MOTHERS TRANSFER INFORMATION VIA EGGS: EFFECT OF MOTHERS' EXPERIENCE WITH PREDATORS ON OFFSPRING

BY

ERIC RICHARD GIESING

THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Natural Resources and Environmental Sciences
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2010

Urbana, Illinois

Master's Committee:

Professor Alison Bell
Professor Richard Warner
Professor Cory Suski

ABSTRACT

Parents play an important role in creating phenotypic variation in their offspring through genetic and environmental mechanisms. For example, mothers can influence their offspring via hormonally-mediated maternal effects. In this study, offspring of mothers that had been exposed to a predator during oogenesis (experimental) were compared to offspring of mothers that had not been exposed to a predator (control). I measured the consequences of maternal exposure to predation risk on the number and size of eggs, egg cortisol content, metabolic rates of eggs, and the growth and behavior of juvenile threespined sticklebacks (*Gasterosteus aculeatus)*. Exposure to a predator during oogenesis caused females to produce larger eggs and heavier clutches, but there was no effect on the number of eggs per clutch. The concentration of cortisol was higher in experimental eggs than control eggs. Experimental eggs also breathed faster soon after fertilization, but the difference between control and experimental eggs in oxygen consumption attenuated over time. Standard length of juveniles increased over the course of the experiment, but there was no effect of treatment on growth during the period when the fry were measured. Shoaling behavior, an antipredator response, was measured from photographs, and was estimated as the nearest neighbor distance between individuals in each tank. Experimental females shoaled more tightly together prior to a mild disturbance. Both control and experimental juveniles shoaled more tightly together immediately following and two minutes after a mild disturbance. Altogether, these results suggest that the effect of mothers on their offspring might depend on a mothers' experience with stressors in the environment, and that mothers might manipulate the development, growth, and behavior of their offspring to match their future environment.

To my wife who kept me sane throughout this process

ACKNOWLEDGMENTS

This project would not have been possible without the support of many people. Many thanks to my co-adviser, Alison Bell, who read numerous revisions, did a lot of heavy lifting, and helped me make sense of it all. Thanks to my co-adviser, Richard Warner for helpful suggestions and encouragement along the way. Also thanks to my committee member, Cory Suski, who so willingly offered his equipment and lab and spent endless hours answering my questions and working out technical kinks. Thanks to the Bell lab for their comments and editing throughout the writing process. Thanks to my wife who endured this process with me and offered unconditional love and support. I could not have done it without you. Lastly, thanks to my parents and siblings who have always encouraged, loved, and supported me no matter what. Thanks for pushing me to succeed.

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

Parents play an important role in creating phenotypic variation in their offspring through both genetic and environmental mechanisms (Mousseau and Fox 1998). Contributions from the mother other than her genotype, such as her phenotype or environment, are known as nongenetic maternal effects (Bernardo 1996, Mousseau and Fox 1998). Maternal effects depend on maternal condition (Mousseau and Fox 1998, Blount 2002) and are modified by the environment she experiences before and during reproduction (Chamber and Leggett 1996). These effects can result from the transfer of materials, such as yolk and maternal hormones (McCormick 1998, McCormick 1999), maternal nutrition (Mather and Jinks 1971), or via behavior (Mather and Jinks 1971, Bernardo 1996, Heath and Blouw 1998). Maternal effects have important evolutionary and ecological consequences (Reznick 1991, Green and McCormick 2005) because they often influence early life history traits of offspring, which can have large effects on survival and reproductive success later in life (Madsen and Shine 2000, Hofer and East 2003).

Among vertebrates, hormonally-mediated maternal effects have been well studied in mammals and birds (Naumenko 1991, Braastad 1998, Sims and Holberton 2000, Groothuis and Schwabl 2007). For example, female birds deposit steroids in the yolk of their eggs, and those steroids influence the behavior, physiology, growth and life history of their offspring (Gil 2008). It is also clear that in mammals, including humans, stressors

during pregnancy can influence the development of offspring in utero via the transfer of steroids from mother to fetus (Talge et al. 2007).

Less is known about hormonally-mediated maternal effects, such as those mediated by cortisol, in fishes. Important studies have shown that cortisol is present in freshly ovulated eggs (Barry et al. 1995; Hwang et al. 1992), and that the cortisol is likely to be maternally-derived (De Jesus et al 1991, Stratholt et al 1997). Unlike mammalian embryos that produce their own endocrine response prior to birth (Sapolsky and Meaney 1986, Matthews and Challis 1996), in fishes, the hypothalamus-pituitary-interrenal (HPI) axis is not fully responsive until after hatching (Barry et al 1995, Feist and Schreck 2001). Therefore, there is a strong positive relationship between maternal stress levels and egg corticosteroid levels (De Jesus et al 1991, Hwang et al 1992, McCormick 1998, Schreck et al 2001).

In fishes, as in other vertebrates, it is likely that maternally derived cortisol has important consequences for offspring because fish embryos are sensitive to the organizational effects of cortisol early in development (Auperin and Geslin 2008). But we know little about natural variation in maternal effects in fishes, and whether exposure to ecologically-relevant conditions during oogenesis affects the behavior, physiology and growth of offspring. Many studies use experimentally-controlled injections or implants to elevate cortisol levels, rather than using an ecologically relevant stressor on mothers and studying naturally occurring effects. Moreover, research on maternal effects also usually focuses on eggs and adulthood (Fox 1997, Heath et al 1999), but little is known about the intermediate stages of progeny development or how progeny compensates for maternal effects from early in their life history.

Another outstanding question about hormonally-mediated maternal effects is whether they might be adaptive. On the one hand, developing embryos are highly sensitive to endocrine perturbations; therefore, we might expect embryos to possess mechanisms that buffer the embryo against hormonal fluctuations in utero. On the other hand, the conditions experienced by a mother during oogenesis or pregnancy might be predictive of the future environment of her offspring. Therefore it might be adaptive for mothers to hormonally program (Weaver et al. 2004) their offspring to be suited for particular environments (Mousseau and Dingle 1991, Galloway 1995, Agrawal 2002, Einum and Fleming 2002).

Recent studies have shown the transfer of maternal effects across generations due to exposure to predators. When fall field cricket *Gryllus pennsylvanicus* mothers were exposed to wolf spiders *Hogna helluo*, their offspring spent significantly more time immobile, exhibiting anti-predator behavior, than offspring from mothers not exposed when encountering spider chemical cues (Sheriff et al 2010). In a similar study, snowshoe hare mothers had fluctuating levels of stress hormones in response to predation risk and these levels were mirrored in their offspring, along with a highly responsive HPA axis, allowing their offspring to have increased anti-predator behavior (Storm and Lima 2010). These generational effects due to predation risk appear to transmit advantages to offspring and may potentially affect other traits as well.

In this experiment, I exposed mothers to an ecologically relevant predator stressor to investigate the consequences of maternal exposure to predation risk for offspring in threespined sticklebacks (*Gasterosteus aculeatus*). I tested the hypothesis that maternally-derived cortisol mediates the effect of maternal stress on offspring phenotypes. Previous

studies have shown that early exposure to cortisol influences egg size and survival of embryos (Campbell et al 1994, McCormick 1998, Eriksen et al 2006), growth and development (Eriksen et al 2006, 2007) and behavior (Espmark et al 2008). To my knowledge, effects of early exposure to cortisol on metabolic rates in eggs have never been explored, despite cortisol affecting metabolic rates in adults (Chan and Woo 1978, Barton and Schreck 1987, Wendelaar Bonga 1997). Therefore, I measured all of these traits in order to gain insight into the mechanisms by which mothers influence their offspring. In their natural habitat, sticklebacks are subject to predation by piscivorous fishes, birds, odonates and snakes, and have numerous behavioral (Huntingford et al. 1994) and morphological (Reimchen 1994) defenses against predators. Other studies have shown that sticklebacks show significant changes in levels of cortisol in response to acute and chronic stressors (Pottinger et al 2002), including predators (Bell et al 2007), making the stickleback an ideal organism for this study.

CHAPTER 2

METHODOLOGY

Overview

In order to determine whether exposure to a predator stress during oogenesis affects the development of her offspring, I exposed gravid female sticklebacks to a model predator (experimental) and left others alone (control). I compared the egg size and number, cortisol content of eggs, and the oxygen consumption of eggs from control and experimental mothers prior to hatching. Then, I monitored the growth and shoaling behavior of the juveniles until approximately 23 weeks after hatching.

Detailed methods

Sticklebacks used in this experiment were F1 generation offspring from a natural population in Putah Creek, CA. Stickleback were lab reared and maintained in the Bell Lab at the University of Illinois at Urbana-Champaign under Division of Animal Research standards and protocols (Protocol # 09204, 8/5/09). Fish were raised in 26.50 L recirculating tanks (36L x 33Wx 24H cm) on a natural photoperiod at 20 C and fed approximately 10% of their body weight once a day with a mixture of frozen bloodworms, mysis shrimp, cyclopleze, and spirulina cubes, except as noted.

In August 2009, female sticklebacks were randomly assigned to one of nine different 26.5 L tanks (36L x 33W x 24H cm), with five fish per tank. I assigned each fish within a tank a unique mark via spine clipping in order to determine the length of time each female had spent in the experimental or control treatment before being stripped

for eggs. Three tanks were used for the control treatment and six for the experimental treatment. I assigned more fish to the experimental group based on a pilot study which detected greater variation in maternally-derived egg cortisol among mothers exposed to a predator (mean=194.20, SE=51.50, n=7) compared to the eggs of mothers not exposed to a predator (mean=89.72, SE=7.76, n=7).

Opaque plastic coverings on the outside of each tank minimized exposure of fish to external visual stressors. One small window with a removable covering allowed me to monitor the gravidity of females with minimal disturbance to fish. Females were provided with additional food (bloodworm cube) every day in order to encourage them to become gravid.

I chased fish in the experimental treatment once daily at a randomly chosen time with a clay Northern Pike model (23L x 1W x 6H cm, painted to match natural markings) a natural predator, for approximately 30 seconds to stress the fish. This protocol of random, repeated acute stress was chosen in an effort to imitate natural conditions where sticklebacks in high predation populations are regularly confronted by the threat of predation (Giles and Huntingford 1984, Bell et al 2010). Fish in the control treatment remained undisturbed. When a female became gravid, judged by a swollen abdomen and characteristic sharp angle near the cloaca, she was measured for standard length and weight while still bearing eggs. She was then stripped for eggs. Fully gravid females readily released their eggs with little applied pressure. Eggs that were dark yellow or non-aggregated were not developmentally ready and were discarded (Vines and Schluter 2006). Stripping continued until all eggs had been released and clutch mass for each female was determined by weighing the entire clutch of eggs for each female. Egg size

(mass) was calculated for each clutch by weighing a subset of eggs and dividing the subset mass by the number of eggs weighed. Total number of eggs was estimated by dividing entire clutch mass by egg size.

On average, females in the experimental and control groups spent the same amount of time in the treatment prior to being stripped for eggs (experimental: mean= 24 days (range= 2-57), control: mean= 28 days (range= 7-46)). Each female contributed one clutch of eggs to the experiment. I artificially fertilized a subset (n=30-40) of stripped eggs from females with a male's sperm. The remaining eggs were stored in 1.5 ml microcentrifuge tubes at -80 C for later cortisol determination. I removed testes from a male, and placed each testis in a separate weighing boat with approximately five ml of tank water. The two different testis of each male fertilized a batch of 20-30 eggs from a mother in an experimental and control treatment. I macerated the testis with fine forceps and a batch of 30-40 eggs was placed in the weighing boat. I gently mixed the eggs and sperm and waited one hour for the sperm to fertilize the eggs. Then, subsets (n=19 to 23) of fertilized eggs were immediately measured for respiration rate (see below), while remaining eggs (n=7 to 21) were artificially incubated and reared in the lab (see below).

After I stripped females of their eggs, I returned them to an 83.28L holding tank (107L x 33W x 24Hcm) where they remained undisturbed for the rest of the experiment. I added new replacement marked females in their place in order to maintain a constant density of five fish/tank. I continued sampling for 10 weeks until clutches from 30 different experimental mothers and 15 different control mothers were collected.

Measuring cortisol in eggs

In order to test the hypothesis that maternal stress influences the amount of cortisol released into eggs, eggs from both the experimental and control treatment were measured for cortisol content. I used a cortisol enzyme-linked immunosorbent assay (ELISA) kit from Assay Designs (Ann Arbor, MI, Cortisol EIA kit #900-071) to determine cortisol concentrations in clutches of eggs. The intra-assay coefficient of variation was 0.93. I weighed 15-25 stickleback eggs from each clutch (n=30 experimental and n=15 control) and homogenized each clutch in 200 l of assay buffer provided in the kit. All following steps were done according to the kit protocol (Assay Design, Catalog #25-0359).

Measuring the oxygen consumption of eggs

In order to test the hypothesis that maternal stress influences respiration rates in eggs, a subset of fertilized eggs from both treatments described above were measured for respiration rate. The oxygen consumption rate (MO2 in mg/O2/g/hr) of six batches of 19-23 eggs from each treatment at different stages of development were measured using intermittent flow respirometry (Loligo Systems, Hobro, Denmark) (Steffensen 1989). Methods were adapted from Gingerich et al 2009.

The respirometry system was a four (725 l) glass chambered fiber optic system with a peristaltic pump. I set up the system in a 120L cooler of oxygenated water with air stones at 20 C (±0.5 C). Temperature was kept constant and temperature probe was calibrated throughout the experiment. One of four chambers was used as a blank to correct for background noise and three were used for measuring oxygen consumption of

eggs. A peristaltic pump re-circulated water through the chambers across the oxygen probes to ensure proper mixing, and the fiber optic oxygen probes were calibrated with both sodium sulfite oxygen free water and strongly aerated water throughout the experiment.

Oxygen consumption of eggs in each chamber was determined with a 28 minute cycle, which consisted of a 13 minute measurement period, a 15 minute flush period to re-oxygenate water, and a two second waiting period following the flush until the measurements were resumed. Oxygen consumption in each chamber was recorded every second, but only coefficient of determination (r^2) values >0.93 were kept. All data was recorded using AutoResp software (Version 1.4).

Measuring the growth and behavior of juveniles

One hour after fertilization, I placed each subset of eggs (n=7-21) in a cup with a mesh screen bottom in a two-liter bottle with tank water, 0.25 ml of methylene blue (to prevent fungus) and an air stone until hatching, approximately six days post fertilization. Newly-hatched fry were placed in four 83.28 L tanks (107L x 33W x 24H cm), two experimental and two control tanks, with their siblings and were fed newly hatched *Artermia* once daily. When fry were approximately 2.5cm standard length, varying in age from one to three months old, I combined family groups and transferred them to 26.50L tanks (36L x 33W x 24H, nine experimental and six control at a density of five fish per tank). Fish were matched for size within each tank in order to avoid dominance interactions over access to food. A grid consisting of cells (3.7 x 3.7 cm) was placed on the bottom of each tank for reference.

Because handling could influence the development of the HPI axis (Auperin and Geslin 2008), I measured standard length and growth noninvasively, by photographing each tank from above. The first photograph of each tank was taken on 7 December 2009, when the fry were two – four months of age. I continued to take photographs of each tank on six different occasions over the course of 12 weeks, with one to two weeks between measurements. I took three pictures of each tank at each age. The first ("before") photograph was of the undisturbed tank. Then, I simulated a mild disturbance by gently moving fish that were in the corner or otherwise not visible in the main body of the tank with a 26mm piece of plastic tubing, and took another photograph immediately following this disturbance ("immediately following"). I took one more photograph two minutes following the mild disturbance ("after"). I used Image J (Version 1.38) to estimate standard length from the photographs on each occasion; the grid on the bottom of each tank was used as a reference. I calculated the average length of fish in each tank at each age in order to monitor average growth rate within a tank (n=9 experimental, n=6 control tanks). On average, the fry in the experimental tanks were older than the fry in the control tanks, so growth was analyzed as a function of age rather than a function of the day or measurement.

The photographs taken to monitor growth rates also allowed me to measure average nearest neighbor distance within a tank. Nearest neighbor distance is a measure of schooling tendency (Azuma and Iwata 1994, Masuda et al 2003) and was measured before, immediately following and after the mild disturbance on all six occasions. Nearest neighbor distance was calculated for each fish in each tank, and was estimated from the center of the head of each fish in the tank to the center of the head of the nearest fish in

each picture. A reference line half-way between two fish was used to account for accurate distance/distortion of water.

Data analysis

Cortisol in eggs

I compared differences in cortisol concentrations in eggs from experimental and control mothers using ANCOVA. Two control clutches were removed from the analysis due to an insufficient volume of sample obtained from the clutch, causing error in the plate results. Cortisol concentrations were not normally distributed; therefore I transformed them using an *ln* transformation. I tested for effect of treatment (experimental vs. control) as a fixed factor, tank (nested within treatment) as a random factor, female body size (length and weight) and time in treatment as covariates, and looked at all interactions on *ln*-transformed cortisol values. Female body size, time spent in treatment and tank were non-significant and therefore removed in subsequent analyses. Effect of treatment on egg size, egg number, and clutch mass were analyzed similarly. All statistical analyses were two-tailed and were performed using SPSS (Version 17).

Oxygen consumption of eggs

Change in oxygen concentration (α) for each chamber was calculated as the slope ($\Delta O_{2saturation}/\Delta t$), and oxygen consumption rate (MO_2, mg O_2 kg^{-1} h^{-1}) for each clutch of eggs was calculated by $MO_2 = \alpha V_{resp} \beta M_b^{-1}$, where V_{resp} is the volume of each chamber minus number of eggs by volume of eggs in chamber (l), β is oxygen solubility (adjusted for temperature and barometric pressure), and M_b^{-1} is the clutch mass (kg)

before placing in chamber (Gingerich et al 2009). I compared differences in the oxygen consumption rate in the eggs from experimental and control mothers using ANCOVA, where treatment was a fixed factor, clutch nested within treatment was a random factor, time since fertilization was a covariate, and the interaction between treatment and time since fertilization.

Growth of juveniles

In order to avoid pseudoreplication (Alvarez & Metcalfe 2005), I analyzed the average growth rate of each tank. I excluded cases where not all of the fish were visible in the photograph. The density of fish in the tanks varied between 2-6 individuals (mean=4.51, SE=0.986, n=75). I analyzed the average standard length of each tank at each age using ANCOVA, with treatment as a fixed factor, tank nested within treatment as a random factor, age and density as covariates, and the interaction between age and treatment.

Behavior of juveniles

Nearest neighbor distance was not normally distributed and I therefore square root transformed average nearest neighbor distance within a tank at each age. Because the average nearest neighbor distance differed before (mean=108.76, SE=11.471, n=58), immediately following (mean=84.33, SE=7.165, n=84), and after the mild disturbance (mean=74.439, SE=5.9336, n=85), I analyzed the before, immediately following and after data separately. I tested for the effect of treatment on nearest neighbor distance, with treatment as a fixed factor, tank nested within each treatment as a random effect, and

density (number of fish in the tank), averaged standard length of fish in each tank, and age as covariates.

CHAPTER 3

RESULTS

Egg size and clutch mass

Exposure to predator stress during oogenesis caused females to produce heavier clutches (Table 1, Figure 3). The clutches of experimental females were larger because they produced bigger eggs (Table 2, Figure 1), not because they produced more eggs (Table 3, Figure 3). Tank and the time spent in treatment did not affect the clutch mass, egg size or number of eggs (Figure 2). Larger females produced heavier clutches (Table 1).

Egg cortisol content

Mothers exposed to a predator stressor (experimental mothers, n=30) had significantly higher concentrations of cortisol in their eggs than mothers that received no physical stressor (n=13) ($F_{1,9} = 5.68$, $P < 0.05$, Figure 4). This effect was independent of mother's standard length ($F_{1,31} = 1.49$, $P = 0.23$). There was also no effect of days spent in the treatment before being stripped of eggs ($F_{1,31} = 1.00$, $P = 0.33$) or the tank she resided in throughout the experiment ($F_{7,31} = 1.59$, $P = 0.18$) on egg cortisol content.

Oxygen consumption of eggs

Clutches of eggs from mothers exposed to a predator stressor (experimental n=6) consumed more oxygen that control clutches (n=6) soon after fertilization, but the difference between treatments attenuated over time. Regardless of treatment, the oxygen

consumption of eggs (MO_2 in mgO_2/g/hr) steadily increased with hours post fertilization (Table 4, Figure 5).

Juvenile growth

Standard length increased over the course of the experiment (Table 5, Figure 6). Tanks differed in standard length. I did not detect an effect of maternal stress on growth rate or standard length during the period when fry were measured. It is unknown if there were differences in growth rates or standard length before or after I measured the body size of the offspring. I did detect a marginally significant negative effect of density on standard length (Table 5).

Nearest neighbor distance of juveniles

I did not detect a significant difference in nearest neighbor distance between the control and experimental groups immediately following (control mean=8.14, SE=.587, n=33, experimental mean=8.76, SE= .493, n=51) or after (control mean=8.38, SE=.578, n=33, experimental mean=7.73, SE=.452, n=52) the mild disturbance.

However, prior to the mild disturbance, the fish in the experimental group were closer together compared to fish in the control group (control mean=10.53, SE=.799, n=20, experimental mean=9.01, SE=.738, n=38, Table 6, Figure 7). There was not an effect of density, standard length, age or tank on nearest neighbor distance (Table 6).

CHAPTER 4

DISCUSSION

When mothers were exposed to the threat of predation, they produced larger eggs with higher cortisol content, which consumed more oxygen. As juveniles, the offspring of maternally-stressed mothers exhibited tighter shoaling behavior, an antipredator defense. These results show that there can be important consequences of maternal experience for their offspring, and suggest that female sticklebacks might prepare their offspring for survival in a high predation environment, possibly via cortisol.

Consistent with other studies (Bernardo 1996, Taborsky 2006a, 2006b, Taborsky et al 2007, Donelson et al 2008), mothers influenced egg size: experimental mothers had significantly larger eggs than control mothers, regardless of maternal size. Other studies have found positive effects of egg size on feeding (Knutsen and Tilseth 1985), swimming abilities (Ojanguren et al 1996), survival rates in the absence of food (Blaxter 1988), and survival rates in the presence (Lillelund and Lasker 1971) or absence (Henrich 1988) of a predator (Leggett and Deblois 1994) in fry. Indeed, this result challenges the idea that stressors cause females to be unable to produce high-quality eggs, as has been suggested in other studies (Campbell et al 1992, Campbell et al 1994, Contreras-Sanchez et al 1998, McCormick 1998, McCormick 1999). Therefore, my results prompt that the increased egg size might give fry an advantage in a high predation environment (Marshall and Uller 2007).

While treatment caused females to produce larger eggs, surprisingly, there was no effect of treatment on the total number of eggs. It is possible that there are other, unmeasured tradeoffs with egg size, such as a cost to future production or later fecundity

(Fletcher et al 1994, Mayhew 2001). Perhaps experimental females invested all of their resources into their current reproduction since they were living in a dangerous, predator filled environment, and may not have the chance to reproduce again in the future.

Eggs of mothers exposed to a predator stressor had higher concentrations of cortisol than eggs from mothers not exposed to a predator. A similar study on coho salmon found that daily chasing during oogenesis caused elevated concentrations of cortisol in both maternal plasma and eggs (Stratholt et al 1997). These results, along with other studies, have shown strong positive relationships between maternal and egg cortisol levels in unfertilized (Hwang et al 1992, Simontacchi et al 2009) and fertilized eggs (De Jesus et al 1991, De Jesus and Hirano 1992). The amount of time that a female was exposed to predator-induced stress had no effect on cortisol concentrations, suggesting that females did not acclimate to the repeated predator stressor.

Oxygen consumption of the eggs increased over time, regardless of treatment. This finding is supported by various studies showing metabolic rates of eggs increase over ontogeny (Berg et al 2001), most likely due to growth and increased embryonic activity (Pakkasmaa et al 2006). Soon after fertilization, eggs from experimental mothers consumed more oxygen. The larger size of experimental eggs likely did not affect the increased oxygen consumption as mass-specific metabolic rates decline with increasing body mass (Makarieva et al 2005). Therefore, I suggest that the mechanism behind this increase in oxygen consumption is due to the direct effect of increased cortisol, as cortisol increases metabolic rates in individuals (Chan and Woo 1978, Barton and Schreck 1987, Wendelaar Bonga 1997) to help evade predation risks. In addition, the attenuating difference between experimental and control clutches prior to hatching could

reflect buffering mechanisms in the egg that develop soon before hatching (Paitz and Bowden 2009), but should be further explored.

I did not detect an effect of treatment on the growth of fry between three to ~ six months of age. I am cautious about interpreting the significance of this result because it is likely that if there was an effect of treatment on offspring growth, it is likely that it would be most evident very early in life, when the fish were too small to be measured reliably and noninvasively. In addition, although I did not consistently record hatching time, anecdotally I observed that experimental clutches tended to hatch faster (~5 days) than control clutches (~6 days). Therefore I suspect that the experimental fry might have hatched earlier, or may have been larger soon after hatching. This is a promising area for future investigation.

Fry from experimental mothers had smaller nearest neighbor distances than fry from control mothers prior to a mild disturbance, regardless of size. This behavior is representative of shoaling behavior, an anti-predator strategy (Magurran 1990, Queiroz and Magurran 2005), where individuals hide behind others to reduce the chance of being caught by a predator (Hamilton 1971). Shoaling also "dilutes" the risk of predation by lessening the probability of running into or becoming the singled out prey (Turner and Pitcher 1986), allows for enhanced coordination among individuals (Pitcher and Parrish 1993), and provides early response to potential threats (Bertram 1980).

These nearest neighbor results give further support for the hypothesis that mothers detected environmental cues of predation and manipulated the behavior of their offspring to be better adapted for their future environment. Guppies originating from high predation streams showed higher levels of grouping than those from low predation

streams (Seghers 1974), and prenatal stress has led to mutual clinging, closer proximity to others during stressful conditions, and the avoidance of open locations (Clarke and Schneider 1993, Koffman 2002).

Although there was a significant treatment effect on distance between individuals prior to the disturbance, there was no difference immediately following or after a mild disturbance. However, regardless of maternal treatment, distance between fish decreased after fish were lightly moved by tubing and after given a time to rest in response to this move. This suggests that all fry, not just fry from experimental mothers, reacted to the mild disturbance by moving closer together.

The transmission of predator response behavior from mothers to offspring is exemplary of maternal programming. Anti-predator behavior is epigenetically inherited by offspring in various organisms, including snowshoe hare (Sheriff et al 2010), field crickets (Storm and Lima 2010), and phantom midges (Agrawal et al 1999). However, these defensive responses to predation risk are not the only behavioral responses mothers are able to program. Maternal licking and grooming influenced the development of the HPA axis, producing a reduced stress response and differences in DNA methlyation, as well as increased licking and grooming behaviors in offspring for generations to follow (Meaney 2001, Weaver et al 2004). These studies show that maternal experience can be inherited by offspring and can result in adaptive responses across multiple generations. This is important as stressors early in life can be transmitted to future generations through changes in maternal behavior and maternal effect.

In summary, cortisol concentrations in eggs, egg size, metabolic rates, and behavior of prenatally stressed individuals were affected by the experience of the mother.

To my knowledge, no previous work has looked at the effects of prenatal stress on so many aspects of the development of progeny in fish, especially through ecologically relevant means. The findings from this study suggest that maternal effects may be in response to predation and prepare their offspring for their future environment, and has important implications for many species, including humans. Mothers exposed to famine, an environmental stressor, during pregnancy, produced smaller offspring which were more susceptible to disease and health problems, and this effect was passed down for generations (Painter et al 2005). This suggests an epigenetic transfer of maternal experience on her progeny, and future research needs to investigate how these maternal effects persist in progeny. If females detect environmental cues of a given predator, then mothers may manipulate the development, growth, and behavior of her offspring to match their future environment, and these mechanisms leave plenty to be discovered.

CHAPTER 5

REFERENCES

References are in *Journal of Experimental Biology* Format

Agrawal, A.A., Laforsch, C. and Tollrian, R. (1999). Transgenerational induction of defenses in animals and plants. *Nature*. 401, 60-63

Agrawal, A. A. (2002). Herbivory and maternal effects: mechanisms and consequences of transgenerational induced plant resistance. *Ecology* 83, 3408-3415.

Alvarez, D. and Metcalfe, N. B. (2005). Catch-up growth and swimming performance in threespine sticklebacks (*Gasterosteus aculeatus*): seasonal changes in the cost of compensation. *Can. J. Fish. Aquat. Sci*. 62, 2169-2176.

Auperin, B. and Geslin, M. (2008). Plasma cortisol response to stress in juvenile rainbow trout is influenced by their life history during early development and by egg cortisol content. *Gen. Comp. Endocrinol*. 158, 234-239.

Azuma, T. and Iwata, M. (1994). Influences of illumination intensity on the nearest neighbor distance in Coho salmon *Oncorhynchus kisutch*. *J. Fish. Biol*. 45, 1113-1118.

Barry, T.P., Malison, J.A., Held, J.A. and Parrish, J.J. (1995). Ontogeny of the cortisol stress response in larval rainbow trout. *Gen. Comp. Endocrinol*. 97, 57-65.

Barton, B. A. and Schreck, C. B. (1987). Metabolic cost of acute physical stress in juvenile steelhead. *Trans. Am. Fish. Soc*. 116, 257-263.

Bell, A. M., Backstrom, T., Huntingford, F. A., Pottinger, T. G. and Winberg, S. (2007). Variable neuroendocrine responses to ecologically-relevant challenges in sticklebacks. *Physiol. Behav*. 91, 15-25.

Bell, A. M., Henderson, L. and Huntingford, F. A. (2010). Behavioral and respiratory responses to stressors in multiple populations of three-spined sticklebacks that differ in predation pressure. *J. Comp. Physiol. B. Biochem. Syst. Environ. Physiol*.180, 211-220.

Berg, O. K., Hendry, A. P., Svendsen, B., Bech, C., Arnekleiv, J. V. and Lohrmann, A. (2001). Maternal provisioning of offspring and the use of those resources during ontogeny: variation within and between Atlantic salmon families. *Funct. Ecol*. 15, 13-23.

Bernardo, J. (1996). Maternal effects in animal ecology. *Am. Zool*. 36, 83-105.

Blaxter, J.H.S. (1988). Pattern and variety of development. In *Fish physiology (*eds. W.S. Hoar and D.J. Randall) pp. 177-252. New York: Academic Press.

Blount, J. D., Surai, P. F., Nager, R. G., Houston, D. C., Moller, A. P., Trewby, M. L. and Kennedy, M. W. (2002). Carotenoids and egg quality in the lesser black-backed gull Larus *fuscus*: a supplemental feeding study of maternal effects. *Proc. Roy. Soc. Lond. B. Biol. Sci.* 269, 29-36.

Braastad, B.O. (1998). Effects of prenatal stress on behaviour of offspring of laboratory and farmed mammals. *Appl. Anim. Behav. Sci.* 61, 159–180.

Campbell, P. M., Pottinger, T. G. and Sumpter, J.P. (1992). Stress reduces the quality of gametes produced by rainbow trout. *Biol. Reprod.* 47, 1140-1150.

Campbell, P.M., Pottinger, T.G. and Sumpter, J.P. (1994). Preliminary evidence that chronic confinement stress reduces the quality of gametes produced by brown and rainbow trout. *Aquaculture* 120,151–169.

Chambers, R. C. and Leggett, W. C. (1996). Maternal influences on variation in egg sizes in temperate marine fishes. *Am. Zool.* 36, 180-196.

Chan, D. K. O. and Woo, N. Y. S. (1978). Effect of cortisol on metabolism of eel, *Anguilla japonica*. *Gen. Comp. Endocrinol.* 35, 205-215.

Clarke, A. S. and Schneider, M. L. (1993). Prenatal stress has long-term effects on behavioral responses to stress in juvenile rhesus monkeys. *Dev. Psychobiol.* 26, 293-304.

Contreras-Sanchez, W. M., Schreck, C. B., Fitzpatrick, M. S. and Pereira, C. B. (1998). Effects of stress on the reproductive performance of rainbow trout (*Oncorhynchus mykiss*). *Biol. Reprod.* 58, 439-447.

De Jesus, E.G., Hirano, T. and Inui, Y. (1991). Changes in cortisol and thyroid hormone concentrations during early development and metamorphosis in Japanese Flounder, *Paralichthys olivaceus*. *Gen. Comp. Endocrinol.* 82, 369-376.

De Jesus, E.G. and Hirano, T. (1992). Changes in whole body concentrations of cortisol, thyroid hormones, and sex steroids during early development of the chum salmon, *Oncorhynchus keta*. *Gen. Comp. Endocrinol.* 85, 55-61.

Donelson, J. M., McCormick, M. I. and Munday, P. L. (2008). Parental condition affects early life-history of a coral reef fish. *J. Exp. Mar. Biol. Ecol.* 360, 109-116.

Einum, S. and Fleming, I. A. (2002). Does within-population variation in fish egg size reflect maternal influences on optimal values? *Am. Nat.* 160, 756-765.

Eriksen, M.S., Bakken, M., Espmark, A., Braastad, B.O. and Salte, R. (2006). Pre-spawning stress in farmed Atlantic salmon, *Salmo salar*: maternal cortisol exposure and hyperthermia during embryonic development affect offspring survival, growth and incidence of malformations. *J. Fish Biol*. 69, 114–129.

Eriksen, M. S., Espmark, A., Braastad, B. O., Salte, R. and Bakken, M. (2007). Long-term effects of maternal cortisol exposure and mild hyperthermia during embryogeny on survival, growth and morphological anomalies in farmed Atlantic salmon *Salmo salar* offspring. *J. Fish. Biol*. 70, 462-473.

Espmark, A. M., Eriksen, M. S., Salte, R., Braastad, B. O. and Bakken, M. (2008). A note on pre-spawning maternal cortisol exposure in farmed Atlantic salmon and its impact on the behaviour of offspring in response to a novel environment. *Appl. Anim. Behav. Sci*. 110, 404-409.

Feist**,** G. and Schreck, C. B. (2001). Ontogeny of the stress response in chinook salmon, *Oncorhynchus tshawytscha*. *Fish. Physiol. Biochem*. 25, 31-40.

Fletcher, J. P., Hughes, J. P. and Harvey, I. F. (1994). Life expectancy and egg load affect oviposition decisions of a solitary parasitoid. *Proc. Roy. Soc. Lond. B. Biol. Sci*. 258, 163-167.

Fox, C. W. (1997). The ecology of body size in a seed beetle, *Stator limbatus*: Persistence of environmental variation across generations? *Evolution* 51, 1005-1010.

Galloway, L. F. 1995. Response to natural environmental heterogeneity: Maternal effects and selection on life-history characters and plasticities in *Mimulus guttatus*. *Evolution* 49, 1095-1107.

Gil, D. (2008). Hormones in avian eggs: Physiology, ecology and behavior. *Adv. Stud. Behav*. 38, 337-398.

Giles, N. and Huntingford, F. A. (1984). Predation risk and inter-population variation in anti-predator behavior in the 3-spined stickleback, *Gasterosteus aculeatus*. *Anim. Behav*. 32, 264-275.

Gingerich, A. J., Philipp, D. P. and Suski, C. D. (2010). Effects of nutritional status on metabolic rate, exercise and recovery in a freshwater fish. *J. Comp. Physiol. B. Biochem. Syst. Environ. Physiol*. 180, 371-384.

Green, B. S. and McCormick, M. I. (2005). Maternal and paternal effects determine size, growth and performance in larvae of a tropical reef fish. *Mar. Ecol. Progr*. 289, 263-272.

Groothuis, T.G. and Schwabl, H. (2007). Hormone-mediated maternal effects in birds: mechanisms matter but what do we know of them? *Philos. Trans. R. Soc. Lond., B*. 363, 1647-1661.

Hamilton, W. D. (1971). Geometry for selfish herd. *J. Theor. Biol*. 31, 295-311.

Heath, D.D. and Blouw, D.M. (1998). Maternal effects in fish: are they adaptive or merely physiological side-effects? In *Adaptive maternal effects*. (eds. T.A. Mousseau and C.W. Fox). pp. 178-201. Oxford: Oxford Univ. Press.

Heath, D. D., Fox, C. W. and Heath, J. W. (1999). Maternal effects on offspring size: Variation through early development of chinook salmon. *Evolution* 53, 1605-1611.

Henrich, S. (1988). Variation in offspring sizes of the poeciliid fish *Heterandria formosa* in relation to fitness. *Oikos* 51, 13-18.

Hofer, H. and East, M. L. (2003). Behavioral processes and costs of co-existence in female spotted hyenas: a life history perspective. *Evol. Ecol*. 17, 315-331.

Huntingford, F. A., Lazarus, J., Barrie, B. D. and Webb, S. (1994). A dynamic analysis of cooperative predator inspection in sticklebacks. *Anim. Behav* 47, 413-423.

Hwang, P., Wu, S., Lin, J. and Wu. L. (1992). Cortisol content of eggs and larvae of teleosts. *Gen. Comp. Endocrinol*. 86, 189-196.

Knutsen, G. M. and Tilseth, S. (1985). Growth, development, and feeding success of Atlantic cod larvae *Gadus morhua* related to egg size. *Trans. Am. Fish. Soc*. 114, 507-511.

Leggett, W. C. and Deblois, E. (1994). Recruitment in marine fishes: Is it regulated by starvation and predation in the egg and larval stages. *Neth. J. Sea. Res*. 32, 119-134.

Lillelund, K. and Lasker, R. (1971). Laboratory studies of predation by marine copepods on fish larvae. *Fish. Bull. U.S*. 69, 655-667.

Madsen, T. and Shine, R. (2000). Silver spoons and snake body sizes: prey availability early in life influences long-term growth rates of free-ranging pythons. *J. Anim. Ecol*. 69, 952-958.

Magurran, A. E. (1990). The inheritance and development of minnow anti-predator behavior. *Anim. Behav*. 39, 834-842.

Makarieva, A. M., Gorshkov, V. G. and Li, B. L. (2005). Energetics of the smallest: do bacteria breathe at the same rate as whales? *Proc. Roy. Soc. Lond. B. Biol. Sci.*272, 2219-2224.

Marshall, D. J. and Uller, T. (2007). When is a maternal effect adaptive? *Oikos* 116, 1957-1963.

Masuda, R., Shoji, J., Nakayama, S. and Tanaka, M. (2003). Development of schooling behavior in Spanish mackerel, *Scomberomorus niphonius,* during early ontogeny. *Fish. Sci.* 69, 772-776.

Mather, K. and Jinks, J.L. (1971) *Biometrical genetics*. Ithaca, New York: Cornell University Press.

Matthews, S. G. and Challis, J. R. G. (1996). Regulation of the hypothalamo-pituitary-adrenocortical axis in fetal sheep. *Trends. Endocrinol. Metabol.* 7, 239-246.

Mayhew, P. J. and Glaizot, O. (2001). Integrating theory of clutch size and body size evolution for parasitoids. *Oikos* 92, 372-376.

McCormick, M.I. (1999). Experimental test of the effect of maternal hormones on larval quality of a coral reef fish. *Oecologia* 118, 412-422.

McCormick, M.I. (1998). Behaviourally induced maternal stress in a fish influences progeny quality by a hormonal mechanism. *Ecology* 79, 1873–1883.

Meaney, M. J. (2001). Maternal care, gene expression, and the transmission of individual differences in stress reactivity across generations. *Annu. Rev. Neurosci.* 24, 1161-1192.

Mousseau, T. A. and Dingle, H. (1991). Maternal effects in insect life histories. *Annu. Rev. Entomol.* 36, 511-534.

Mousseau, T. A. and Fox, C. W. (1998). The adaptive significance of maternal effects. *Trends. Ecol. Evol.* 13, 403-407.

Naumenko, E. V. (1991). Modification in early ontogeny of the stress response of adults. *News. Physiol. Sci.* 6, 219-223.

Ojanguren, A. F., ReyesGavilan, F. G. and Brana, F. (1996). Effects of egg size on offspring development and fitness in brown trout, *Salmo trutta. Aquaculture* 147, 9-20.

Painter, R.C., Roseboom, T.J. and Bleker, O.P. (2005). Prenatal exposure to the Dutch Famine and disease in later life: an overview. *Reprod Toxicol.* 20, 345-352

Paitz, R. T. and Bowden, R. M. (2009). Rapid decline in the concentrations of three yolk steroids during development: Is it embryonic regulation? *Gen. Comp. Endocrinol.* 161, 246-251.

Pakkasmaa, S., Penttinen, O. P. and Piironen, J. (2006). Metabolic rate of Arctic charr eggs depends on their parentage. *J. Comp. Physiol. B. Biochem. Syst. Environ. Physiol.* 176, 387-391.

Pottinger, T. G., Carrick, T. R. and Yeomans, W. E. (2002). The three-spined stickleback as an environmental sentinel: effects of stressors on whole-body physiological indices. *J. Fish. Biol.* 61, 207-229.

Queiroz, H. and Magurran, A. E. (2005). Safety in numbers? Shoaling behaviour of the Amazonian red-bellied piranha. *Biol. Lett.* 1, 155-157.

Reimchen, T. E. (1994). Predators and morphological evolution in threespine stickleback. In *The evolutionary biology of the threespine stickleback*. (eds. M.A. Bell and S.A. Foster). pp. 240-273. Oxford: Oxford.

Reznick, D.N. (1991). Maternal effects in fish life histories. In *The unity of evolutionary biology*. (ed. E.C. Dudley). pp. 780-793. Portland, OR: Dioscorides Press.

Sapolsky, R. M. and Meaney, M. J. (1986). Maturation of the adrenocortical stress response: Neuroendocrine control mechanisms and the stress hyporesponsive period. *Brain. Res. Rev.* 11, 65-76.

Schreck, C. B., Contreras-Sanchez, W. and Fitzpatrick, M. S. (2001) Effects of stress on fish reproduction, gamete quality, and progeny. *Aquaculture* 197, 3-24.

Seghers, B. H. (1974). Schooling behavior in guppy (*Poecilia reticulate*): Evolutionary response to predation. *Evolution* 28, 486-489.

Sheriff, M.J., Krebs, C.J. and Boonstra, R. (2010). The ghosts of predators past: population cycles and the role of maternal programming under fluctuating predation risk. *Ecology e-View*.

Simontacchi, C., Negrato, E., Pazzaglia, M., Bertotto, D., Poltronieri, C. and Radaelli, G. (2009). Whole-body concentrations of cortisol and sex steroids in white sturgeon (*Acipenser transmontanus*, Richardson 1836) during early development and stress response. *Aquaculture. Int.* 17, 7-14.

Sims, C. G. and Holberton, R. L. (2000). Development of the corticosterone stress response in young Northern Mockingbirds (*Mimus polyglottos*). *Gen. Comp. Endocrinol.* 119, 193-201.

Spicer, J. I. and Gaston, K. J. (1999). Amphipod gigantism dictated by oxygen availability? *Ecol. Lett.* 2, 397-401.

Steffensen, J. F. (1989). Some errors in respirometry of aquatic breathers: how to avoid and correct them. *Fish. Physiol. Biochem.* 6, 49-59.

Storm, J.J. and Lima, S.L. (2010). Mothers forewarn offspring about predators: A transgenerational maternal effect on behavior. *Am. Nat.* 175, 382-390.

Stratholt, M.L., Donaldson, E.M. and Liley, N.R. (1997). Stress induced elevation of plasma cortisol in adult female coho salmon (*Oncorhynchus kisutch*), is reflected in egg cortisol content, but does not appear to affect early development. *Aquaculture* 158, 141-153.

Taborsky, B. (2006a). Mothers determine offspring size in response to own juvenile growth conditions. *Biol. Lett.* 2, 225-228.

Taborsky, B. (2006b). The influence of juvenile and adult environments on life-history trajectories. *Proc. Roy. Soc. Lond. B. Biol. Sci.*273, 741-750.

Taborsky, B., Skubic, E. and Bruintjes, R. (2007). Mothers adjust egg size to helper number in a cooperatively breeding cichlid. *Behav. Ecol.* 18, 652-657.

Talge, N. M., Neal, C. and Glover, V. (2007). Antenatal maternal stress and long-term effects on child neurodevelopment: how and why? *J. Child. Psychol. Psychiatr.* 48, 245-261.

Turner, G.F. and Pitcher, T.J. (1986). Attack abatement: a model for group protection by combined avoidance and dilution. *Am. Nat.* 128, 228–240.

Vines, T. H. and Schluter, D. (2006). Strong assortative mating between allopatric sticklebacks as a by-product of adaptation to different environments. *Proc. Roy. Soc. Lond. B.* 273, 911–916.

Weaver, I. C. G., Cervoni, N., Champagne, F. A., D'Alessio, A. C., Sharma, S., Seckl, J. R., Dymov, S., Szyf, M. and Meaney, M. J. (2004). Epigenetic programming by maternal behavior. *Nat. Neurosci.* 7, 847-854.

Wendelaar Bonga, S. E. (1997). The stress response in fish. *Physiol. Rev.* 77, 591-625.

Willmer, P., Stone, G. and Johnston, I. (2004). *Environmental physiology of animals*. London: Blackwell.

CHAPTER 6

FIGURES

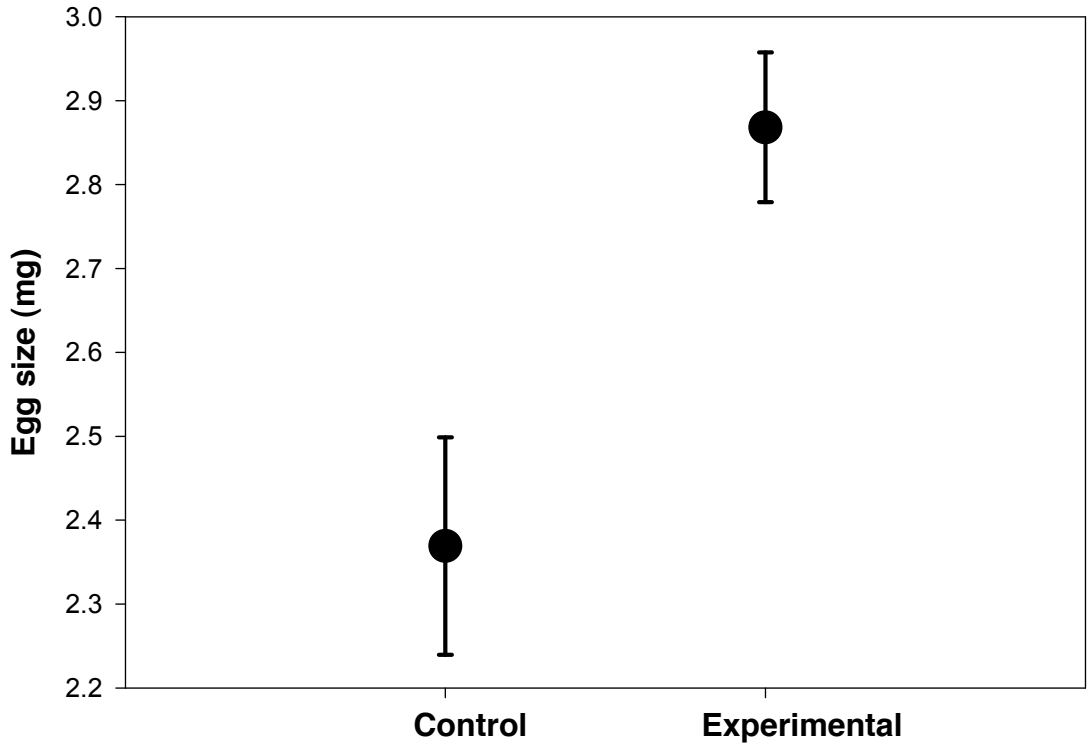

Figure 1: Size of unfertilized three-spined stickleback eggs (mg) from experimental and control mothers were measured. Egg size from clutches of mothers exposed to a predator stressor (experimental, n=30) was significantly larger than eggs from clutches of mothers not exposed to a predator stressor (control, n=15). Values show mean ± SE.

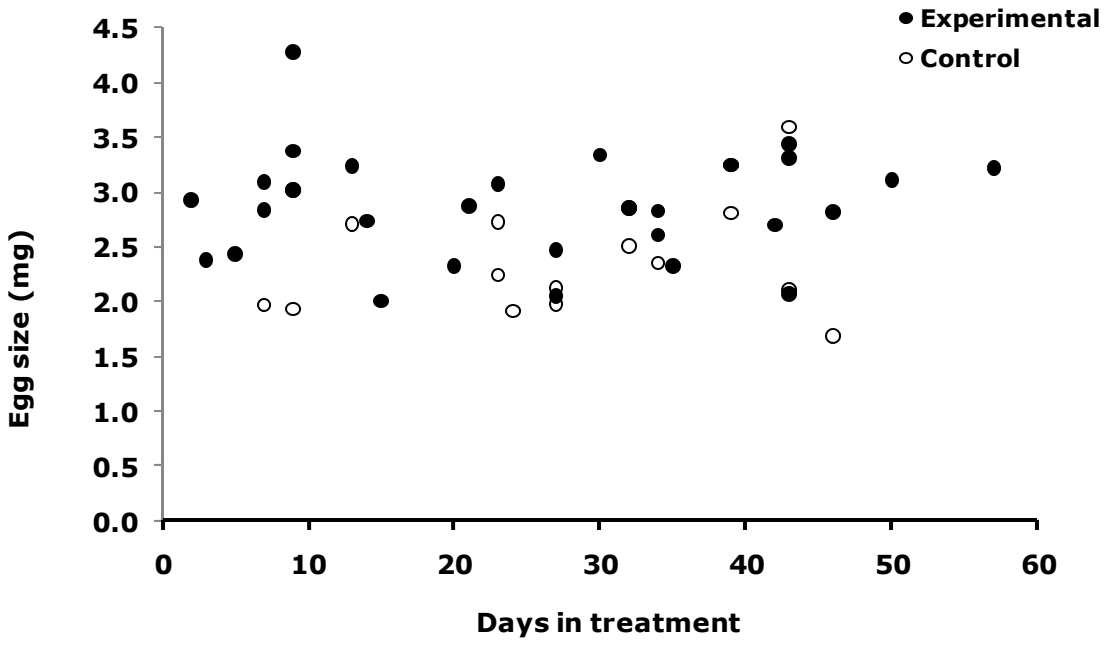

Figure 2: Size of unfertilized three-spined stickleback eggs (mg) were measured in relation to amount of time mothers spent in treatment. Size of eggs was not affected by number of days mothers spent in treatment.

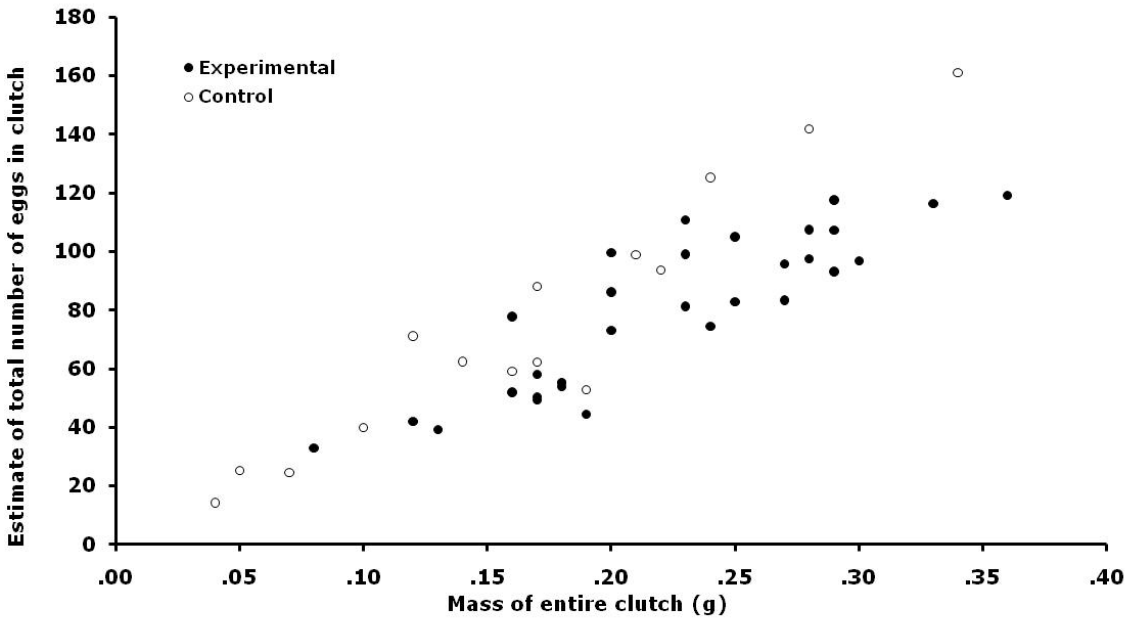

Figure 3: Number of unfertilized three-spined stickleback eggs and total clutch mass (mg) from experimental and control mothers was measured. Estimated number of eggs did not significantly differ between clutches from mothers exposed (experimental, n=30) and not exposed (control, n=15) to a predator stressor. However, total clutch mass was larger in clutches from experimental mothers; note fewer filled circles on left hand side of graph.

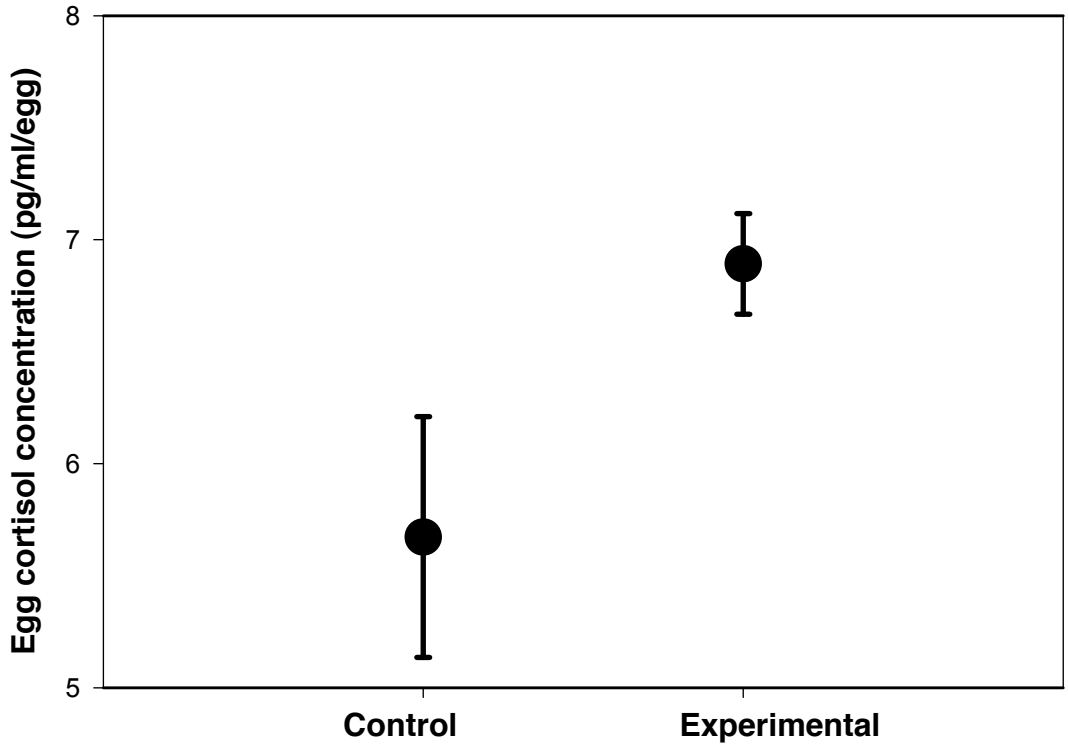

Figure 4: Unfertilized three-spined stickleback mothers were exposed to a predation risk and cortisol concentrations (pg/ml/egg) in their eggs were measured. Cortisol concentrations in eggs from mothers exposed to a predator stressor (experimental, n=30) were significantly higher than eggs from mothers not exposed to a predator stressor (control, n=13). Values show means ± SE.

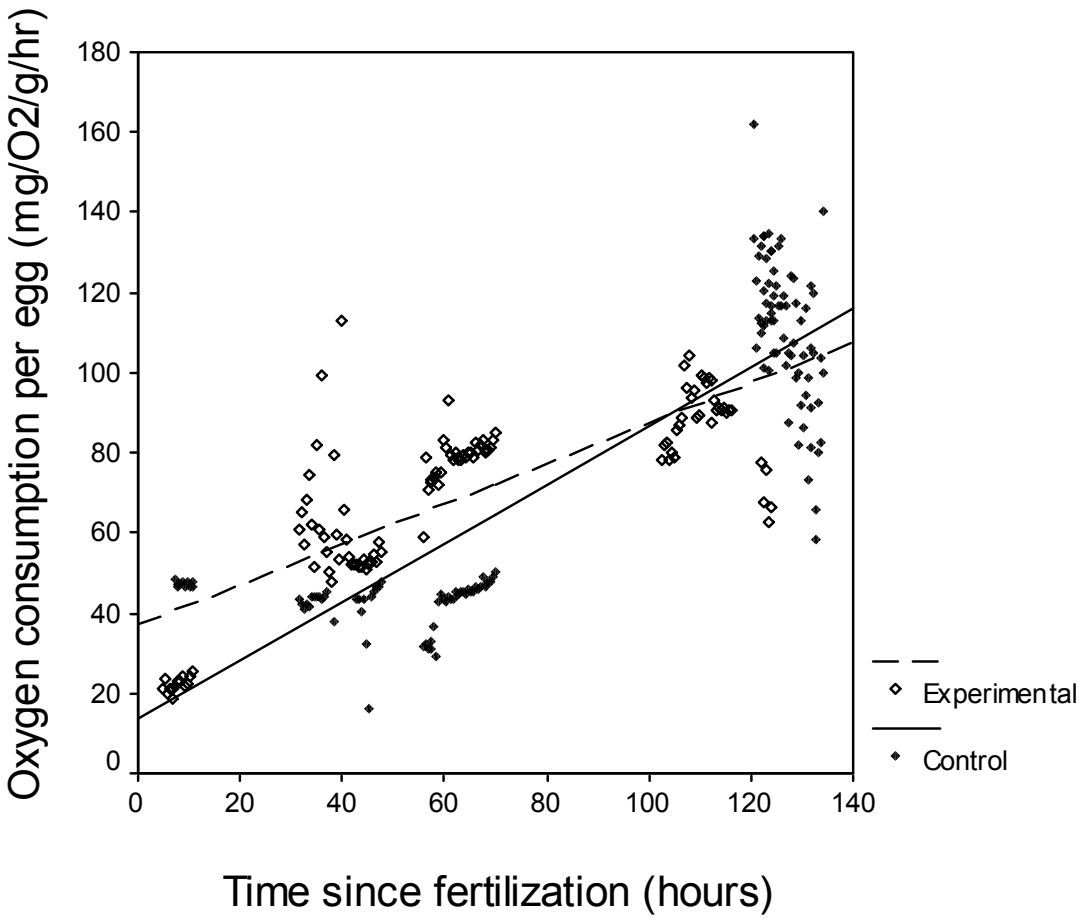

Figure 5: Oxygen consumption (mg/O2/g/hr) of fertilized threespined stickleback eggs from experimental and control mothers was measured. Oxygen consumption of eggs increased over time regardless of treatment. Eggs from mothers exposed to a stressor (experimental, n=6) consumed more oxygen than eggs from mothers not exposed to a predator stressor (control, n=6) soon after fertilization, but the difference attenuated close to hatching.

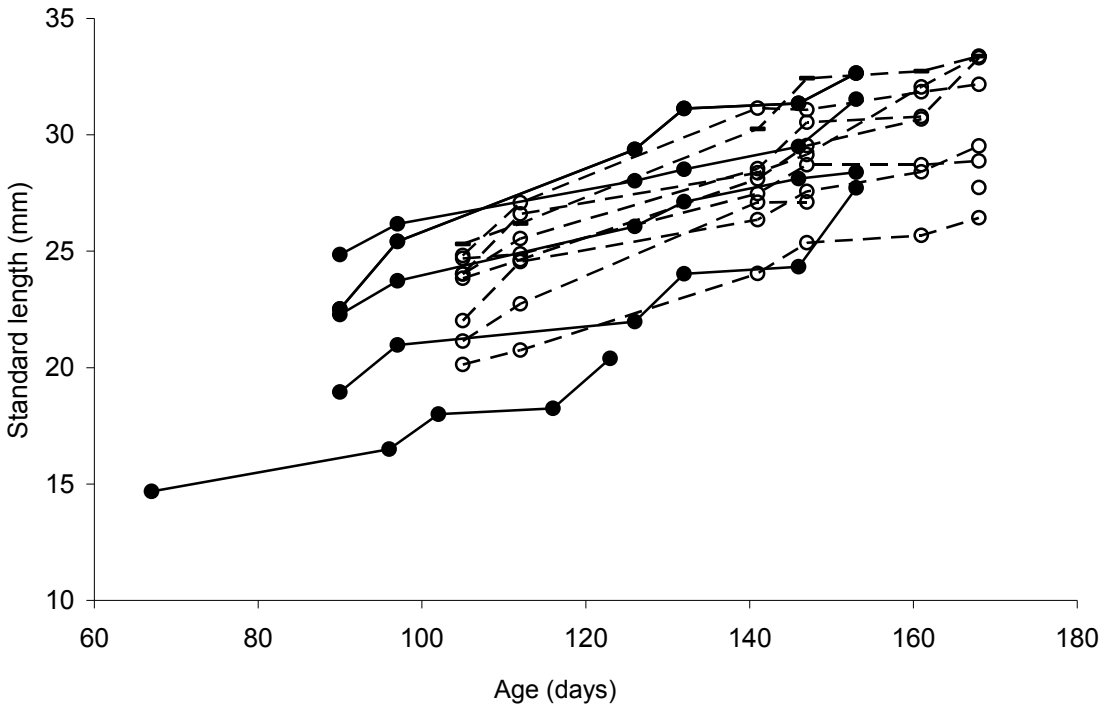

Figure 6: Standard length (mm) of threespined stickleback juveniles was measured, regardless of maternal treatment. Standard length increased over time for all individuals (n=75), regardless of whether mothers were exposed to a predator or not (experimental or control). Standard length also differed according to tank.

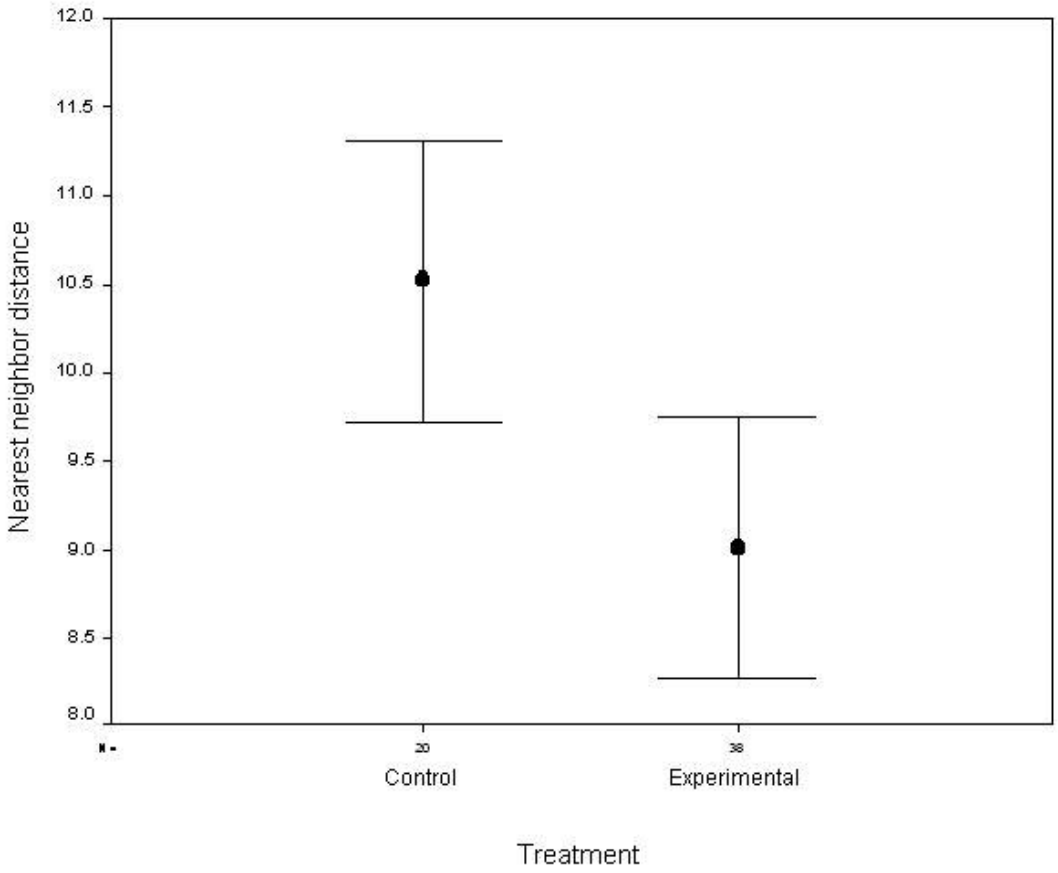

Figure 7 A: Shoaling behavior, an antipredator response, was measured from photographs, and was estimated as the nearest neighbor distance between individual threespined sticklebacks in each tank. Figure A: Nearest neighbor distance before experiencing a mild disturbance was significantly closer for fry from mothers exposed to a predator stressor (experimental, n=45) than from mothers not exposed to a predator stressor (control, n=30). Values show means ± SE.

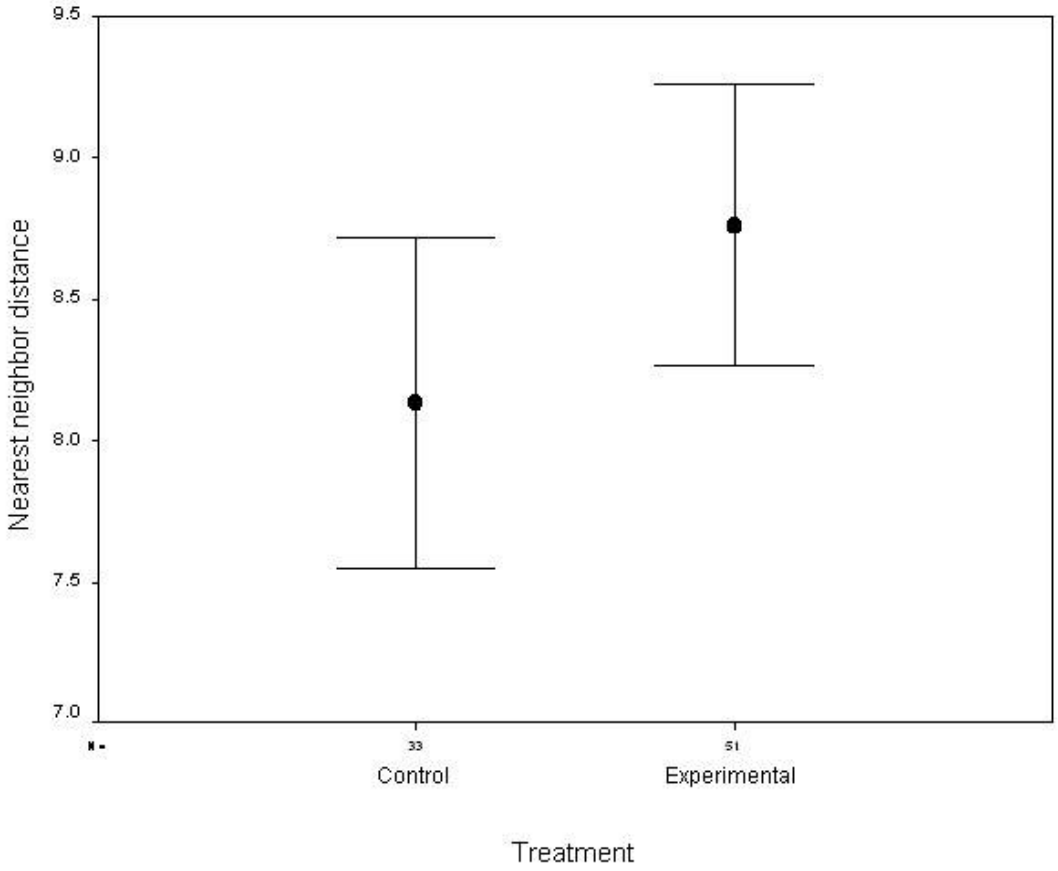

Figure 7 (cont.) B: Shoaling behavior, an antipredator response, was measured from photographs, and was estimated as the nearest neighbor distance between individual threespined sticklebacks in each tank. Figure B: Nearest neighbor distance immediately following a mild disturbance was not significantly closer for fry from mothers exposed to a predator stressor (experimental, n=45) than from mothers not exposed to a predator stressor (control, n=30). Values show means ± SE.

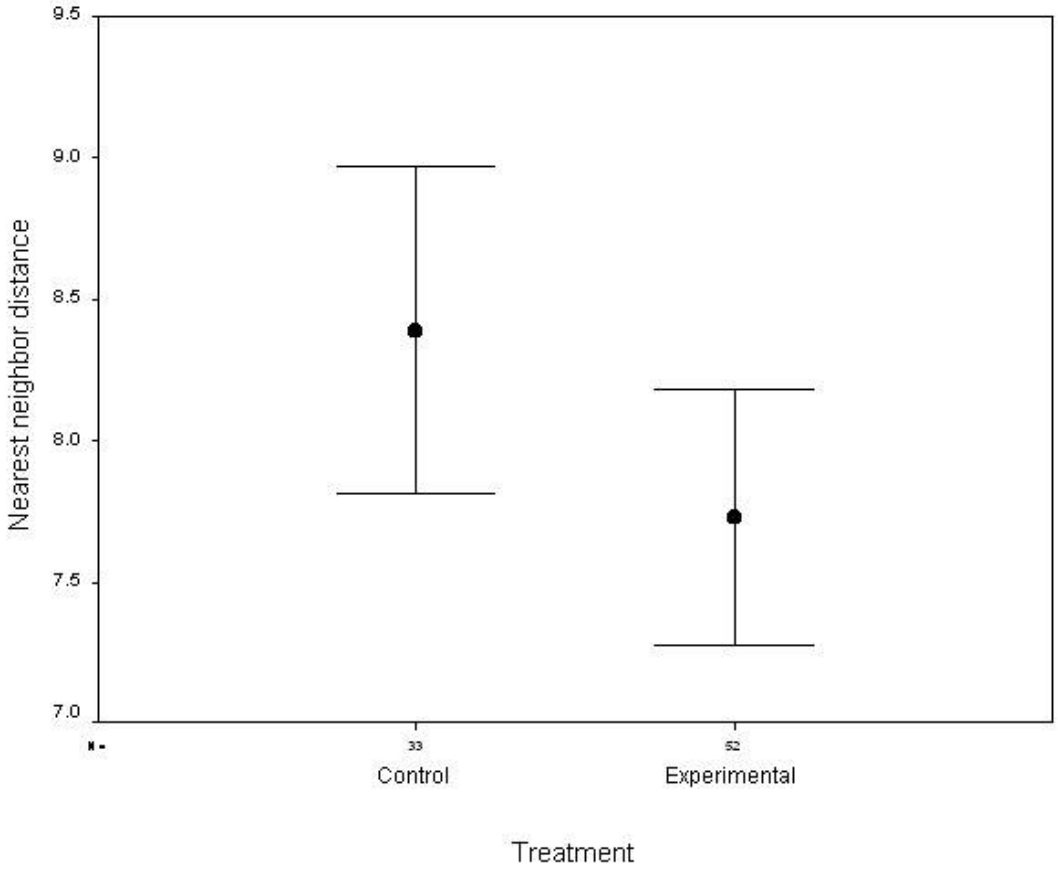

Figure 7 (cont.) C: Shoaling behavior, an antipredator response, was measured from photographs, and was estimated as the nearest neighbor distance between individual threespined sticklebacks in each tank. Figure C: Nearest neighbor distance after a mild disturbance was not significantly closer for fry from mothers exposed to a predator stressor (experimental, n=45) than from mothers not exposed to a predator stressor (control, n=30). Values show means ± SE.

CHAPTER 7

TABLES

	df	*MS*	*F*	*P*
Female standard length	1,34	0	4.9	.033
Days in treatment	1,34	0	0	.978
Treatment	1,12	0	5.5	.038
Tank(Treatment)	7,34	0	.7	.686
Error	34	0		

Table 1: ANOVA table of between-subject effects on clutch mass

	df	*MS*	*F*	*P*
Female standard length	1,34	0.4	1.5	.231
Days in treatment	1,34	.1	.4	.526
Treatment	1,11	2.7	12.6	.005
Tank(Treatment)	7,34	.2	.8	.595
Error	34	.3		

Table 2: ANOVA table of between-subject effects on egg size

	df	*MS*	*F*	*P*
Female standard length	1,34	7118.4	6.8	.013
Days in treatment	1,34	29.4	0	.868
Treatment	1,15	1.6	0	.958
Tank(Treatment)	7,34	447.6	.4	.877
Error	34	1043.5		

Table 3: ANOVA table of between-subject effects on number of eggs

	df	*MS*	*F*	*P*
Time since fertilization	1,241	1108.1	9.7	0.002
Treatment	1,186	2439.6	18.1	0.000
Clutch(Treatment)	10,241	4345.2	38.1	0.000
Treatment*Time since fertilization	1,241	1191.7	10.4	0.001
Error	241	114.1		

Table 4: ANOVA table of the between-subject effects on oxygen consumption per egg

	df	*MS*	*F*	*P*
Density	1,69	2.7	3.3	0.072
Age	1,69	494.2	618.4	0.000
Treatment	1,69	0.9	0.8	0.373
Tank(Treatment)	13,69	30.0	37.5	0.000
Treatment*Age	1,69	1.5	1.9	0.172
Error	69	0.8		

Table 5: Anova table of the between-subject effects on length of fry

	df	*MS*	*F*	*P*
Density	1,40	31.2	2.0	0.163
Age	1,40	9.1	0.6	0.448
Standard length	1,40	.9	0.1	0.815
Treatment	1,51	68.7	4.5	0.038
Tank(Treatment)	13,40	13.8	0.9	0.565
Error	40	15.4		

Table 6: Anova table of the between-subject effects on nearest neighbor distance