Variability in Egg Hatching Time as Evidence of a Bet-Hedging Strategy in Japanese Medaka (*Oryzias latipes)*

by

Kelsey L. Mercer

A Thesis Submitted to
Saint Mary's University, Halifax, Nova Scotia
In Partial Fulfillment of the Requirements for
the Degree of Bachelor of Science with Honours

April, 2021, Halifax, Nova Scotia

Approved: _____
Dr. Laura Weir
Supervisor

Approved: _____
Dr. David Dansereau
Examiner

Date: April 27th, 2021

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Abstract

Understanding life-history strategies is essential for developing a clear picture of how organisms have evolved and how they may perform under future conditions. Bet-hedging is a life-history strategy that allows populations to persist in unpredictable, variable conditions. It is a risk-reduction strategy that lowers generational fitness while decreasing fitness variability across generations, enabling lineage survival when conditions are not optimal. As such, it may be a favourable life-history strategy as conditions become increasingly variable due to climate change. Japanese medaka *(Oryzias latipes*) is a small fish that lives in an extremely variable habitat in the wild. It is commonly used as a model organism for toxicology, oncology, and developmental studies. A robust understanding of their life-history parameters is therefore necessary to determine the effects of different treatments in these studies. These fish demonstrate significant variability in egg hatching times, which does not appear to be influenced by external conditions. The goal of this study was to quantify within-clutch and within-population variability and to determine whether both were consistent across different environments, which would support a bet-hedging theory in medaka egg hatching time. For this study I isolated clutches and determined the hatching dates of the eggs to quantify variability. 24 experimental tanks contained four operational sex ratios (OSR; sexually active males:sexually active females), which allowed for comparison of higher-stress vs lower-stress environments. Maternal size and clutch size were also considered. The observed similarity in variability across all treatments provides support for a bet-hedging theory.

April, 2021

Table of Contents

Acknowledgments

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Introduction

1.1 What is a Bet-Hedging Strategy?

Understanding how organisms evolve to react to changes in their environment has always been important, as the natural world is a stochastic place. It is increasingly critical to learn how organisms will adapt to unpredictable conditions in the face of climate change, which introduces novel variability to natural environments (Simons, 2011; Haaland *et al.*, 2018). Life-history traits such as how and when an organism reproduces, develops, matures, and dies are fundamental to fitness. These traits determine the organism's overall reproductive success throughout its life (Flatt & Heyland, 2011). Evolutionary strategies for life-history traits are adopted over large time scales in response to climactic conditions, predation pressures, and resource availability, among other factors (Haaland *et al.* 2018; Bruijning *et al.* 2019). The strategies that are selected for with respect to life-history traits increase reproductive success over these larger timescales, and they should evolve to increase the fitness of a lineage (Haaland *et al.* 2018).

One of these evolutionary strategies is bet-hedging. In organisms that adopt a bet-hedging strategy there is increased variance in life history traits (when compared to a strategy such as canalization, in which stabilizing selective pressures promote one optimal phenotype; Lips, 2001). Bet-hedging is typically adopted for traits related to reproduction and development; for example, variability in seed germination time, clutch size, and egg size. Bet-hedging develops in unstable environments that have unpredictable conditions (Lips, 2001; Haaland *et al.* 2018; Haaland *et al.* 2019). Because it is an evolutionary strategy that affects life history traits, selection acts on these traits over a large time scale in environments that have random fluctuations in their conditions to produce a bet-hedging response.

In a bet-hedging strategy many different phenotypes are expressed within a generation (e.g., large eggs, moderately sized eggs, and small eggs; Philippi & Seger, 1989). This phenotypic diversification is seen on an individual level – all potential phenotypes may be expressed by the offspring of one individual, and, when applicable, within one reproductive event (Philippi & Seger, 1989). Following the example above, this means that one individual, if using a bet-hedging strategy, would lay large, moderate, and small eggs all within the same generation (across reproductive events), or within the same clutch (within reproductive events).

Bet-hedging is beneficial in environments that do not have predictable conditions; maximum offspring survival is not expected, but some offspring should survive under any potential condition due to the diversity of phenotypes among the offspring (Lips, 2001; Bruijning *et al.* 2019). Thus, bet-hedging is a risk reduction strategy. Mean fitness within a generation is decreased compared to the mean generational fitness of organisms in more predictable environments where all offspring are likely to survive. Organisms with variable reproductive and developmental traits do not, however, risk losing all of their offspring if conditions are not optimal for one expressed phenotype (Bruijning *et al.* 2019). This increases the likelihood that some of an individual's offspring will survive in every generation.

Moderate offspring survival in every generation reduces fitness variance over a larger time scale (Bruijning *et al.* 2019). Although the fitness of each generation is reduced and no fitness "peaks" or highly successful years are observed, there are also fewer reproductive events with offspring mortality that is detrimental to the lineage (Bruijning *et al.* 2019). The result of decreased fitness variance in a lineage is increased fitness over generations in the face of constantly changing conditions; this is the advantage gained by populations that exhibit a bet-hedging strategy (Bruijning *et al.* 2019).

Phenotypic plasticity is another life-history strategy where there is increased variance as a result of an unpredictable environment; therefore, variance in life history traits on its own is not bet-hedging (Lips, 2001; Haaland *et al.*, 2019). Phenotypic plasticity is typically seen in variable environments that have predictable conditions, which provide a set of environmental cues to trigger differences in phenotypic expression (Lips, 2001). In environments that do not have these short-term, reliable cues, differences in phenotype should be genetically driven rather than influenced by environment (Lips, 2001). The variability observed in bet-hedging is, as a result, more innate, and should persist even in optimal conditions (Lips, 2001).

The defining feature of a bet-hedging strategy is the reduced fitness variance achieved by decreasing arithmetic mean fitness (fitness within a generation) while increasing geometric mean fitness (fitness across generations; Haaland *et al.* 2019). Random variability in the environment affects the survival of offspring. Differential offspring survival influenced by environmental variability encourages increased variability in reproductive output, because no one phenotype will guarantee survival. Increased variability in life history traits in unpredictable environments decreases fitness variance over generations. Over time, this increases geometric mean fitness at the expense of arithmetic mean fitness, and the innate variability in life history traits persists in populations as a bet-hedging strategy.

1.2 Environmental Variability

Variability in the environment can manifest as multiple stressors that have significant effects on organisms, leading to behavioural and physiological adaptations, as well as genetic adaptation through natural selection over time (Hoffmann & Hercus, 2000; Bijlsma & Loeschcke, 2005). This occurs not only when stress is extreme, but also under moderate stressor

intensities (Hoffmann & Hercus, 2000). Biotic factors (such as inter- and intra-species competition) have long been recognized as stressors that influence adaptation in populations (Bijlsma & Loeschcke, 2005). Abiotic environmental stressors also have significant effects on behavioural, physiological, and genetic adaptations, particularly in regions with variable climates that lack predictability (Bijlsma & Loeschcke, 2005). Direct stress on a population can have large scale effects, leading to genotypic/evolutionary adaptation within populations (Bijlsma & Loeschcke, 2005). Direct stress on individuals can alter behavioural and physiological characteristics of the individual, and can potentially have effects on offspring that have not been exposed the stressors themselves (Andersen *et al.*, 2005; Bijlsma & Loeschcke, 2005). For example, maternal effects produce differential characteristics in offspring as a result of direct stress on the reproducing female. In organisms that exhibit this type of plasticity, offspring express different phenotypes despite developing in the same conditions and displaying a lack of genetic variability (Andersen *et al.*, 2005). Abiotic and biotic stresses both contribute to adaptation and typically have compounded rather than independent effects (Bijlsma & Loeschcke, 2005).

1.2.1 Abiotic Stressors

The physical characteristics of an environment act as abiotic selective agents. Temperature/climate and chemical composition of the environment are considered to be some of the most significant stressors with respect to selection (Clarke, 2003; Bijlsma & Loeschcke, 2005; Sulmon *et al.*, 2015). Temperature affects metabolism and all physiological processes within an organism, and changes in temperature force organisms to adopt strategies to maintain their physiological rates (Clarke, 2003; Claireaux & Lefrançois, 2007). Adaptations to temperature changes are typically genotypic, but can be attained through acclimation as well

(Clarke, 2003). In aquatic environments temperature influences metabolism and growth rates, as well as larval development and mortality, and water temperature can be a key determinant in habitat selection (Svärdson, 1949; Pepin, 1991; Claireaux & Lefrançois, 2007; Pörtner, 2012; Freitas *et al,* 2016). Chemical stressors can cause metabolic imbalances and affect osmoregulation, as well as altering biochemical structures and producing toxic metabolites, which negatively affect physiological processes (Sulmon *et al.*, 2015). Salinity and pH are critical parameters in aquatic environments, and changes in these factors can cause extreme stress (or be detrimental) to fish populations (Yao *et al.*, 2010; Kültz, 2015). Significant energy is required to maintain homeostasis in response to thermal and chemical stressors, increasing an organism's metabolic demands (Sulmon *et al.*, 2015). It is critical for genotypes to adapt to the potential abiotic conditions in their environment, particularly climactic and chemical factors, in order to remain fit.

1.2.2 Biotic Stressors

Interactions among and within species also influence behaviour and genetic makeup. Inter- and intra-species competition, predation, and parasitism are common biotic stressors (Bijlsma & Loeschcke, 2005). Stress as a result of intraspecies competition has a higher relevance for the purposes of this paper than interspecies stressors. Individuals of the same species living in the same space compete directly for resources. Intraspecific competition occurs for a number of factors, including habitat/nesting sites, food (as a response to food availability), and reproduction (Svärdson, 1949; Emlen & Oring, 1977; Kirkpatrick, 1982). Reproduction is a fundamental attribute of fitness, and as a result strong competition for reproductive opportunities is a driver of behavioural and morphological adaptations (Emlen & Oring, 1977; Kirkpatrick, 1982). When there is a "limiting" sex, meaning that one sex becomes a limiting factor for the

others' reproduction, competition increases (Emlen & Oring, 1977). This leads to adaptations such as sexual dimorphism (morphological adaptations) and competitive interactions (behavioural adaptations). Sexual dimorphism leads to exaggerated traits in the non-limiting sex that are not present in the limiting sex and in some cases these traits hinder survival, which highlights the importance of sexual selection as a biotic stressor (Kirkpatrick, 1982).

1.3 Japanese Medaka: A Model Organism & Life in the Wild

Japanese medaka (*Oryzias latipes*), commonly known as Japanese rice fish, are small freshwater fish that inhabit slow-moving, shallow bodies of water in Eastern Asia (Leaf *et al.* 2011; Hilgers & Schwarzer, 2019). Their preferred habitats are marshes, ponds, and rice paddies, which is where their common name originates (Leaf *et al.* 2011; Hilgers & Schwarzer, 2019). They are hardy fish that can survive in a wide range of conditions; they are active in temperatures ranging from 4-40°C, are resilient to disease, and are able to survive and spawn in acidic water (to a pH of approximately 4.1; Yada & Ito, 1998; Wittbrodt *et al.*, 2002; Hilgers & Schwarzer, 2019). Although they are commonly reared as freshwater fish, *O. latipes* is considered euryhaline and through acclimation can tolerate salinities up to 80ppt (Inoue & Takei, 2002; Yao *et al.*, 2010)

Japanese medaka are commonly used as a model organism for various types of research; endocrinology, toxicology and oncology, among others (Wittbrodt *et al.*, 2002; Leaf *et al.* 2011; Hilgers & Schwarzer, 2019). Their clear eggs, which allow for visual observation of embryonic development, make them an excellent model for developmental studies (Wittbrodt *et al.*, 2002; Iwamatsu, 2004). Other factors such as their simple habitat and dietary requirements, size, and

ease of husbandry contribute to their popularity (Wittbrodt *et al.*, 2002; Hilgers & Schwarzer, 2019).

In the wild Japanese medaka live in an environment that is highly variable. The marshes and rice paddies that they inhabit are shallow, slow-moving, and can be subject to periodic agricultural disturbances (Hata, 2002; Hilgers & Schwarzer, 2019). In Japan, inland waters are susceptible to acidic conditions due to runoff from mineral springs and volcanic eruptions, and effluent from industrial activity is an increasing source of acidity in aquatic environments (Jozuka & Adachi, 1979). Because of its high surface area to volume ratio, shallow water is more prone to changing conditions than deeper water (McKee *et al.* 2002). Fluctuations in temperature are common; these changes can be observed in even a 24 hour period as there is warming during the day and significant nocturnal cooling (MacIntyre & Melack, 1995). Wind and thermal currents have more pronounced effects on shallower water, allowing them to easily resuspend sediment and change the chemical composition of the habitat (MacIntyre & Melack, 1995). In addition, shallow pools are prone to evaporation. Evaporation affects chemical composition by removing water from a system, and slow-moving currents reduce nutrient and chemical export, so these factors increase stochasticity in water composition (Schindler, 1997). Increased evaporation can also change the size, shape, and distribution of the medaka's environment and lead to short-term fragmentation.

Large-scale changes have occurred over time due to the development of the natural environment for agricultural purposes. Rice paddies are an optimal habitat for medaka – one that they will choose to stay in throughout the year if seasonal draining does not force them to move into streams during the non-breeding season (Hata, 2002). Unfortunately, some agricultural advances are threatening to these fish. Mid-season draining and field irrigation add periodic

disturbances to rice paddy habitats (Hata, 2002). Concrete irrigation channels increase current velocity in the habitat, forcing medaka to congregate near the shore rather than maintaining an even distribution throughout the paddy (Fuduka *et al.* 2005). Due to the unpredictable, highly variable conditions in the medaka's natural habitat, it is expected that they will exhibit bet-hedging with respect to their life history traits to maximize their fitness over generations.

Few studies have focused on medaka in the wild (Hilgers & Schwarzer, 2019). While much is known about characteristics that affect their use in the lab, comparatively less is known about life in their natural habitat. The life history characteristics and evolutionary history of Japanese medaka have not yet been thoroughly investigated (Hilgers & Schwarzer, 2019). Both short and long term stochasticity are common in the natural habitats of Japanese medaka. The evolution of characteristics to deal with short term environmental variability, such as their acidity and temperature tolerances, have been observed (Yada & Ito, 1998; Wittbrodt *et al.*, 2002; Hilgers & Schwarzer, 2019). Strategies related to the life history traits of Japanese medaka, or those that would develop in response to changes over a longer time scale, need to be studied in more depth (Leaf *et al.*, 2011).

1.4 Reproduction and Development in Japanese Medaka

The breeding season for Japanese medaka occurs from April until October in the wild (Iwamatsu, 2004). It is influenced by photoperiod and, to a lesser extent, temperature. Japanese rice fish begin spawning under a 14:10 light/dark photoperiod; oviposition typically occurs from one hour before to one hour after the onset of the light period (Iwamatsu, 2004). Spawning occurs daily during the breeding season (Leaf *et al.* 2011). On average, female clutches range from 10-30 eggs, however smaller and larger clutches have also been observed (Leaf *et al.*

2011). Unfertilized eggs adhere in clumps to the ventral surface of the female fish via sticky attachment filaments (Iwamatsu, 2004; Hilgers & Schwarzer, 2019). After external fertilization occurs, female medaka deposit their eggs on substrate (typically vegetation) in the environment (Hirshfield, 1980). There is no parental investment in *Oryzias latipes*, so individual fitness is determined through survival of offspring based on maternal and genetic factors alone (Hirshfield, 1980).

1.4.1 Operational Sex Ratio (OSR)

Operational sex ratio (OSR) is a biotic source of variability that affects reproductive behaviour in Japanese medaka. OSR is the ratio of sexually active males to sexually active females in a population (Grant & Foam, 2002). Female medaka are the limiting sex, because they can produce one clutch of eggs per day, but males have the ability to fertilize up to 30 females per day (Grant & Foam, 2002). Because males have the capacity for a higher potential rate of reproduction, females tend to be choosier while males compete for access to the female fish (Clark & Grant, 2010). In more competitive environments (higher OSRs where there are fewer female than male fish), male competition increases as opportunities to mate with females become more limited (Grant & Foam, 2002; Clark & Grant, 2010). Due to increased competition, both male-male (intrasexual) and male-female (intersexual) aggression typically increase with increasing OSR (Clark & Grant, 2010; Weir, 2013). Male aggression appears to be based on genetics, and increased individual aggression leads to increased reproductive success (Fujimoto *et al.*, 2015; Sasaki & Yamahira, 2016).

Intersexual aggression is a direct stressor on spawning females. As outlined in section 1.2, direct stress on the spawning females has the potential to produce differential phenotypic expression in offspring as a plastic response. Simultaneous following, defined as two or more

males following directly behind a female fish within one body length, is the most common form of intrasexual aggression observed in medaka mating behaviour (Weir, 2013). Another form of intrasexual aggression is disruption, where a second male physically separates or disturbs a spawning pair (Weir, 2013). Female resistance to courtship attempts increases as the number of males in the environment increases (when female abundance is not changed; Weir, 2013).

1.4.2 Variability in Hatching Time

Significant variability has been observed in egg hatching time of Japanese medaka in a laboratory setting (Teather *et al.*, 2000). In their study examining the early life history parameters of *Oryzias latipes*, Teather *et al.* (2000) found that eggs reared under the same conditions hatched as early as 8 days post spawning and as late as 35 days post spawning. Hatching time was not related to egg volume, nor was it related to maternal characteristics such as size or condition. Some of the variation observed was within-clutch variation, meaning that the phenotypic diversity was expressed at an individual level. Leaf *et al.* (2011) reported all eggs hatching before 7 days post spawning, which is significantly different from the results reported by Teather *et al* (2000).

Embryonic development of Japanese Medaka has been thoroughly documented (Iwamatsu, 2004). Their development has been divided into 39 stages based on diagnostic features including number/size of blastomeres, development of the central nervous system, development of eyes, heart, tail, and fins, and body size and movement (Iwamatsu, 2004). *Oryzias latipes* follow the typical teleostean developmental pattern (Iwamatsu, 2004). The fish reach their hatching stage (stage 39) 9-10 days post spawning at 26°C (Iwamatsu, 2004). If development follows these stages as reported, the variability observed should not be related to developmental synchrony.

1.5 Research Goals

Despite the high variability observed in medaka egg-hatching time in a controlled environment, no studies have examined this effect as a potential bet-hedging response to date. Due to their widespread use as a model organism, it is extremely important to have a comprehensive understanding of the life-history characteristics of Japanese medaka. In addition to understanding more about *Oryzias latipes* on its own, studies evaluating evolutionary strategies that enable organisms to effectively cope with random, unpredictable variability in the environment are increasingly important. Climate change threatens to introduce new variability to natural environments, and aquatic environments are particularly susceptible due to changes in temperature and chemical composition. This study will quantify the variability in egg hatching time in Japanese medaka, both within-population (among-clutch) and within-clutch variability, as well as the persistence of within-clutch variability in the population. It will also assess whether individuals in more stressful environments (those with higher OSRs) exhibit the same degree of variability as those in less stressful environments (those with lower OSRs). Due to the innate nature of the variability in a bet-hedging strategy (as opposed to a plastic strategy), it is expected that there will not be significant differences in variability across OSRs. This study will also look at other potential influences on variability (maternal length and clutch size) to determine if there is a relationship between these factors and observed variability. I predict that there will be no significant relationship between the studied factors and the within or among-clutch variability. Quantifying the variability in *Oryzias latipes* egg hatching time to provide evidence of a bet-hedging strategy will help to develop a more robust understanding of the life history characteristics of Japanese medaka and how they have evolved in the wild.

Materials and Methods

2.1 Experimental animals

Two-hundred and eighty-eight Japanese medaka (*Oryzias latipes)* were used in this experiment. These fish were third generation lab-bred fish from Saint Mary's University, Halifax, Nova Scotia. Fish were anaesthetized with MS222 (Tricaine methanesulfonate) buffered with sodium bicarbonate and tagged for individual identification prior to the experiment. Visual Implant Elastomer Tags (NorthWest Marine Technology) were used for tagging. Each fish was tagged in two of four possible locations on the dorsal surface; either anterior or posterior to the dorsal fin on the left or right side. Tags chosen were one or two of eight possible colours (black, blue, brown, green, orange, pink, red, or yellow).

Fish were housed in ten-gallon tanks measuring 20 inches x 10 inches x 12 inches. Tanks contained an undergravel filter and aeration device. The tanks were heated and kept between 23-28°C and temperatures were taken daily with a surface thermometer. The tanks contained fresh water with a pH of 7.4-8.4. The fish were kept under a 14:10 hour light:dark photoperiod with the light period beginning at 0700h. There were a total of 24 experimental tanks, each containing 12 adult fish housed at four operational sex ratios (0.5, 1, 2, and 5). The OSR of 0.5 contained eight females and four males, the OSR of 1 contained 6 females and 6 males, the OSR of 2 contained four females and eight males, and the OSR of 5 contained two females and ten males. Six shelves in the lab held four tanks each. Each shelf had one tank of each OSR and the order of OSRs on the shelves was randomized to minimize bias based on proximity to the entryway of the room and position of the shelf.

The fish were fed once per day. The adult medaka were fed commercial flakes (TetraMin Tropical Flakes) and a mixture of frozen adult brine shrimp and live *Artemia* nauplii (re-hydrated

brine shrimp cysts which were hatched in the lab). Water quality tests (nitrate, nitrite, ammonia, and pH) and water changes (25% of tank volume) were conducted weekly on the experimental tanks. During water changes, tanks were topped up with RODI (reverse osmosis deionized) water. The RODI water used in the tanks was 0.042% red sea salt, 0.004% alkaline buffer (to meet pH parameters of 7-8), and 0.013% stress coat.

2.2 Experimental design

In this experiment I collected eggs from all 24 experimental tanks. There were four experimental treatments. Each treatment comprised one of the four OSRs outlined in section 2.1. The eggs were collected between 0730h and 1000h; earliest collected eggs were taken at least half an hour after the onset of the light period to allow time for fertilization. The eggs were collected early in the day to increase the likelihood that the entire clutch was intact, because females will begin to remove their eggs from their abdominal surfaces, or other fish start to consume them, later in the morning. Clutches that were visibly disturbed (eggs trailing behind the female or being eaten) were not collected.

2.3 Egg collection and hatching

Female fish with clutches adhered to their ventral surface were removed from the experimental tank one at a time and returned immediately after egg collection. These females were caught and isolated in a small Tupperware container to reduce the amount of time that was spent out of water, which limits stress on the fish. The isolated female was removed from the Tupperware by hand and eggs were brushed off of the ventral surface with a paintbrush. The eggs were transferred from the brush to a small petri dish containing a rearing solution made up

of 1.0g sodium chloride, 0.03g potassium chloride, 0.04g calcium chloride dihydrate, 0.163g magnesium sulfate heptahydrate, and 10mL 0.01% methylene blue in one litre of deionized water. The eggs were examined under a dissection microscope to determine the number of eggs in the clutch and number of eggs that were viable/fertilized. Fertilized eggs can be identified by an increase in the perivitelline space as well as differences in the cytoplasm (see Figure 1; Iwamatsu, 2004). After collection, the clutches were incubated and monitored and hatching dates were recorded. Maternal tags were recorded and each clutch was isolated during incubation to allow for observation of within-clutch variability.

Each clutch was transferred to its own unique cell in an ice cube tray. The cells were filled with rearing solution and labelled with the tank of origin (1-24) and maternal tags. The ice cube trays were kept on a shelf with lights set to the same photoperiod as the experimental tanks. The temperature of the cells was monitored and remained between 19-23°C; each cell was checked daily for hatched fish. The hatched fish were removed from the cell once their date of hatching was recorded. The cells were regularly topped up with rearing solution or RODI water meeting tank water requirements to avoid dessication.

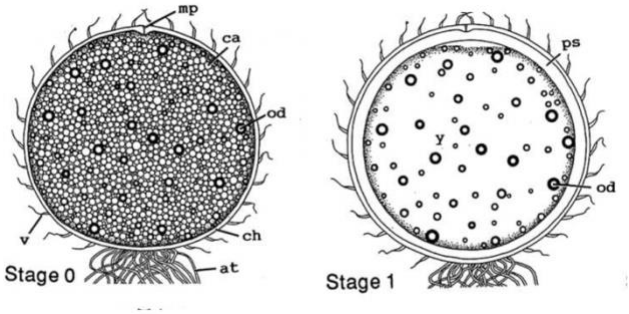

Figure 1. Unfertilized *O. latipes* egg (Stage 0) and fertilized egg (Stage 1). Visible differences are present in the cytoplasm (concentrated oil droplets (od)) and the perivitelline space. Iwamatsu, 2004.

2.4 Statistical analysis

Data analysis was carried out using Microsoft Excel version 16.16.21, Python version 3.8.5 (with Atom 1.54.0 using matplotlib.pyplot and seaborn), and R Studio version 1.3.1093. Prior to analysis, the dates that the fish hatched were converted to "days post spawning" to create a uniform, quantifiable metric. Pivot tables were used in Excel to calculate a weighted mean "days to hatch" (based on how many fish hatched on a given day) for each clutch. These were also used to calculate standard deviation for each clutch. The coefficient of variation (standard deviation divided by the mean) was calculated for each clutch and converted to a percentage value to create a more standardized metric for analyzing within-clutch variability.

Statistical analysis was carried out in R Studio. The package "lme4" was used for linear mixed models (LMM) to determine the influence of OSR on mean days to hatch and CV. Mean days to hatch and CV were fixed effects with females as random effects to avoid pseudoreplication. Linear mixed models were also used for maternal length and clutch size in relation to mean days to hatch and CV. Data were averaged per female data for graphing. All figures were created using Python.

2.5 Visual Observation

Visual observations were done throughout the incubation period on a smaller sample size (n=~20 clutches) to keep track of development/developmental synchrony. Observations of whole clutches were done at ~24 hour intervals using a Wild Heerbrugg dissecting microscope. The clutches were transferred to petri dishes for this process to enable clear visual observation. Iwamatsu (2004) was used as the staging reference.

Results

3.1 Within and Among-Clutch Variation and OSR Differences

Within and among-clutch variation was quantified and differences across OSRs (different stress levels in the environment) were compared to determine if there is evidence of bet-hedging in medaka egg hatching time. The mean days to hatch for each clutch, representing among-clutch variation, ranged from 17.45 days to 40.18 days (Figure 1). Within-clutch variation, measured as the coefficient of variation (CV; standard deviation/mean) ranged from 1.65% to 38.41% of the mean. There were no differences between the four OSR treatments for mean days to hatch ($F_{3,85}$ = 1.15, p = 0.33; Figure 1) or the within-clutch CV ($F_{3,83}$ = 0.19, p = 0.90; Figure 2). The overall standard deviation in mean days to hatch ranged from 0.50 to 9.96 days, with a mean of 3.59 days (Figure 3). Days to hatch for individual eggs ranged from 15 to 52 days (Figure 4).

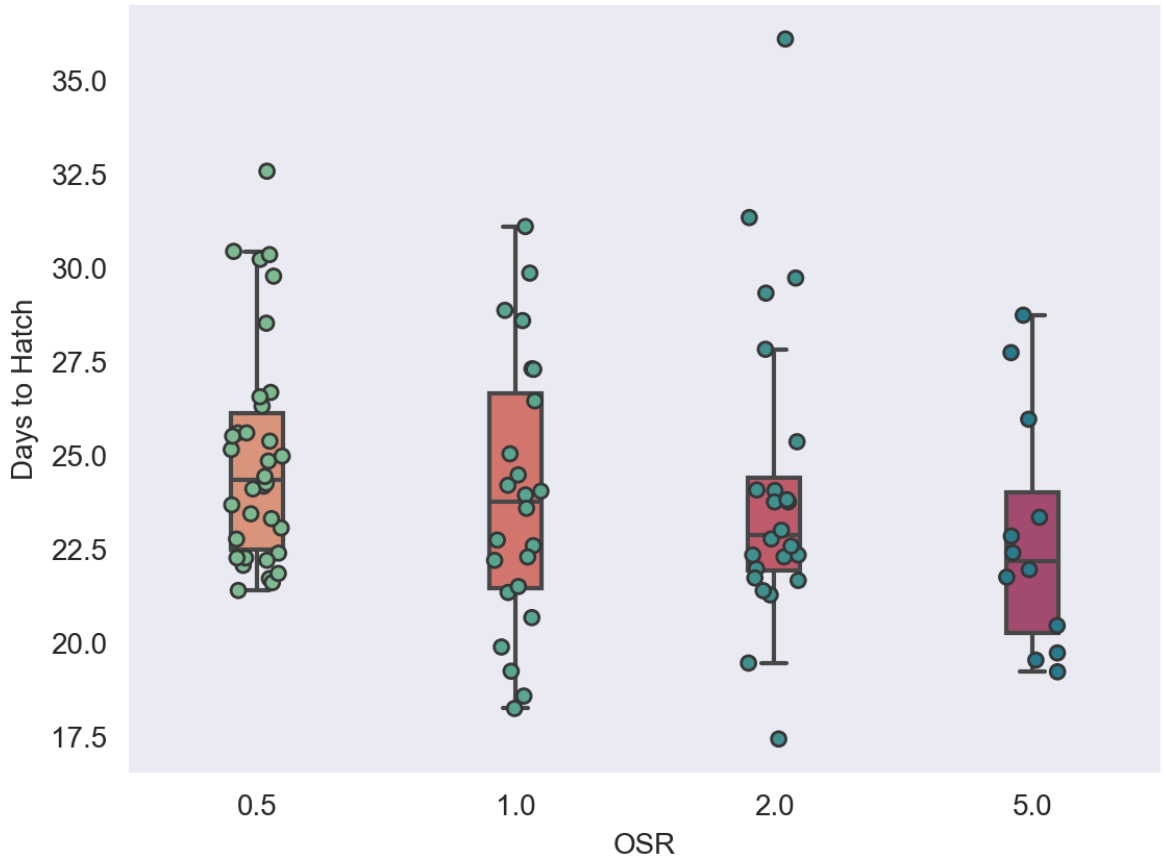

Figure 1. Mean days to hatch for each clutch (blue) and boxplots showing average days to hatch for OSR treatments of 0.5, 1, 2, and 5. n=150, averaging female data reduced observations to 92. Visual representation of among-clutch variability. There were no significant differences between OSRs, indicating that short-term stressors do not impact variability in among-clutch egg hatching time.

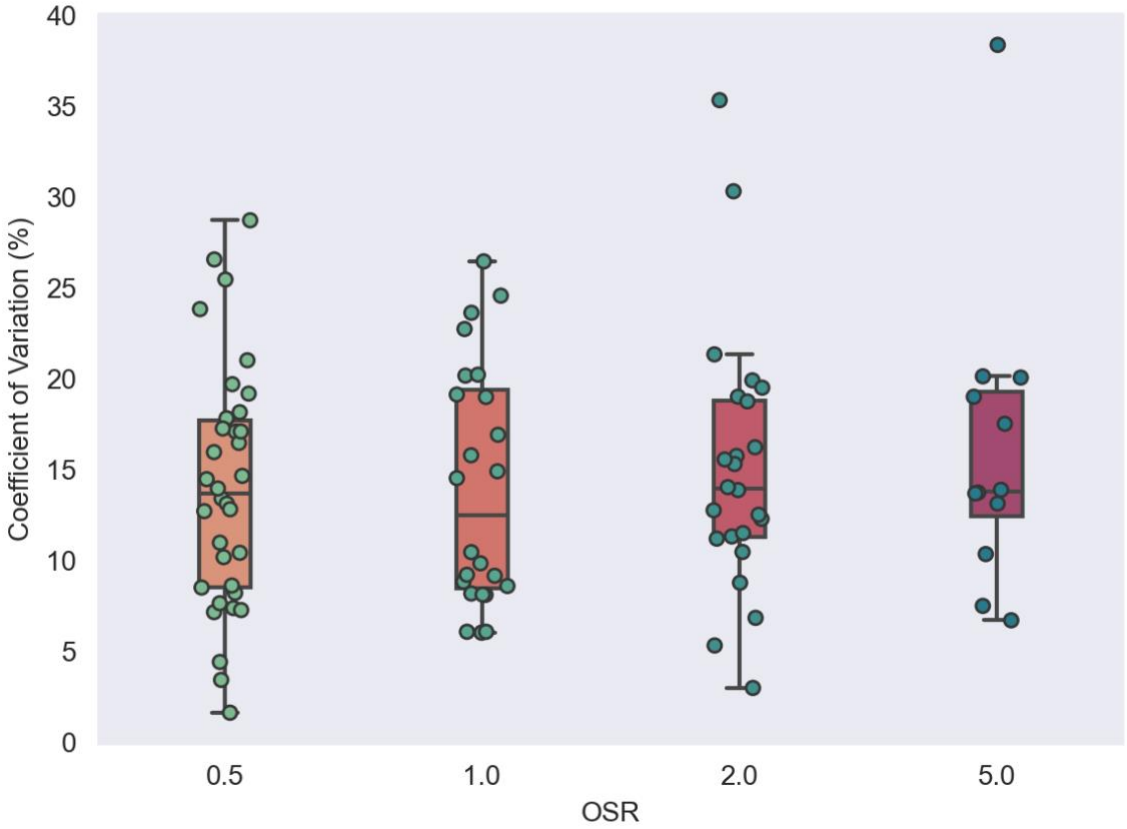

Figure 2. Coefficient of variation for each clutch (blue) and boxplots for CV across the OSR treatments of 0.5, 1, 2, and 5. n=150, averaging female data reduced observations to 92. Visual representation of within-clutch variability. There were no significant differences between OSRs, indicating that short-term stressors do not impact variability in within-clutch egg hatching time.

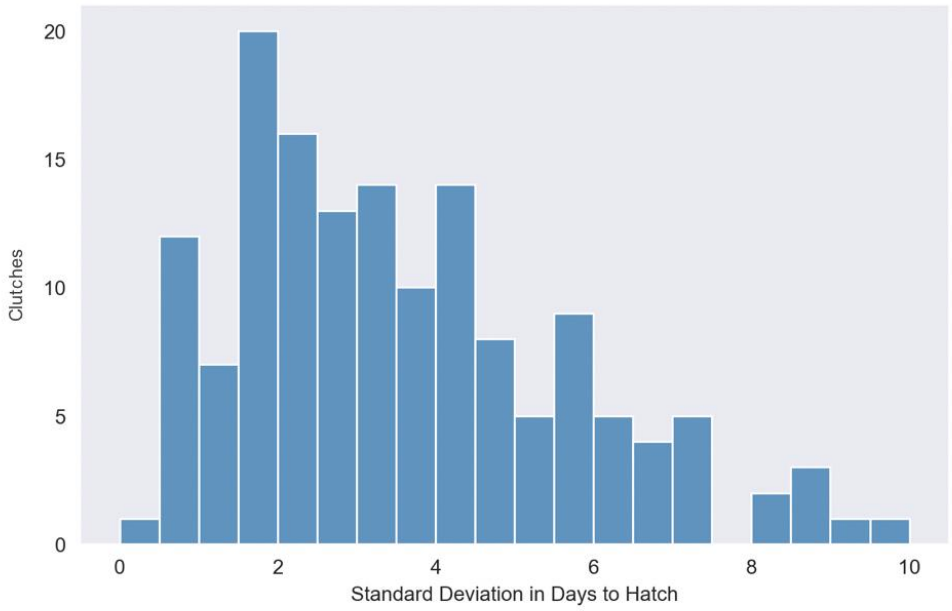

Figure 3. Histogram of standard deviation in days to hatch across all clutches. n=150. Values range from 0.50 days to 9.96 days with a mean deviation of 3.59 days.

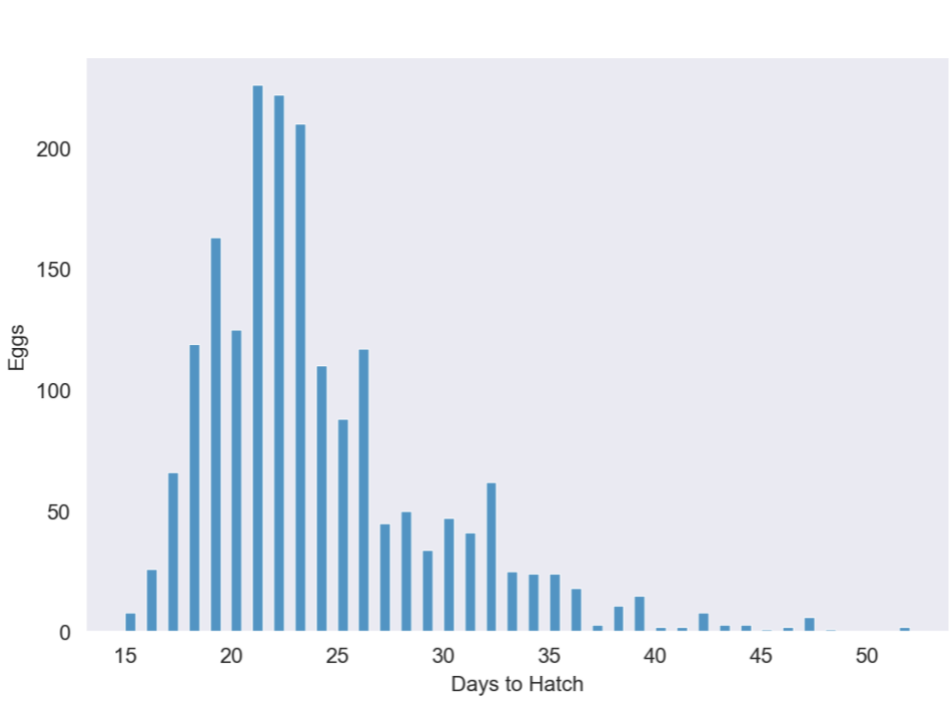

Figure 4. Days to hatch for individual eggs from all clutches. n=1910. Values range from 15 to 52 days, with most eggs hatching between 15 and 35 days.

3.2 Effects of Maternal Length and Clutch Size

To examine the relationship between other reproductive parameters and variability in egg hatching time, maternal length and clutch size were compared to mean days to hatch and CV. There were no significant relationships observed between maternal length and average days to hatch ($F_{1,100} = 0.02$, $p = 0.89$) or maternal length and CV ($F_{1,105} = 0.22$, $p = 0.63$; Figure 5). No relationship was found between clutch size and CV (Figure 6A; $F_{1,119} = 1.15$, $p = 0.28$). There was a significant negative relationship found between clutch size and average days to hatch (Figure 6B; $F_{1,120} = 5.40$, $p = 0.022$).

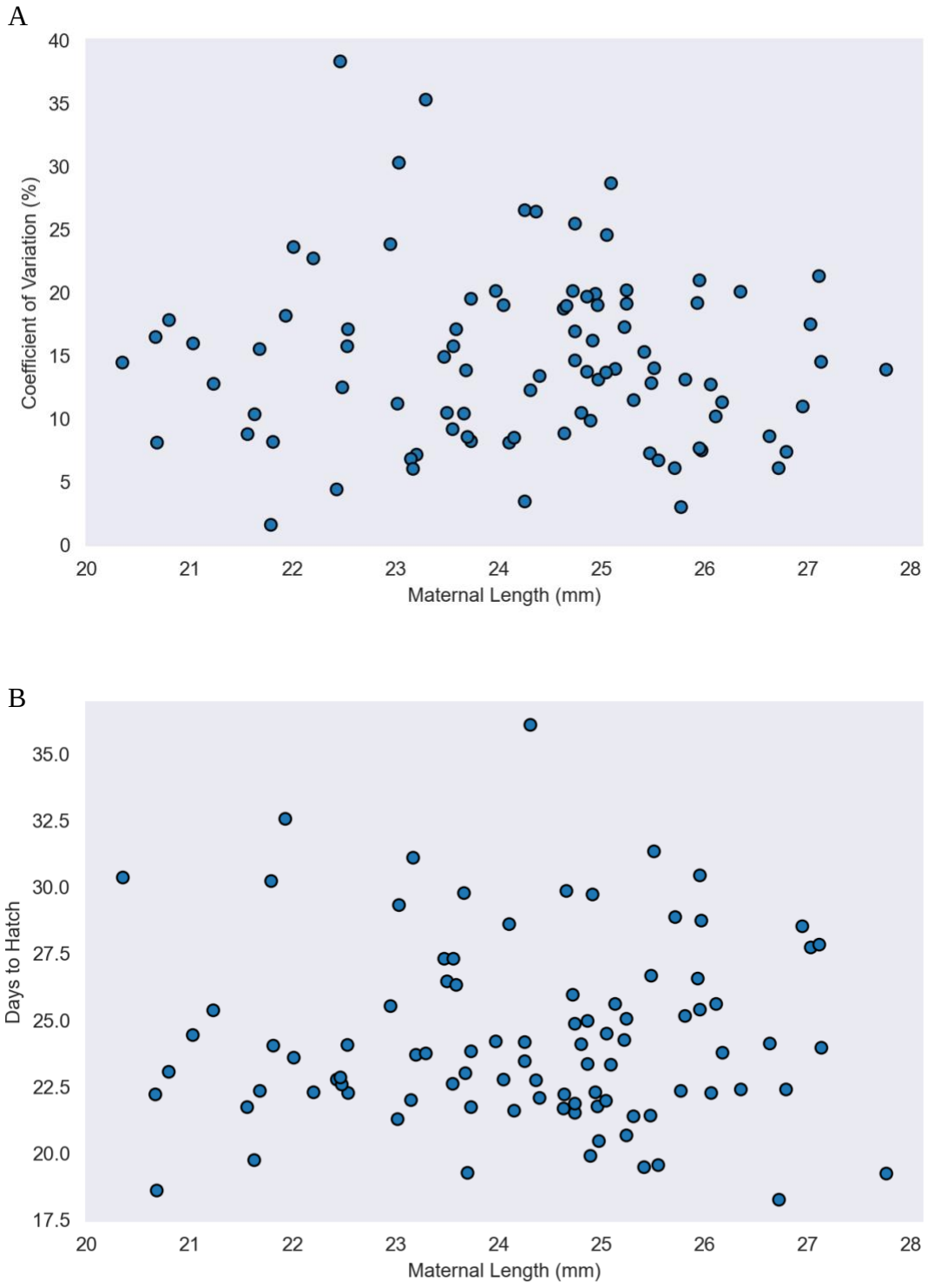

Figure 5. Plot of the relationship between maternal length in millimetres and CV (A) and between maternal length and mean days to hatch (B). n=148, averaging female data reduced observations to 90. No significant relationship is observed between maternal length and CV or mean days to hatch.

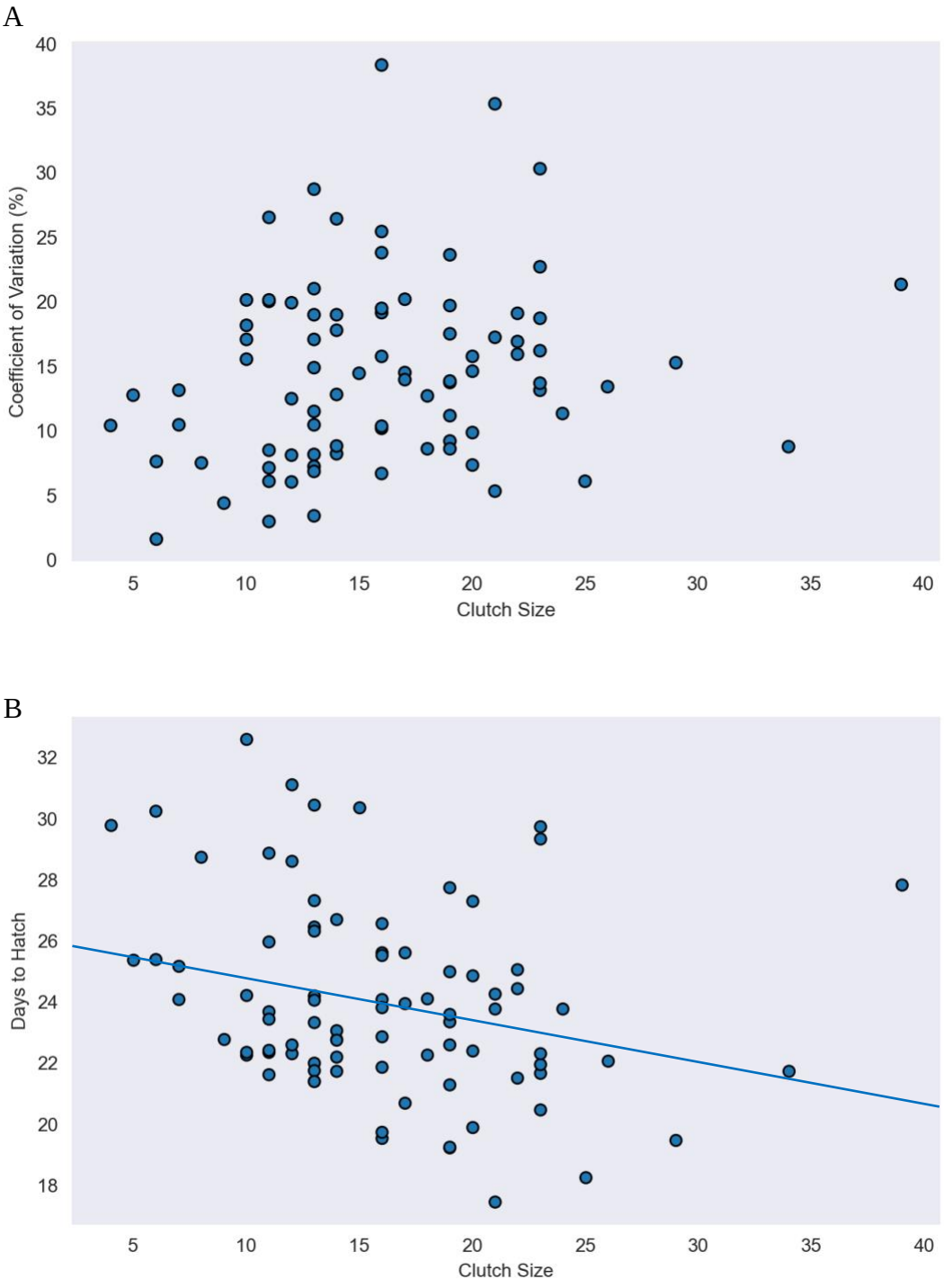

Figure 6. Plot of the relationship between clutch size and CV (A) and between clutch size and mean days to hatch with regression (B). n=128, averaging female data reduced observations to 85. No significant relationship is observed between clutch size and CV. An inverse relationship was found between clutch size and mean days to hatch.

Discussion

The purpose of this study was to determine if there is evidence of bet-hedging in medaka egg hatching time by quantifying within and among-clutch variability and by comparing variability across OSRs. With a bet-hedging strategy, variation should be innate and therefore consistent across different environments (Lips, 2001; Bruijning *et al.* 2019), so it was expected that all four OSRs would have similar among and within-clutch variation. Thus, my data support that this is a bet-hedging strategy rather than a plastic response to the environmental stressors related to differences in OSR. I collected data on maternal length and clutch size to look for relationships between these factors and the variability observed. No relationship was expected between maternal length or clutch size and either among or within-clutch variation. This prediction was true for all but the relationship between clutch size and mean days to hatch, which did have a significant negative relationship.

4.1 Among-clutch variation

I observed significant among-clutch variability, measured as the difference in the weighted mean days to hatch for each clutch. Mean days to hatch for each clutch was 22 days, and ranged from 18 to 40.18 days at temperatures ranging from 19-23°C. The majority of the clutches fell between 18 and 35 days to hatch, which gives a span of over two weeks (17 days). Laurel *et al.* (2008) note that in larger marine fish, which typically develop at lower temperatures, differences of seven days are considered to be a bet-hedging response. They suggest that monomorphic differences at the time of hatching (such as larval length or yolk size) could lead to differential survival in offspring. The range of variability found in this study supports the theory of a bet-hedging strategy in the population. The similarity between OSRs is

consistent with the predicted results, indicating that the among-clutch variability may be innate and not influenced by short-term stressors in the environment. Further research on the effects of different stressors (e. g. salinity, temperature, food availability) on variability would provide more insight on this point.

At 26°C ± 1, medaka embryos will develop to the hatching stage within ten days of fertilization (Iwamatsu, 2004). Rosemore & Welsh (2012) reported that as rearing temperature decreases from 28°C to 24°C, the rate of development slows. Their study found that embryos reared at 24°C took 11.5 hours longer, on average, to develop a heartbeat compared to embryos reared at 28°C or 32°C (53.5 and 42 hours, respectively). The rearing solution that the eggs were incubated in for this study remained at an ambient temperature of 21°C ± 2. This likely explains the difference in hatching time observed between this study and previous work. Teather *et al.* (2000) reported individuals hatching between 8 and 35 days post spawning at 27°C ± 2, while my results found a range for individual hatching time of 15 to 52 days post spawning. Because all of the eggs were reared under the same conditions, it should not significantly affect developmental synchrony, only the period over which hatching occurred. Leaf *et al.* (2011) reported that in contrast to the results of Teather *et al.* (2000), all of their eggs hatched to the larval stage before 7 days post spawning, despite being incubated at approximately the same temperature (26.5°C ± 2.2). Given the scale of the difference between those two papers, it is unclear whether the observed results across these studies is primarily a result of environmental factors or whether the genetic makeup of the *O. latipes* populations is playing a role as well.

4.2 Within-clutch variation

Within-clutch variability was quantified as coefficient of variation. The average CV across all OSRs was approximately 15% of the mean. Despite being reared under the same conditions, significant variability (0.50-9.96 days) was observed within individual clutches. This is consistent with the expected results. It is interesting to note that the CV varied widely within the population (1.65%-38.41%); although all individual females displayed some degree of variability, the rate of within-clutch variability was not consistent across the population. The within-clutch variability did not have a relationship with OSR, so, as with among-clutch variability, it appears to be innate and not affected by short-term environmental changes. At 9.56 days, the range for within-clutch variability was lower than the range for among-clutch variability. While within-clutch variability is present, at this temperature it appears to be less significant than among-clutch variability. Overall, the within and among clutch variability support a potential bet-hedging mechanism in *Oryzias latipes* egg hatching time.

4.3 Effects of maternal length and clutch size on hatching time

I predicted for this study that external factors would not have a relationship with the observed variability, and I did not find any effect of maternal length on the parameters I measured. There was no relationship between maternal length and either within or among-clutch variability, which follows the predicted results. This is consistent with the results found by Teather *et al.* (2000), and suggests that variability in medaka egg hatching time is independent of maternal size. Clutch size was not related to the variation seen within individual clutches, as expected. However, there was a negative relationship observed between clutch size and mean days to hatch, whereby as clutch size increases, the clutch tends to hatch earlier on average.

More work in this area may provide insight on how egg hatching variability is influenced by clutch size, as well as potential mechanisms for hatch time variability.

4.4 Future directions

In this study, visual observations of a smaller sample size (~20 clutches) under a dissection microscope seemed to confirm within-clutch developmental synchrony. This indicates that even when the developmental rate is consistent across all embryos within a clutch, within-clutch hatch time is still variable. These observations were done looking at the entire clutch at 24-hour intervals, so some differences in the developmental stages of the eggs may have been missed. Observations of individual eggs to identify developmental stages at more frequent intervals could confirm developmental synchrony in medaka embryo development. This project provides groundwork for more research into bet-hedging and potential mechanisms for hatch time variability in *Oryzias latipes.* Future research could include work on developmental synchrony and its relationship to variability, differences in variability and hatch period at different temperatures, examining the relationship between clutch size and time to hatch, and looking at different mechanisms that enable the observed within and among-clutch variability.

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