of development. This partially supports my hypothesis that adult rivulus deposit embryos out of water as a strategy to exploit the greater oxygen levels in air. The decreased energetic cost of aerial development was interesting and requires further investigation. A decreased metabolic cost of development could be advantageous if an embryo needs to delay hatching until environmental conditions are suitable. Alternatively, greater yolk reserves could be advantageous if hatchlings enter a habitat with limited food resources. My results do not support the possibility of an embryonic diapause in water-reared embryos. Embryos reared out of water may have displayed a hatching response when returned to water, likely because of differences in dissolved oxygen concentrations in air versus water. Alternatively, they may have increased movement and metabolic rate to maintain oxygen consumption. This study has

examined terrestrial incubation from a novel angle and has shown new implications for growth in an aerial environment. Terrestrial incubation introduces embryos to an environment rich in oxygen, which can accelerate growth and avoid harsh aquatic conditions if embryos are able to remain in a moist environment.

GENERAL DISCUSSION

The results of my thesis demonstrate the unique ability of *K. marmoratus* to recognize kinship of a single embryo. I also showed that terrestrial incubation in embryonic *K. marmoratus* is advantageous because developmental rate is accelerated. This is important because rivulus inhabit an unpredictable environment and it is important to develop quickly. Furthermore, I found that the terrestrial release of embryos eliminated embryonic predation by conspecifics. Together, the findings of my thesis demonstrated strategies to maximize embryo survival (through kin recognition and terrestrial spawning) and increase embryonic development. It also provided new information on the biology of the mangrove rivulus and potentially insights into the invasion of land by ancestral vertebrates.

Kin recognition of embryos has not been heavily studied in fishes; only the three-spined sticklebacks have previously been found to determine relative kinship of clutches of embryos (Mehlis et al. 2010). Further research is required before any conclusions are made about the strategies used by *K. marmoratus* to recognize kin, however the results of this study indicate that odour cues are probably involved. Y-maze or fluviarium experiments would be good methods to test the hypothesis that *K. marmoratus* use odour cues to recognize kin (Olsén et al. 1998). This would involve exposing an adult to two streams of water scented by related and unrelated embryos and quantifying the amount of time spent at each stream. If odour cues are used to discriminate between kin and non-kin, fish are predicted to spend the majority of their time in the non-kin stream of water.

To further investigate the importance of olfaction in kin recognition, exposing adults to sublethal concentrations of copper would be informative. Previous research has found that copper can inhibit olfaction in salmonids (Baldwin et al. 2003) by degenerating olfactory

epithelium receptors (Julliard et al. 1996; Hansen et al. 1999). The effects of acute or low dose exposures (4 h) are transient, as the olfactory epithelium can regenerate within 1 day, as seen in chum salmon (*Oncorhynchus keta*) (Sandahl et al. 2006). An ideal experimental setup would involve testing the ability of adults to recognize kin prior to a copper exposure (assuming rivulus are affected by copper in a similar manner as salmonids), after acute copper exposure, and finally after a two week recovery period. If olfaction is important in kin recognition, then I would predict that adults will recognize kin before the copper exposure and after the recovery period, but would lose the ability after the two week copper exposure.

The strain differences in embryonic cannibalism suggested that the two strains of *K. marmoratus* tested use different methods of recognition. There are no other reports of intraspecific differences in strategies used to recognize kin, although there have been reports of different rates of cannibalism based on feeding quantities. Fish that were fasted early in the larval stages became cannibalistic while individuals that were fed never consumed conspecifics (Cuff 1977). Some species also have cannibalistic and non-cannibalistic phenotypes, such as larval Arizona tiger salamanders, *Ambystoma tigrinum* (Pfennig et al. 1994). I have suggested two different explanations for why these differences exist in my thesis, however there could be several more, such as differences in environmental conditions experienced by the animals prior to being held in captivity. Previous research on *K. marmoratus* has demonstrated other phenotypic differences between strains, such as aggression and hormone levels (e.g. cortisol and 11-ketotestosterone, Earley and Hsu 2008). Steroid hormone differences between Strain 1 and 2 in my study could also contribute to the observed differences in behaviour. It would be useful to measure circulating hormone levels, such as 11–ketotestosterone, to provide insight into possible sources of the differences in kin recognition.

K. marmotatus embryos developed in a terrestrial environment without severe water loss, i.e. there was no difference in mass beween air- vs. water-reared embryos. How do *K. marmoratus* embryos avoid desiccation? In other species that incubate embryos on land, the expression of aquaporin mRNA was decreased in the skin (*F. heteroclitus*, Tingaud-Sequeira et al. 2009) or there was increased concentration of protein elements in the chorion (*Austrofundulus limnaeus*, Podrabsky et al. 2001). Further research on the ability of rivulus embryos to reduce water loss and possibly survive dehydration as seen in other species would provide important details on their ability to develop terrestrially. It would also be informative to follow these embryos through to maturity, measuring their growth rates after hatching, reproductive output and ability to survive air exposure. Developmental plasticity occurs in species of cichlids when exposed to different oxygen regimes during early development (Chapman et al. 2008). Therefore, it is possible that early development out of water could produce adults that are better able to cope with further bouts of air-exposure.

It would be interesting to further investigate the observed delay in hatching in mangrove rivulus. Mangrove rivulus embryos show large variability in hatch time, ranging from 20 days to 90 days (Sakakura and Noakes 2000). I found that embryos exposed to a terrestrial environment were hatching competent at 15 dpr but had not hatched by 30 dpr. Embryos reared in water were also hatching competent at 30 dpr but had consumed their yolk reserves, while air-reared embryos had a significant portion of their yolk remaining. Delayed hatching has been observed in many fish and amphibian species, which have differential hatching rates depending on length and time of air exposure (Bradford and Seymour 1988; Martin 1999; Tingaud-Sequeira et al. 2009; Podrabsky et al. 2010). I would therefore hypothesize that delayed hatching in mangrove rivulus grants terrestrially incubated embryos the ability to survive extended periods of

unfavourable conditions. This would be imperative for development in such a variable ecosystem, where environmental conditions can change rapidly (e.g. rainfall) or slowly (e.g. droughts, Taylor 2012).

One constraint of the experimental protocol was that adults were unable to choose the environment for the deposition of embryos. To explicitly test for a preference for the deposition of embryos, an experimental design where adults are exposed to both air and water would be required. A semi-terrestrial environment, where adults are allowed to select between an aquatic or a terrestrial environment, would allow us to test whether there is an element of choice to *K. marmoratus* spawning sites. In addition, using environmental conditions more similar to those an embryo would experience in the wild may provide a more accurate estimate of natural spawning preferences. Aquatic conditions used in this study ([H_2S] = 0 ppb, 15‰, T = 25ºC, >90% DO sat.) were different from water conditions in the wild, which are both extremely harsh and variable ([H_2S] = 50–700 ppb, Abel et al. 1987; 0–68‰, T=7-32ºC Taylor et al. 1995; < 1 mg O_2/L, Dunson and Dunson 1999). Adults can sense environmental conditions and emerse if they become unfavourable (H_2S in Abel et al. 1987; hypoxia in Regan et al. 2011). Therefore, it would be expected that they would actively avoid unfavourable environments for the deposition of embryos. If the experiment was repeated with low oxygen and high [H_2S] in water, I would predict that adults would only release embryos in an aerial environment. Additionally, the number of embryos released in an aquatic environment may be artificially inflated relative to wild adults. Strain 1 have been held in captivity since 1991 (Strain 1 or 50.91, Tatarenkov et al. 2010) and it is possible that chronic exposure to a higher quality aquatic environment may have selected for the release of embryos in water. The observed difference in embryos released in air and water could be much greater with wild caught fish as the confounding effects of captivity

would be eliminated. If this experiment was repeated with wild animals, I would predict that the number of embryos released in a terrestrial environment would be higher than the number released by adults held in captivity.

The ability of anamniotic embryos to develop in a moist terrestrial environment may have been an adaptation required for early tetrapods to leave the water. Similar to my study, ancestral vertebrates may have exploited the higher oxygen levels of an aerial environment for early development. My results also suggest that embryonic predation may have been reduced if embryos were released out of water. Kin recognition of embryos also reduces predation. The presence of homologous MHC genes in both teleosts (Kaastrup et al. 1989; Klein et al. 1993) and extant tetrapods (Yamaksi et al. 1976) suggests that the MHC gene family would probably have been present in the common ancestor. Although a number of studies have been done on species that incubate embryos out of water (Kramer 1978; Geiser and Seymour 1989; Smyder and Martin 2002; McDowall and Charteris 2006), few discuss how these environmental conditions can relate to the evolution of terrestriality. Graham and Lee (2004) argued that marine amphibious fishes and their associated reproductive strategies cannot provide information about the transition of ancestral vertebrate reproduction. They explain that because marine species that incubate embryos terrestrially require well-oxygenated water after hatch, teleost reproduction is a dead end for the study of ancestral vertebrates. However, previous studies have not examined a species of fish that experiences hypoxia in the larval stage and are amphibious as an adult. This is important as the environmental conditions that are thought to have selected for life out water include aquatic hypoxia (Clack 2012). *K. marmoratus* is a fully amphibious species and can survive out of water for at least 66 days (Taylor 1990).

My results demonstrate that *K. marmoratus* have the capacity survive early life stages out of water. Survival of embryos on land may have been an important step in the transition to life on land that is frequently overlooked. The variable and harsh environmental conditions that cause mangrove rivulus to leave the water are likely similar to what ancestral vertebrates experienced during the transition from an aquatic to a terrestrial life. Ultimately, the unique reproductive strategies used by the rivulus lend them to the study of the environmental factors that drove the transition from an aquatic to a terrestrial lifestyle in the early development of ancestral vertebrates.

REFERENCES

Abel, D., Koenig, C., and Davis, W. (1987) Emersion in the mangrove forest fish Rivulus marmoratus: a unique response to hydrogen sulfide. Environmental Biology of Fishes. 18, 67-72.

Alderdice, D.F., Wickett, W.P., and Brett, J.R. (1958) Some effects of temporary exposure to low dissolved oxygen levels on Pacific salmon eggs. Journal of the Fisheries Board of Canada. 15, 229-250.

Arnold, K.E. (2000) Kin recognition in rainbowfish (*Melanotaenia eachamensis*): sex, sibs and shoaling. Behavioral Ecology and Sociobiology. 48, 385-391.

Baldwin, D.H., Sandahl, J.F., Labenia, J.S., and Scholz, N.L. (2003) Sublethal effects of copper on coho salmon: Impacts on nonoverlapping receptor pathways in the peripheral olfactory nervous system. Environmental Toxicology and Chemistry. 22, 2266-2274.

Barimo, J.F., Steele, S.L., Wright, P.A., and Walsh, P.J. (2004) Dogmas and controversies in the handling of nitrogenous wastes: ureotely and ammonia tolerance in early life stages of the gulf toadfish, *Opsanus beta*. The Journal of Experimental Biology. 207, 2011-2020.

Barrionuevo, W., and Burggren, W. (1999) O_2 consumption and heart rate in developing zebrafish (*Danio rerio*): influence of temperature and ambient O_2. American Journal of Physiology: Regulatory, Integrative and Comparative Physiology. 276, R505-R513.

Bianchini, K., and Wright, P.A. (2013) Hypoxia delays hematopoiesis: retention of embryonic hemoglobin and erythrocytes in larval rainbow trout, *Oncorhynchus mykiss*, during chronic hypoxia exposure. The Journal of Experimental Biology. 216, 4415-4425.

Bland, M.J., Bielmyer, G.K., and Ring, B.C. (2001) Toxic effects of hygromycin B on *Kryptolebias marmoratus* early development. Georgia Academy of Science. 68, 140-148.

Bradford, D., and Seymour, R. (1985) Energy conservation during the delayed-hatching period in the frog *Pseudophryne bibroni*. Physiological Zoology. 58, 491-496.

Bradford, D., and Seymour, R. (1988) Influence of environmental PO_2 on embryonic oxygen consumption rate, rate of development, and hatching in the frog *Pseudophryne bibroni*. Physiological Zoology. 61, 475-482.

Brown, G., and Brown, J. (1996) Kin discrimination in salmonids. Reviews in Fish Biology and Fisheries. 219, 201-219.

Brown, G., Brown, J., and Crosbie, A. (1993) Phenotype matching in juvenile rainbow trout. Animal Behaviour. 46, 1223-1225.

Brown, J.L. (1997) A theory of mate choice based on heterozygosity. Behavioral Ecology. 8, 60-65.

Burggren, W.W., Infantino, R.L., and Townsend, D.S. (1990) Developmental changes in cardiac and metabolic physiology of the direct-developing tropical frog *Eleutherodactylus coqui*. The Journal of Experimental Biology. 152, 129-147.

Carls, M., Rice, S., and Hose, J. (1999) Sensitivity of fish embryos to weathered crude oil: part I. Low-level exposure during incubation causes malformations, genetic damage, and mortality in larval pacific herring (*Clupea pallasi*). Environmental Toxicology and Chemistry. 18, 481-493.

Chadwick, T.D., and Wright, P.A. (1999) Nitrogen excretion and expression of urea cycle enzymes in the Atlantic cod (*Gadus morhua* L.): a comparison of early life stages with adults. The Journal of Experimental Biology. 202, 2653-2662.

Chapman, L.J., Albert, J., and Galis, F. (2008) Developmental plasticity, genetic differentiation, and hypoxia-induced trade-offs in an African cichlid fish. The Open Evolution Journal. 2, 75-88.

Clack, J.A. (1988) New material of the early tetrapod *Acanthostega* from the upper Devonian of east Greenland**.** Palaeontology. 31, 699-724.

Clack, J.A. (2002a) An early tetrapod from 'Romer's Gap'. Nature. 418, 72-76.

Clack, J.A. (2002b) The dermal skull roof of *Acanthostega gunnari*, an early tetrapod from the Late Devonian. Earth and Environmental Science Transactions of the Royal Society of Edinburgh. 93, 17-33

Clack, J.A. (2012) Gaining ground, second addition: the origin and evolution of tetrapods. Indiana University Press.

Coates, M.I., and Clack, J.A. (1991) Fish-like gills and breathing in earliest known tetrapod. Nature. 352, 234-236.

Costa, W.J. (2011) Identity of *Rivulus ocellatus* and a new name for a hermaphroditic species of *Kryptolebias* from south-eastern Brazil (Cyprinodontiformes: Rivulidae). Ichthyological Exploration of Freshwaters. 22, 185-192

Courtenay, S.C., Quinn, T.P., Dupuis, H.M.C., Groot, C., and Larkin, P. A. (2001) Discrimination of family-specific odours by juvenile coho salmon: roles of learning and odour concentration. Journal of Fish Biology. 58, 107-125.

Cuff, W.R. (1977) Initiation and control of cannibalism in larval walleyes. The Progressive Fish-Culturist. 39, 29-32.

Cunningham, J.E., and Balon, E.K. (1985) Early ontogeny of *Adinia xenica* (Pisces, Cyprinodontiformes): 1. The development of embryos in hiding. Environmental Biology of Fishes. 14, 115-166.

Daeschler, E.B., Shubin, N.H., and Jenkins, F.A. (2006) A Devonian tetrapod-like fish and the evolution of the tetrapod body plan. Nature. 440, 757-63.

Darken, R., Martin, K.M., and Fisher, M. (1998) Metabolism during delayed hatching in terrestrial eggs of a marine fish, the grunion *Leuresthes tenuis*. Physiological and Biochemical Zoology. 71, 400-406.

Davis, W.P., Taylor, D.S., and Turner, B.J. (1990) Field observations of the ecology and habits of mangrove rivulus (*Rivulus marmoratus*) in Belize and Florida. Ichthyological Explorations of Freshwaters. 1, 123-134.

Dejours, P (1988) Respiration in water and air: adaptations, regulation, evolution. New York: Elsevier.

DeMartini, E.E. (1987) Paternal defence, cannibalism and polygamy: factors influencing the reproductive success of painted greenling (Pisces, Hexagrammidae). Animal Behaviour. 35, 1145-1158.

Dhiyebi, H.A, O'Donnell, M.J., and Wright, P.A. (2013) Water chemistry in the microenvironment of rainbow trout *Oncorhynchus mykiss* embryos is affected by development, the egg capsule and crowding. Journal of Fish Biology. 82, 444-457.

Diaz, R.J., and Rosenberg, R. (2008) Spreading dead zones and consequences for marine ecosystems. Science. 321, 926-929.

Dimichele, L., and Taylor, M.H. (1980) The environmental control of hatching in *Fundulus heteroclitus.* Journal of Experimental Zoology. 214, 181-187.

Dimichele, L., and Taylor, M.H. (1981) The mechanism of hatching in *Fundulus heteroclitus*: development and physiology. Journal of Experimental Zoology. 217, 73-79.

Downs, J.P., Daeschler, E.B., Jenkins, F.A, and Shubin, N.H. (2008) The cranial endoskeleton of *Tiktaalik roseae*. Nature. 455, 925-929.

Dunson, W.A., and Dunson, D.B. (1999) Factors influencing growth and survival of the killifish, *Rivulus marmoratus*, held inside enclosures in mangrove swamps. Copeia. 1999, 661-668.

Earley, R.L., and Hsu, Y. (2008) Reciprocity between endocrine state and contest behavior in the killifish, *Kryptolebias marmoratus*. Hormones and behavior. 53, 442-451.

Earley, R.L., Hanninen, A.F., Fuller, A., Garcia, M.J., and Lee, E.A. (2012) Phenotypic plasticity and integration in the mangrove rivulus (*Kryptolebias marmoratus*): a prospectus. Integrative and Comparative Biology. 52, 814-827.

Edenbrow, M., and Croft, D.P. (2012) Kin and familiarity influence association preferences and aggression in the mangrove killifish *Kryptolebias marmoratus*. Journal of Fish Biology. 44, 503-518.

Ellison, A., Allainguillaume, J., Girdwood, S., Pachebat, J., Peat, K. M., Wright, P.A., and Consuegra, S. (2012a) Maintaining functional major histocompatibility complex diversity under inbreeding: the case of a selfing vertebrate. Proceedings of the Royal Society B: Biological Sciences. 279, 5004-5013.

Ellison, A., Wright, P.A., Taylor, D.S., Cooper, C., Regan, K., Currie, S., and Consuegra, S. (2012b) Environmental diel variation, parasite loads, and local population structuring of a mixed-mating mangrove fish. Ecology and Evolution. 2, 1682-1695.

Forsgren, E., Karlsson, A., and Kvarnemo, C. (1996) Female sand gobies gain direct benefits by choosing males with eggs in their nests. Behavioral Ecology and Sociobiology. 39, 91-96.

Frick, N.T., and Wright, P.A. (2002) Nitrogen metabolism and excretion in the mangrove killifish *Rivulus marmoratus*. I. The influence of environmental salinity and external ammonia. The Journal of Experimental Biology. 205, 79-89.

Frommen, J.G., Brendler, C., and Bakker, T.C.M. (2007) The tale of the bad stepfather: male three-spined sticklebacks *Gasterosteus aculeatus L*. recognize foreign eggs in their manipulated nest by egg cues alone. Journal of Fish Biology. 70, 1295-1301.

Fu, C., Wilson, J.M., Rombough, P.J., and Brauner, C.J. (2010) Ions first: Na^+ uptake shifts from the skin to the gills before O2 uptake in developing rainbow trout, *Oncorhynchus mykiss*. Proceedings of the Royal Society B: Biological Sciences, 277, 1553-1560.

Garside, E.T. (1959) Some effects of oxygen in relation to temperature on the development of lake trout embryos. Canadian Journal of Zoology. 37, 689-698.

Garside, E.T. (1966) Effects of oxygen in relation to temperature on the development of embryos of brook trout and rainbow trout. Journal of the Fisheries Board of Canada. 23, 1121-1134.

Geiser, F., and Seymour, R.S. (1989) Influence of temperature and water potential on survival of hatched, terrestrial larvae of the frog *Pseudophryne bibronii*. Copeia. 1989, 207-209.

Gibb, A.C., and Ferry-Graham, L. (2005) Cranial movements during suction feeding in teleost fishes: Are they modified to enhance suction production? Zoology. 108, 141-53.

Gibson, R.N. (1982) Recent studies on the biology of intertidal fishes. In "Oceanography and marine biology: an annual review. Volume 20" edits by Barnes, H., and Barnes, M. Aberdeen University Press. 363-414.

Gittleman, J.L. (1981) The phylogeny of parental care in fishes. Animal Behaviour. 29, 936-941.

Graham, J.B., and Lee, H.J. (2004) Breathing air in air: in what ways might extant amphibious fish biology relate to prevailing concepts about early tetrapods, the evolution of vertebrate air breathing, and the vertebrate land transition? Physiological and Biochemical Zoology. 77, 720-31.

Griem, J.N., and Martin, K.L.M. (2000) Wave action: the environmental trigger for hatching in the California grunion *Leuresthes tenuis* (Teleostei: Atherinopsidae). Marine Biology. 137, 177-181.

Grizzle, J.M. and Thiyagarajah, A. (1987) Skin histology of *Rivulus ocellatus marmoratus*: apparent adaptation for aerial respiration. Copeia. 1987, 237-240.

Hamilton, W.D. (1964a) The genetical evolution of social behaviour. I. Journal of Theoretical Biology. 7, 1-16.

Hamilton, W.D. (1964b) The genetical evolution of social behaviour. II. Journal of Theoretical Biology. 7, 17-52.

Hamilton, W.D., Axelrod, R., and Tanese, R. (1990) Sexual reproduction as an adaptation to resist parasites (a review). Proceedings of the National Academy of Sciences. 87, 3566-3573.

Hansen, J.A., Rose, J.D., Jenkins, R.A., Gerow, K. G., and Bergman, H.L. (1999) Chinook salmon (*Oncorhynchus tshawytscha*) and rainbow trout (*Oncorhynchus mykiss*) exposed to

copper: Neurophysiological and histological effects on the olfactory system. Environmental Toxicology and Chemistry. 18, 1979-1991.

Harrington, R.W.Jr (1961) Oviparous hermaphroditic fish with internal self-fertilization. Science. 134, 1749-1750.

Harrington, R.W.Jr (1963) Twenty-four-hour rhythms of internal self-fertilization and of oviposition by hermaphrodites of *Rivulus marmoratus*. Physiological Zoology. 36, 325-341.

Hastings, D., and Burggren, W. (1995) Developmental changes in oxygen consumption regulation in larvae of the South African clawed frog *Xenopus laevis*. The Journal of Experimental Biology. 198, 2465-2475.

Heming, T.A., and Buddington, R.K. (1988) Yolk absorption in embryonic and larval fishes. In "Fish Physiology V11A" edits by Hoar, W.S., and Randall, D.J. Academic Press, Inc. 407-446.

Hernandez, L.P., Gibb, A.C., and Ferry-Graham, L. (2009) Trophic apparatus in Cyprinodontiform fishes: functional specializations for picking and scraping behaviors. Journal of Morphology. 270, 645-61.

Holmes, W., and Sherman, P. (1982) The ontogeny of kin recognition in two species of ground squirrels. American Zoologist. 517, 491-517.

Iles, T.D., and Holden, M.J. (1969) Bi-parental mouth brooding in *Tilapia galilaea* (Pisces, Cichlidae). Journal of Zoology. 158, 327-333.

Incardona, J.P., Collier, T.K., and Scholz, N.L. (2004) Defects in cardiac function precede morphological abnormalities in fish embryos exposed to polycyclic aromatic hydrocarbons. Toxicology and Applied Pharmacology. 196, 191-205.

Ishimatsu, A., Yoshida, Y., Itoki, N., Takeda, T., Lee, H.J., and Graham, J.B. (2007) Mudskippers brood their eggs in air but submerge them for hatching. The Journal of Experimental Biology. 210, 3946-3954.

Jew, C.J., Wegner, N.C., Yanagitsuru, Y., Tresguerres, M., and Graham, J.B. (2013) Atmospheric oxygen levels affect mudskipper terrestrial performance: implications for early tetrapods. Integrative and Comparative Biology. 1-10.

Johnson, A., Carew, E., and Sloman, K.A. (2007) The effects of copper on the morphological and functional development of zebrafish embryos. Aquatic Toxicology. 84, 431-438.

Julliard, A.K., Saucier, D., and Astic, L. (1996) Time-course of apoptosis in the olfactory epithelium of rainbow trout exposed to a low copper level. Tissue and Cell. 28, 367-377.

Kaastrup, P., Stet, R., and Tigchelaar, A. (1989) A major histocompatibility locus in fish: serological identification and segregation of transplantation antigens in the common carp (*Cyprinus carpio* L.). Immunogenetics. 30, 284-290.

Kawano, S.M., and Blob, R.W. (2013) Propulsive forces of mudskipper fins and salamander limbs during terrestrial locomotion: implications for the invasion of land. Integrative and Comparative Biology. 53, 283-294.

Klein, D., Ono, H., O'hUigin, C., and Vincek, V. (1993) Extensive MHC variability in cichlid fishes of Lake Malawi. Nature. 364, 330-334.

Kramer, D. (1978) Terrestrial group spawning of *Brycon petrosus* (Pisces: Characidae) in panama. Copeia. 3, 536-538.

Kristensen, I. (1970) Competition in three cyprinodont fish species in the Netherlands Antilles. Caribbean Marine Biological Institute. 119, 82-101.

Levels, P., Gubbels, R., and Denuce, J. (1986) Oxygen consumption during embryonic development of the annual fish *Nothobranchius korthausae* with special reference to diapause. Comparative Biochemistry and Physiology Part A: Physiology. 84, 1961-1964.

Liss, P. (1973) Processes of gas exchange across an air-water interface. Deep Sea Research and Oceanographic Abstracts. 20, 221-238.

Little, C. (1990) The terrestrial invasion. An ecophysiological approach to origin of land animals. Cambridge University Press.

Long, J.A., and Gordon, M.S. (2004) The greatest step in vertebrate history: a paleobiological review of the fish-tetrapod transition. Physiological and Biochemical Zoology. 77, 700-719.

Mackiewicz, M., Tatarenkov, A., Perry, A., Martin, J.R., Elder, J.F., Bechler, D.L., and Avise, J.C. (2006a) Microsatellite documentation of male-mediated outcrossing between inbred laboratory strains of the self-fertilizing mangrove killifish (*Kryptolebias marmoratus*). Journal of Heredity. 97, 508-513.

Mackiewicz, M., Tatarenkov, A., Taylor, D.S, Turner, B.J., and Avise, J. (2006b) Extensive outcrossing and androdioecy in a vertebrate species that otherwise reproduces as a self-fertilizing hermaphrodite. Proceedings of the National Academy of Science. 103, 9924-9928.

Macro, A. (2001) Effects of prolonged terrestrial stranding of aquatic *Ambystoma gracile* egg masses on embryonic development. Journal of Herpetology. 35, 510-513.

Manica, A. (2002) Filial cannibalism in teleost fish. Biological Reviews. 77, 261-277.

Marliave, J. (1981) High intertidal spawning under rockweed, *Fucus distichus*, by the sharpnose sculpin, *Clinocottus acuticeps*. Canadian Journal of Zoology. 59, 1122-1125.

Martin, K., Bailey, K., Moravek, C., and Carlson, K. (2011) Taking the plunge: California grunion embryos emerge rapidly with environmentally cued hatching. Integrative and Comparative Biology. 51, 26-37.

Martin, K.L., and Carter, A.L. (2013) Brave new propagules: terrestrial embryos in anamniotic eggs. Integrative and Comparative Biology. 1-15.

Martin, K.L.M. (1999) Ready and waiting: delayed hatching and extended incubation of anamniotic vertebrate terrestrial eggs. American Zoologist. 288, 279-288.

Martin, K.L.M., and Swiderski, D.L. (2001) Beach spawning in fishes: phylogenetic tests of hypotheses. American Zoologist. 537, 526-537.

Matschak, T.W., Hhopcroft, T., Smason, P., Crook, A.R., and Stickland, N.C. (1998) Temperature and oxygen tension influence the development of muscle cellularity in embryonic rainbow trout. Journal of Fish Biology. 53, 581-590.

Matschak, T.W., Stickland, N.C., Mason, P.S., and Crook, A.R. (1997) Oxygen availability and temperature affect embryonic muscle development in Atlantic salmon (*Salmo salar* L.). Differentiation. 61, 229-235.

McDowall, R., and Charteris, S. (2006) The possible adaptive advantages of terrestrial egg deposition in some fluvial diadromous galaxiid fishes (Teleostei: Galaxiidae). Fish and Fisheries. 7, 153-164.

Mehlis, M., Bakker, T.C., and Frommen, J.G. (2009) Nutritional benefits of filial cannibalism in three-spined sticklebacks (*Gasterosteus aculeatus*). Naturwissenschaften. 96, 399-403.

Mehlis, M., Bakker, T.C., Engqvist, L., and Frommen, J.G. (2010) To eat or not to eat: egg-based assessment of paternity triggers fine-tuned decisions about filial cannibalism. Proceedings of the Royal Society B: Biological Sciences. 277, 2627-2635.

Middaugh, D. (1981) Reproductive ecology and spawning periodicity of the Atlantic silverside, *Menidia menidia* (Pisces: Atherinidae). Copeia. 1981, 766-776.

Miller, S.C., Reeb, S.E., Wright, P.A., and Gillis, T.E. (2008) Oxygen concentration in the water boundary layer next to rainbow trout (*Oncorhynchus mykiss*) embryos is influenced by hypoxia exposure time, metabolic rate, and water flow. Canadian Journal of Fisheries and Aquatic Sciences. 65, 2170-2177.

Miller, S.C., Gillis, T.E., and Wright, P.A. (2011) The ontogeny of regulatory control of the rainbow trout (*Oncorhynchus mykiss*) heart and how this is influenced by chronic hypoxia exposure. The Journal of Experimental Biology. 214(12), 2065-2072.

Mitchell, N.J. (2002) Low Tolerance of Embryonic Desiccation in the Terrestrial Nesting Frog *Bryobatrachus nimbus* (Anura: Myobatrachinae). Copeia. 2002, 364-373.

Moses, M.E., Hou, C., Woodruff, W.H., West, G.B., Nekola, J.C., Zuo, W., and Brown, J. H. (2008) Revisiting a model of ontogenetic growth: estimating model parameters from theory and data. The American Naturalist. 171, 632-645.

Mourabit, S., Edenbrow, M., Croft, D.P., and Kudoh, T. (2011) Embryonic development of the self-fertilizing mangrove killifish *Kryptolebias marmoratus*. Developmental Dynamics. 240, 1694-1704.

Mueller, C.A., Joss, J.M., and Seymour, R.S. (2011) Effects of environmental oxygen on development and respiration of Australian lungfish (*Neoceratodus forsteri*) embryos. Journal of Comparative Physiology B. 181, 941-952.

Nelson, J.S. (2006) Fishes of the World: Fourth Edition. John Wiley & Sons, Inc., Hoboken, New Jersey.

Niedźwiedzki, G., Szrek, P., Narkiewicz, K., Narkiewicz, M., and Ahlberg, P. E. (2010) Tetrapod trackways from the early Middle Devonian period of Poland. Nature. 463, 43-48.

Nilsson, P.A., and Brönmark, C. (2000) Prey vulnerability to a gape-size limited predator: behavioural and morphological impacts on northern pike piscivory. Oikos. 88, 539-546.

Ninness, M.M., Stevens, E.D., and Wright, P.A. (2006) Removal of the chorion before hatching results in increased movement and accelerated growth in rainbow trout (*Oncorhynchus mykiss*) embryos. The Journal of Experimental Biology. 209, 1874-1882.

Olsén, K.H., Grahn, M., and Lohm, J. (2002) Influence of MHC on sibling discrimination in Arctic char, *Salvelinus alpinus* (L.). Journal of Chemical Ecology. 28, 783-95.

Olsén, K.H., Grahn, M., Lohm, J., and Langefors, Å. (1998) MHC and kin discrimination in juvenile Arctic charr, *Salvelinus alpinus* (L.). Animal Behaviour. 56, 319-327.

Ong, K.J., Stevens, E.D., and Wright, P.A. (2007) Gill morphology of the mangrove killifish (*Kryptolebias marmoratus*) is plastic and changes in response to terrestrial air exposure. The Journal of Experimental Biology. 210, 1109-1115.

Packard, M.J., and Seymour, R.S. (1997) Evolution of the amniote egg. In "Amniote origins. Completing the transition to land" edits by Sumida, S.S. and Martin, K.L.M. Academic Press, Inc. 265-290.

Pelster, B., and Burggren, W.W. (1996) Disruption of hemoglobin oxygen transport does not impact oxygen-dependent physiological processes in developing embryos of zebra fish (*Danio rerio*). Circulation research. 79, 358-362.

Penn, D. (2002) The scent of genetic compatibility: sexual selection and the major histocompatibility complex. Ethology. 108, 1-21.

Pfennig, D.W., Reeve, H., and Sherman, P.W. (1993) Kin recognition and cannibalism in spadefoot toad tadpoles. Animal Behaviour. 46, 87-94.

Pfennig, D.W., Sherman, P.W., Hall, M., and Collins, J.P. (1994) Kin recognition and cannibalism in polyphenic salamanders. Behavioral Ecology. 5, 225-232.

Podrabsky, J.E., Carpenter, J.F., and Hand, S.C. (2001) Survival of water stress in annual fish embryos: dehydration avoidance and egg envelope amyloid fibers. American Journal of Physiology: Regulatory, Integrative and Comparative Physiology. 280, R123-R131.

Podrabsky, J.E., Tingaud-Sequeira, A., and Cerda, J. (2010) Metabolic dormancy and responses to environmental desiccation in fish embryos. In "Dormancy and resistance in harsh environments" edits by Lubzens, E., Cerdáa, J., Clark, M.S. Springer Berlin Heidelberg. 203-226.

Pronko, A.J., Perlman, B.M., and Ashley-Ross, M.A. (2013) Launches, squiggles and pounces, oh my! The water–land transition in mangrove rivulus (*Kryptolebias marmoratus*). The Journal of Experimental Biology. 216, 3988-3995.

Quinn, T.P., and Hara, T.J. (1986) Sibling recognition and olfactory sensitivity in juvenile coho salmon (*Oncorhynchus kisutch*). Canadian Journal of Zoology. 64, 921-925.

Randall, D., Burggren, W., and French, K. (2001) Eckert animal physiology. Mechanisms and adaptation 5th edition. W.H. Freeman and Company, New York, U.S.A.

Reeve, H.K. (1989) The evolution of conspecific acceptance thresholds. American Naturalist. 133, 407-435.

Regan, K.S., Jonz, M.G., and Wright, P.A. (2011) Neuroepithelial cells and the hypoxia emersion response in the amphibious fish *Kryptolebias marmoratus*. The Journal of Experimental Biology. 214, 2560-2568.

Reusch, T., Haberli, M., Aeschlimann, P., and Milinski, M. (2001) Female sticklebacks count alleles in a strategy of sexual selection explaining MHC polymorphism. Nature. 400, 15-17.

Rice, W. (1983) Parent-offspring pathogen transmission: a selective agent promoting sexual reproduction. American Naturalist. 121, 187-203.

Rodela, T.M., and Wright, P.A. (2006) Metabolic and neuroendocrine effects on diurnal urea excretion in the mangrove killifish *Rivulus marmoratus*. The Journal of Experimental Biology. 209, 2704-2712.

Rombough, P.J. (1986) Mathematical model for predicting the dissolved oxygen requirements of steelhead (*Salmo gairdneri*) embryos and alevins in hatchery incubators. Aquaculture. 59, 119-137.

Rombough, P.J. (1994) Energy partitioning during fish development: additive or compensatory allocation of energy to support growth? Functional Ecology. 8, 178-186.

Rombough, P.J. (1999) The gill of fish larvae. Is it primarily a respiratory or an ionoregulatory structure? Journal of Fish Biology. 55, 186-204.

Sakakura, Y., and Noakes, D. (2000) Age, growth, and sexual development in the self-fertilizing hermaphroditic fish *Rivulus marmoratus*. Environmental Biology of Fishes. 59, 309-317.

Sandahl, J.F., Miyasaka, G., Koide, N., and Ueda, H. (2006) Olfactory inhibition and recovery in chum salmon (*Oncorhynchus keta*) following copper exposure. Canadian Journal of Fisheries and Aquatic Sciences. 63, 1840-1847.

Sato, A., Satta, Y., Figueroa, F., Mayer, W.E., Zaleska-Rutczynska, Z., Toyosawa, S., Travis, S., and Klein, J. (2002) Persistence of *Mhc* heterozygosity in homozygous clonal

killifish, *Rivulus marmoratus*: implication for the origin of hermaphroditism. Genetics. 162, 1791-1803.

Sayer, M.D.J. (2005) Adaptations of amphibious fish for surviving life out of water. Fish and Fisheries. 6, 186-211.

Sayer, M.D.J., and Davenport, J. (1991) Amphibious fish: why do they leave water? Reviews in Fish Biology and Fisheries. 181, 159-181.

Schmitt, R.J., and Holbrook, S.J. (1984) Gape-limitation, foraging tactics and prey size selectivity of two microcarnivorous species of fish. Oecologia. 63, 6-12.

Shubin, N.H., Daeschler, E.B., and Jenkins, F.A. (2006) The pectoral fin of *Tiktaalik roseae* and the origin of the tetrapod limb. Nature. 440, 764-71.

Shykoff, J.A., and Schmid-Hempel, P. (1991) Parasites and the advantage of genetic variability within social insect colonies. Proceedings of the Royal Society of London. Series B: Biological Sciences. 243, 55-58.

Skulan, J. (2000) Has the importance of the amniote egg been overstated? Zoological Journal of the Linnean Society. 130, 235-261.

Silver, S.J., Warren, C.E., and Doudoroff, P. (1963) Dissolved oxygen requirements of developing steelhead trout and chinook salmon embryos at different water velocities. Transactions of the American Fisheries Society. 92, 327-343.

Smith, C., and Reay, P. (1991) Cannibalism in teleost fish. Reviews in Fish Biology and Fisheries. 64, 41-64.

Smyder, E.A., and Martin, K.L.M. (2002) Temperature effects on egg survival and hatching during the extended incubation period of California grunion, *Leuresthes tenuis*. Copeia. 2002, 313-320.

Sommerfeld, R., Boehm, T., and Milinski, M. (2008) Desynchronising male and female reproductive seasonality: dynamics of male MHC-independent olfactory attractiveness in sticklebacks. Ethology Ecology and Evolution. 20, 325-336.

Soto, C. G., Leatherland, J. F., and Noakes, D. L. (1992) Gonadal histology in the self-fertilizing hermaphroditic fish *Rivulus marmoratus* (Pisces, Cyprinodontidae). Canadian Journal of Zoology. 70, 2338-2347.

Speer-Blank, T., and Martin, K. (2004) Hatching events in the California Grunion, *Leuresthes tenuis*. Copeia. 2004, 21-27.

Stacey, P.B., and Ligon, J.D. (1991) The benefits-of-philopatry hypothesis for the evolution of cooperative breeding: variation in territory quality and group size effects. American Naturalist. 137, 831-846.

Steffensen, J.F. (1985) The transition between branchial pumping and ram ventilation in fishes: energetic consequences and dependence on water oxygen tension. The Journal of Experimental Biology. 114, 141-150.

Stewart, J.R. (1997) Morphology and evolution of the egg of oviparous amniotes. In "Amniote origins. Completing the transition to land" Edited by Sumida, S.S., and Martin, K.L.M. Academic Press, Inc. 291-326.

Strathmann, R., and Hess, H. (1999) Two designs of marine egg masses and their divergent consequences for oxygen supply and desiccation in air. American Zoologist. 39, 253-260.

Swain, D.P., and Lindsey, C.C. (1986) Meristic variation in a clone of the cyprinodont fish *Rivulus marmoratus* related to temperature history of the parents and of the embryos. Canadian Journal of Zoology. 64, 1444-1455.

Tatarenkov, A., Lima, S., Taylor, D.S. and Avise, J.C. (2009) Long-term retention of self-fertilization in a fish clade. Proceedings of the National Academy of Sciences. 106, 14456-14459.

Tatarenkov, A., Ring, B.C., Elder, J.F., Bechler, D.L., and Avise, J.C. (2010) Genetic composition of laboratory stocks of the self-fertilizing fish *Kryptolebias marmoratus*: a valuable resource for experimental research. PloS one. 5, e12863.

Tattersall, G.J., and Spiegelaar, N. (2008) Embryonic motility and hatching success of *Ambystoma maculatum* are influenced by a symbiotic alga. Canadian Journal of Zoology. 86, 1289-1298.

Taylor, D.S. (1990) Adaptive specializations of the cyprinodont fish *Rivulus marmoratus*. Florida Scientist. 53, 239-248.

Taylor, D.S. (2012) Twenty-four years in the mud: what have we learned about the natural history and ecology of the mangrove rivulus, *Kryptolebias marmoratus*? Integrative and Comparative Biology. 52, 724-736.

Taylor, D.S., Davis, W., and Turner, B. (1995) *Rivulus marmoratus*: ecology of distributional patterns in florida and central indian river lagoon. Bulletin of Marine Science. 57, 202-207.

Taylor, D.S., Davis, W.P. and Turner, B.J. (2004) Groveling in the mangroves: 16 years in pursuit of the cyprinodont fish *Rivulus marmoratus* on the Belize Cays. Atoll Research Bulletin. 525, 1-14.

Taylor, D.S., Turner, B.J., Davis, W.P., and Chapman, B.B. (2008) A novel terrestrial fish habitat inside emergent logs. The American Naturalist. 171, 263-266.

Taylor, M.H. (1999) A suite of adaptations for intertidal spawning. Integrative and Comparative Biology. 39, 313-320.

Taylor, M.H., Di Michele, L., and Leach, G.J. (1977) Egg stranding in the life cycle of the mummichog, *Fundulus heteroclitus*. Copeia. 1977, 397-399.

Tingaud-Sequeira, A., Zapater, C., Chauvigné, F., Otero, D., and Cerdà, J. (2009) Adaptive plasticity of killifish (*Fundulus heteroclitus*) embryos: dehydration-stimulated development and differential aquaporin-3 expression. American Journal of Physiology: Regulatory, Integrative and Comparative Physiology. 296, R1041-R1052.

Touchon, J.C., and Warkentin, K.M. (2010) Short- and long-term effects of the abiotic egg environment on viability, development and vulnerability to predators of a Neotropical anuran. Functional Ecology. 24, 566-575.

Turko, A.J., Earley, R.L., and Wright, P.A. (2011) Behaviour drives morphology: voluntary emersion patterns shape gill structure in genetically identical mangrove rivulus. Animal Behaviour. 82, 39-47.

Turner, B.J., William P.D., and Taylor, **D.S.** (1992) Abundant males in populations of a selfing hermaphrodite fish, *Rivulus marmoratus*, from some Belize cays. Journal of Fish Biology. 40, 307-310.

Van Wassenbergh, S. (2013) Kinematics of terrestrial capture of prey by the eel-catfish *Channallabes apus*. Integrative and Comparative Biology. 53, 258-268.

Villinger, J., and Waldman, B. (2008) Self-referent MHC type matching in frog tadpoles. Proceedings of the Royal Society B: Biological Sciences. 275, 1225-1230.

Von Bertalanffy, L. (1957) Quantitative laws in metabolism and growth. The Quarterly Review of Biology. 32, 217-231.

Vulesevic, B., and Perry, S.F. (2006) Developmental plasticity of ventilatory control in zebrafish, *Danio rerio*. Respiratory Physiology and Neurobiology. 154, 396-405.

Walker, B.W. (1949) Periodicity of spawning by the grunion, *Leuresthes tenuis*, an atherine fish. PhD Thesis, Scipps Institution of Oceanography Technical Report.

Ward, A.J., and Hart, P.J. (2003) The effects of kin and familiarity on interactions between fish. Fish and Fisheries. 4, 348-358.

Warkentin, K.M., Gomez-Mestre, I., and McDaniel, J.G. (2005) Development, surface exposure, and embryo behavior affect oxygen levels in eggs of the red-eyed treefrog, *Agalychnis callidryas*. Physiological and Biochemical Zoology. 78, 956-966.

Winberg, S., and Olsén, K.H. (1992) The influence of rearing conditions on the sibling odour preference of juvenile arctic charr, *Salvelinus alpinus* L. Animal Behaviour. 44, 157-164.

Wood, A.H. (1932) The effect of temperature on the growth and respiration of fish embryos (*Salmo fario*). The Journal of Experimental Biology. 9, 271-276.

Wourms, J.P. (1972) The developmental biology of annual fishes. III. Pre-embryonic and embryonic diapause of variable duration in the eggs of annual fishes. The Journal of Experimental Zoology. 182, 389-414.

Wright, P., Felskie, A., and Anderson, P. (1995) Induction of ornithine-urea cycle enzymes and nitrogen metabolism and excretion in rainbow trout (*Oncorhynchus mykiss*) during early life stages. The Journal of Experimental Biology. 198, 127-135.

Yamazaki, K., Boyse, E., and Mike, V. (1976) Control of mating preferences in mice by genes in the major histocompatibility complex. The Journal of Experimental Medicine. 144, 1324-1335.