Heritability of sexual traits in the common Japanese medaka (*Oryzias latipes*)

By
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Abstract

Adaptation is a process that occurs through natural and sexual selection and can drive variation within species. By showing that certain sexual traits are inherited, I demonstrated that these traits can vary, at least in part, because of adaptation, rather than entirely because of plasticity across abiotic and social environments. Japanese medaka fish (*Oryzias latipes*) have observable and quantifiable morphological traits, making them a good model for monitoring the influence of operational sex ratio, OSR, the ratio of sexually available males to females, on population variability. The fish were isolated in mating pairs in standardized environmental conditions to determine whether variation in traits was because of the OSR or genetic factors. Anal fin area (mm^2), standard body length (mm), and testis weight (mg) were measured for the parental fish and their offspring, which were collected as eggs and raised until sexual maturity. Linear regressions were used to indicate heritability between the parental and offspring traits. Average standard body length, male anal fin area, and testis weight had the highest narrow sense heritability factors (h^2=0.5430, h^2=0.3295, and h^2=0.6286, respectively). The influence of OSR was determined for the parental traits using general linear models, as they had been bred in four independent OSRs (0.5, 1, 2, 5) for three generations previous to this experiment. All traits had a weak influence of OSR except for male and female anal fin area and testis weight, which was stronger only when body length was also factored in. By showing that these traits are inherited, I provide evidence that additive genetic variation is playing a role in the variation between and among populations of medaka.

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1.0 Introduction

1.1 What drives variation?

Acclimation and adaptation are both drivers of variation within species (Darwin, 1859; Kelly, Panhuis, & Stoehr, 2012). Acclimation occurs within individuals, whereby changes in behaviour, physiology, or morphology can occur in an organism as an adjustment to changes in their environment. Phenotypic plasticity is one type of acclimation in which individuals change their phenotypic expression within their lifespan (Kelly et al., 2012). Snowshoe hares are a classic example of a species that do this; they change from thin brown coats in the summer to thick white coats in the winter (Nagorsen, 1983). These changes improve the hares' temperature regulation and camouflage to increase their survival across seasons (Nagorsen, 1983; Zimova, Mills, Lukacs, & Mitchell, 2014). Anadromous fish species also exhibit phenotypic plasticity, as they have the ability to acclimate to marine or freshwater habitats by changing their physiology to maintain osmotic equilibrium (Jensen et al., 2015).

By contrast, adaptation does not occur within individuals, but rather across generations. The variation from adaptation can occur through both natural and sexual selection, and typically takes many generations to establish the variation among populations owing to different selective pressures (Darwin, 1859). For example, morphological and physiological variation has been extensively researched within the three-spined stickleback species; different populations are adapted to either freshwater or marine habitats due to their differing osmoregulation (Currey, Bassham, & Cresko, 2019; Rastorguev et al., 2018). These fish are under the influence of natural selection, which is a force that will eventually lead to speciation in conjunction with other factors such as

geographic separation, therefore slowly drives variation over time (Darwin, 1859; Nosil & Schluter, 2011). However, gene frequencies are always changing across and within populations, causing variation within species as well.

Sexual selection is another mechanism that can contribute to the variation within species that results in adaptation to different conditions that enhance survival and reproductive success (Darwin, 1871). Sexual selection occurs when mates are either chosen through mate competition (intrasexual selection) or mate choice (intersexual selection) (Darwin, 1871). An example of intersexual selection is peahens choosing large and extravagant tails in peacocks because of their implication in evading predators (Yorzinski, Patricelli, Babcock, Pearson, & Platt, 2013). Intrasexual selection is demonstrated by dung beetles; males with larger horns outcompete other males during male-male competition (McCullough & Simmons, 2016). Sexual traits are either those that differ between the sexes and often evolve via sexual selection, causing sexual dimorphism in the species, or are simply traits that contribute to the reproductive success of individuals. Behavioural traits, such as courtship and aggressive behaviours, are examples of observable plasticity in populations that will differ depending on the individuals access to potential mates or competitors (Allen, 2019). In male-biased populations, males are often more aggressive as they need to compete for access to the limited number of females in the population (Allen, 2019; Emlen & Oring, 1977). On the other hand, in female-biased populations, males increase their courtship behaviours to appeal to females; however, there are exceptions to these theories (Allen, 2019; Emlen & Oring, 1977). Therefore, depending on the operational sex ratio (OSR, the ratio of sexually available males to females in a population), the males will have more or less

access to females and sexual traits, including both behaviours and morphological traits, will be affected accordingly.

When measuring continuous phenotypic traits, it can be difficult to determine whether this trait had developed through genetic inheritance or environmental influence; therefore, both plasticity and adaptation should be considered as driving forces of trait variation (Falconer & Mackay, 1996). To measure plasticity of a trait, abiotic and social environments can be manipulated to investigate changes in behaviour, morphology, and physiology (Kelly et al., 2012). When considering adaptation as a process leading to trait divergence among populations, these traits must have an underlying genetic basis, and thus be heritable (Falconer & Mackay, 1996).

The mode of interaction among loci and the alleles contributing to a particular trait influence the ability to measure the heritability of that trait. Dominant and epistatic genetic variation both have differential expression of alleles because of dominance of some alleles over others, whether at a single locus or multiple loci (Falconer & Mackay, 1996). This means that offspring may not phenotypically resemble their parents. However, additive genetic variation, which is the component of variation for which all alleles are equally expressed, makes it possible to predict offspring phenotypes by measuring the parental trait (Falconer & Mackay, 1996). Broad sense heritability is a measure of all three components of genetic variation, therefore cannot be measured quantitatively (Falconer & Mackay, 1996). However, narrow sense heritability is the result of looking exclusively at additive effects of alleles, resulting from a correlation between parent and offspring phenotypes (Falconer & Mackay, 1996). Measuring the narrow sense of heritability only indicates the proportion of heritability that results from

additive effects of alleles, meaning that dominant and epistatic variation could still be playing a role in the inheritance of the trait along with other environmental factors.

Narrow sense heritability is measured using the relationship between quantitative traits across generations (Falconer & Mackay, 1996; Visscher et al., 2006). The slope of the regression line, h^2, provides a number indicating the effects of additive alleles on the inheritance of the trait being measured. The h^2 value can range between 0 and 1, increasing with a higher heritability (Visscher et al., 2006). Some traits have exceptionally high h^2 values, such as human height at approximately 0.8; however, any number that corresponds with a significant linear regression implies some variation from additive genetic variation (Macgregor, Cornes, Martin, & Visscher, 2006; Visscher et al., 2006).

1.2 Medaka fish

Japanese medaka (*Oryzias latipes*) are found in freshwater or brackish rice fields throughout Eastern Asia (Shima & Mitani, 2004). They are easily reared in laboratory conditions, where they spawn daily and take two months to reach sexual maturity (Shima & Mitani, 2004). Although primarily used in genetic and developmental research, these fish also have observable mating behaviours and exhibit quantifiable behavioural and morphological variation throughout their natural range (Leaf et al., 2011; Ono & Uematsu, 1957; Shima & Mitani, 2004). To improve upon the studies that analyze their genetic, morphological, and behavioural characteristics, the drivers of the genetic and phenotypic variation must be explored.

Testis size in fishes has been studied for commercial fisheries to determine interspecies variation and individual responses to sex hormones (Parenti & Grier, 2004;

Sato, Suzuki, Shibata, Sakaizumi, & Hamaguchi, 2008; Yoshikawa et al., 2020). A larger testis is associated with a higher proportion of sperm released, indicating that there could be a correlation between testis size and fertilization rate (Lüpold, Linz, Rivers, Westneat, & Birkhead, 2009; Ramm & Schärer, 2014; Rowe & Pruett-Jones, 2011). Previous studies on medaka have shown that testis size decreases as the OSR becomes increasingly male-biased across experimental populations (Allen, 2019).

Medaka anal fins are sexually dimorphic; males have larger fins than the females (Kawajiri, Kokita, & Yamahira, 2009; Koseki, Takata, & Maekawa, 2000). During reproduction, males use their anal fins to wrap around a clutch of eggs on a female so that when they release their sperm their fertilization success rate will be increased and to block sperm from competing males (Koseki et al., 2000; Ono & Uematsu, 1957). Therefore, having a larger anal fin is beneficial in male-biased populations where intrasexual competition is intense, which has been documented in wild populations (Fujimoto, Miyake, & Yamahira, 2015). However, laboratory experiments that have replicated these social environments have found opposite results—that males in male-biased populations actually developed smaller anal fins (Allen, 2019). This was unexpected, but the discrepancy could have resulted from an increase in energy allocated to mating behaviours (Allen, 2019). Also, females do not select for males with larger anal fins, body size is much more important to their selection (Howard, Martens, Innis, Drnevich, & Hale, 1998). Larger males are also shown to be more aggressive (Allen, 2019; Howard et al., 1998).

Medaka growth rate depends on abiotic factors in their environment such as temperature, population density, and resource availability (Dhillon & Fox, 2007). They

undergo gradual senescence, not indeterminate growth like some other bony fish species (e.g., Goss, 1974; Patnaik, Mahapatro, & Jena, 1994). Female fish choose to mate with larger males, but males may also choose to mate with larger females (Grant, 1995; Howard et al., 1998). In many fish species, such as salmonids, larger females have a higher fecundity, meaning they produce bigger clutches and/or larger eggs (de Eyto et al., 2015; Thorpe, Miles, & Keay, 1984). Although medaka produce larger clutches with smaller eggs and vice versa, fecundity has been a contentious point in studying medaka life-history (Leaf et al., 2011; Teather, Boswell, & Gray, 2000). There has been some evidence of intra-clutch egg size variation, a mechanism used by some bird and fish species to increase the survival rate of their whole clutch (Bernardo, 1996; C. Chambers & Leggett, 1996; R. C. Chambers & Trippel, 1997; Leaf et al., 2011; Poisbleau, Dehnhard, Demongin, Quillfeldt, & Eens, 2015).

1.3 Study objectives

The primary objective of this study is to determine whether the observed variability in anal fin size, body length, and testis size are the result of additive genetic variation or under influence of the environment. To do this, the fish were removed from their social environments so that a clear indication of the effect of additive alleles could be determined with a controlled environment. Alongside this, exploring the relationship between female medaka and their eggs will expand the life-history of medaka that we currently know.

2.0 Materials and Methods

2.1 Rearing conditions for generation two medaka

The Japanese medaka (*Oryzias latipes)* used in this experiment were originally obtained from Aquatic Research Organisms (New Hampshire, USA) and had been bred for two generations at Saint Mary's University for use in a larger, ongoing project. From this point onwards, the adult fish will be referred to as generation 2 and the offspring as generation 3. The previous generations were reared in the Aquatic Facilities at Saint Mary's University; 12 mating adults were held in 10-gallon tanks (50 cm x 25 cm x 30 cm) that were kept between 24°C and 28°C. They were equipped with an under-gravel filter, air stones for aeration, and artificial plants for habitat enrichment. There were 24 tanks in total, with fish held at 4 different OSRs (0.5, 1, 2, 5), replicated six times each. Each generation was strategically mixed to minimize inbreeding while still implementing the OSRs once each generation had reached sexual maturity. The photoperiod throughout the experiment was set to 16 hours of light and 8 hours of dark.

2.2 Experimental set-up

Second generation adults were selected from the 24 stock tanks just after they reached sexual maturity. Thus, the fish had been in rearing tanks with their siblings prior to the experiment and had not been in specific OSRs. There were two replicates from each stock tank, resulting in a total number of 48 mating pairs housed in 1-litre containers. To account for the OSR and to minimize inbreeding, the males were rotated within the OSR to prevent them from mating with their potential sibling.

Before being transferred to their new environments, the fish were anaesthetized in 0.15 g/L Tricane methanesulfonate (MS-222) and 0.3 g/L sodium bicarbonate ($NaHCO_3$).

Once unresponsive, they were placed on a paper towel saturated with water and measured using electronic calipers. They were then placed under a dissecting microscope (Wild Heerbrugg) using a lamp as a light source and had their fins gently splayed using a paint brush. Their pictures were taken at 6X under the microscope with a ruler in frame as a size reference for future measurements.

After being placed in the containers with mating pairs, the fish were given a two-week period to acclimate to their new environment prior to egg collections to ensure that any eggs the females produced had been fertilized by the male in the container with her, as to avoid the possibility of internal fertilization. Throughout the experiment, the container water was changed every week with a mixture of water from the stock tanks and Reverse Osmosis Deionization (RODI) System water (1.55 ml/gal reef salt (Instant Ocean), 0.5 ml/gal stress coat (API), and 0.15 ml/gal alkaline buffer (Seachem)). Before use, the ion concentration, pH, and oxygen concentration of the RODI water were measured to ensure they were at safe levels for the fish. Each week, prior to changing the container water, the nitrogen cycle was monitored using pH, nitrite, nitrate, and total ammonium/ammonia test kits (API). This ensured that there were bacteria in the containers that were cycling the fishes' waste. The containers for the generation 2 fish were equipped with gravel and fake plants for habitat enrichment.

2.3 Egg collections and rearing generation three

Eggs were collected in the mornings; females with eggs were isolated in a small container and their eggs were gently removed using a small paint brush. On average, eggs were collected from 7 of the 48 females in a given day. The eggs were placed in a petri dish full of a rearing solution (1.0 g/L NaCl, 0.03 g/L KCl, 0.04 g/L $CaCl_2 \bullet 2\ H_2O$, 0.163 g/L

$MgSO_4 \bullet 7\ H_2O$, and 10 ml 0.01% Methylene Blue) and a photograph was taken under a microscope with a 12X lens; a ruler was in frame as a size reference for future measurement and assessment of fertilization success. Once the photo was taken, the eggs and rearing solution were placed in labelled ice cube trays where they would hatch approximately nine days later. Once hatched, the larval medaka were placed in labelled 1-litre containers where they remained until sexually mature adults.

2.4 Euthanization and testis dissections

Once the required number of offspring were collected and reared from generation 2, they were euthanized in 0.6 g/L MS-222 and 1.2 g/L $NaHCO_3$. To ensure that the fish had been euthanized, their spinal cords were severed using a razor blade by making a cut on the dorsal side between their eye and operculum. This procedure was done in small batches, approximately twenty fish at one time, to minimize deterioration as the fish had to be sampled and immediately dissected. The fish were measured for standard body length using electronic calipers and their photograph was taken under a 6X lens, with their fins splayed with a paint brush prior to any dissections.

Male fish were dissected with micro-dissecting scissors (Fine Science Tools). First, a fish was longitudinally cut from the anus to the throat on the ventral side of the fish using the micro-dissecting scissors; the cut was shallow to avoid damage to organs. Following this, the fish was pinned to a small piece of rubber using dissection pins, with the anterior facing left. Two transverse cuts were made, one from the anus to the dorsal side of the fish and one from the throat to the dorsal side of the fish, cutting posterior to the pectoral fin and gills. This, and the proceeding dissection steps, were done under a 12X lens for easier viewing. Any connective tissue holding the skin onto the organs was

scraped away using a pair of forceps and the piece of skin was removed from the fish by cutting longitudinally along the dorsal side of the fish. This effectively left a window in the fish to see its organs.

Using two pairs of forceps, the intestines were carefully removed from the fish in a downward motion. The testis are located behind the other internal organs, so care had to be taken when removing the other tissues as to not puncture any of the organs. Once exposed, another picture of the testis inside the male was taken under the 12X lens of the microscope. Using both pairs of forceps, the testis was pinched on the caudal and cranial ends and removed from the body of the fish; forceps were used to remove any debris, such as adipose tissue, from the testis while being careful not to puncture the gonads. After all debris was removed, a picture of the gonads was taken under a 24X lens on the microscope after which the testis were carefully placed high up on the side of a labelled 1.5 ml microcentrifuge tube. All of the labelled tubes, each containing one testis, were left open and placed in a large container with a petri dish of drying agent on the side. The large container was closed and sealed with parafilm to prevent any moisture from entering the container. The testis were left to dry for approximately 8 weeks and were then weighed to the nearest 0.001mg to determine testis size.

2.5 Statistical analysis

Image J 1.52q, an imaging software, was used to analyze the photos of the fish and eggs that were taken throughout the experiment. A global scale was set using the ruler in the photos and the measurements were taken by hand. Anal fins were traced using the freehand selection tool, and their area (mm^2) was taken while only the diameter (mm) of the eggs was measured using the freehand lines tool. While measuring the eggs, the clutch

size was also recorded. The testis were weighed using an analytical balance (Perkin Elmer AD6 Autobalance) to the nearest 0.001mg.

All statistical analysis was completed in R, version 3.6.1 (R Core Team, 2019). Correlations between sexual traits (anal fin area, standard body length, and testis weight) were modelled using correlation plots, but the correlation and heritability factors were determined using linear regressions (LMs). The slope of the regression provided the narrow sense heritability factor, (h^2).

The influence of operational sex ratio (OSR) on parental traits was modeled using boxplots (five number summaries; the median is represented with a bold horizontal line, the 25^{th} and 75^{th} quartiles are the boxes on either side, and the whiskers represent the minimum and maximum values), and analyzed with general linear models (GLMs) and Tukey post-hoc tests. Operational sex ratio and standard body length were used as predictors of variation in the different sexual traits. Tukey post-hoc tests were run to determine if there were pairwise differences between groups.

2.6 Ethical statement

All procedures were carried out in accordance with the Standard Operating Procedures outlined by Saint Mary's University Animal Care Committee under protocol 17-04.

3.0 Results

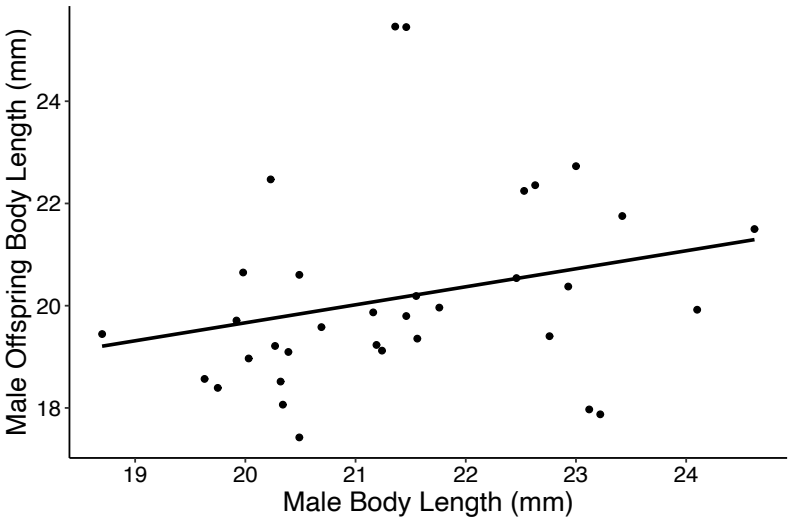
3.1 Determining heritability of sexual traits

Offspring body lengths were averaged for each tank and compared to the average parental lengths which resulted in a significant linear regression and considerable heritability factor (R^2=0.1100, p=0.0254, h^2=0.5430, Figure 3.1.1 c). Body length was also separated by sex and analyzed in the same manner. Male and female body lengths had much weaker relationships on their own (R^2=0.0234, p=0.1354, h^2=0.2916, and R^2=0.1019, p=0.0262, h^2=0.3925, respectively, Figure 3.1.1 a and b).

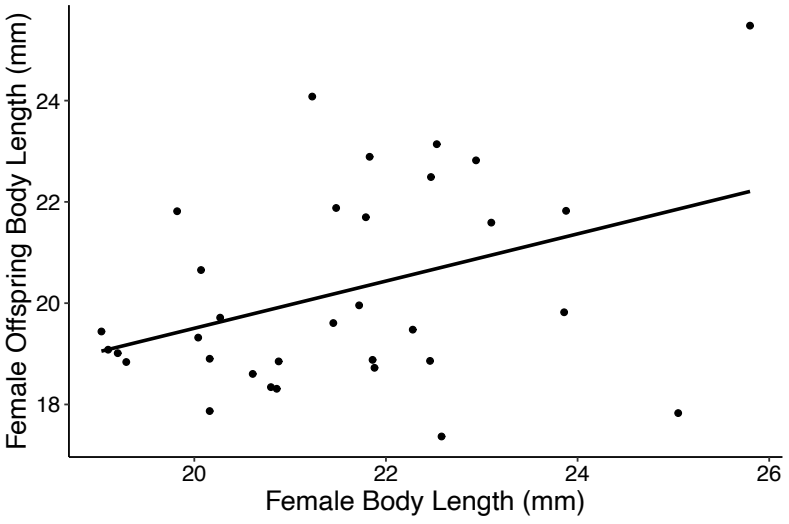
Anal fins are sexually dimorphic in medaka, so male offspring were compared with their male parent and female offspring were compared to their female parent (Figure 3.1.2 a and b). Neither male nor female anal fin area had a significant adjusted correlation coefficient (R^2=0.0332, p=0.1728 and R^2=-0.0201, p=0.5083, respectively); however, the linear regression indicated heritability factors of h^2=0.3295 for the males and h^2=0.1004 for the females (Figure 3.1.2 a). When offspring were compared to the averaged parents with no regard to sex, there was also no significant prediction and the linear regression was intermediate (R^2=-0.0104, p=0.4161, h^2=0.2455, Figure 3.1.2 c). Testis weight had the highest heritability factor and had a significant adjusted correlation coefficient (R^2=0.3446, p=0.0010, h^2=0.6286, Figure 3.1.3).

Larger males were found to have larger testis (R^2=0.1904, p=0.0014, Figure 3.1.4 a) but body size was not associated with anal fin size (R^2=-0.0212, p=0.7992, Figure 3.1.4 a). A correlation between male testis weight and male anal fin size was conducted but there was no relationship found between the two traits (r=0.044, p=0.7711, Figure 3.1.5).

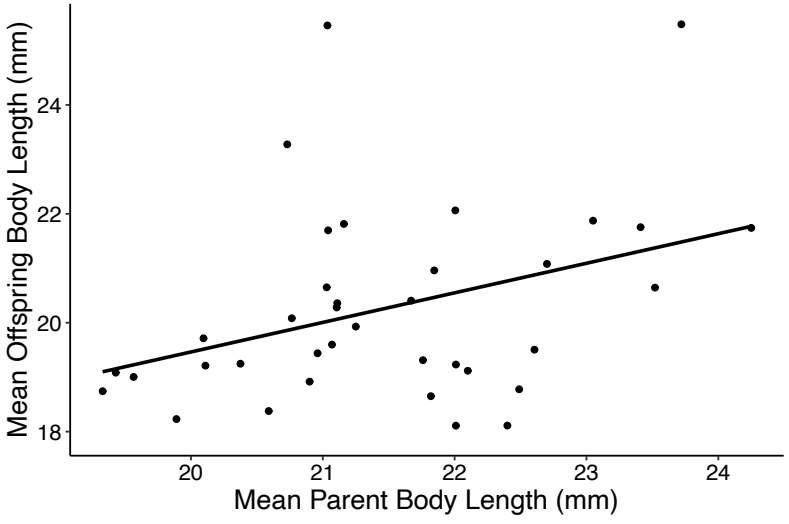
Figure 3.1.1: Heritability of body length categorized by sex and averaged for each tank, analyzed using linear regressions. A) Average male offspring body length (mm) with respect to their parental male body length (R^2=0.0234, p=0.1354, h^2=0.2916). B) Average female offspring body length (mm) with respect to parental female body length (R^2=0.1019, p=0.0262, h^2=0.3925). C) Mean offspring body length for each tank with respect to mean parent body length per tank (R^2=0.1100, p=0.0254, h^2=0.5430).


Male Offspring Body Length (mm)
24
22
20
18
19
20
21
22
23
24
Male Body Length (mm)

A)

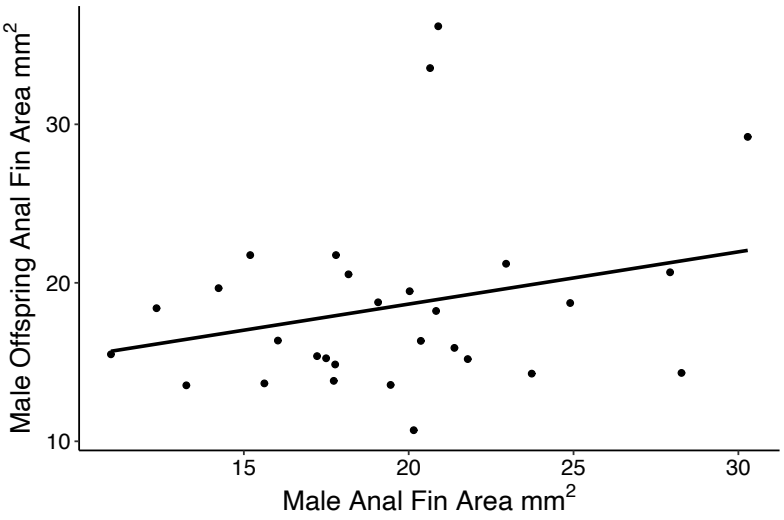

Female Offspring Body Length (mm)
24
22
20
18
20
22
24
26
Female Body Length (mm)

B)

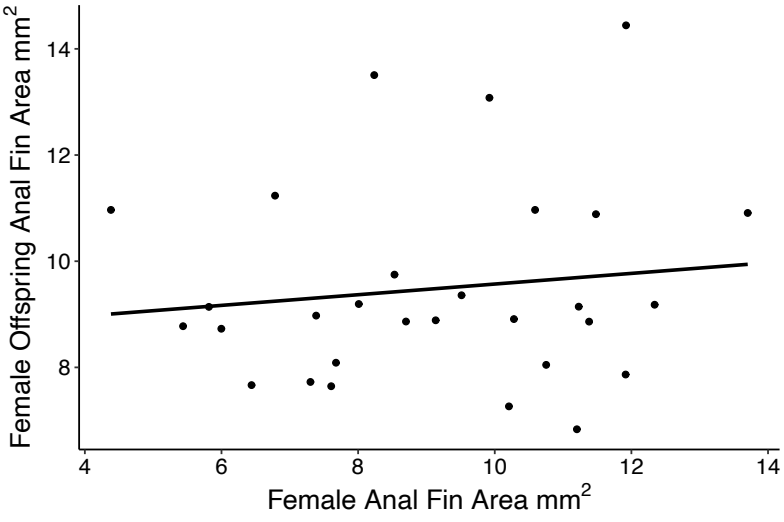

Mean Offspring Body Length (mm)
24
22
20
18
20
21
22
23
24
Mean Parent Body Length (mm)

C)

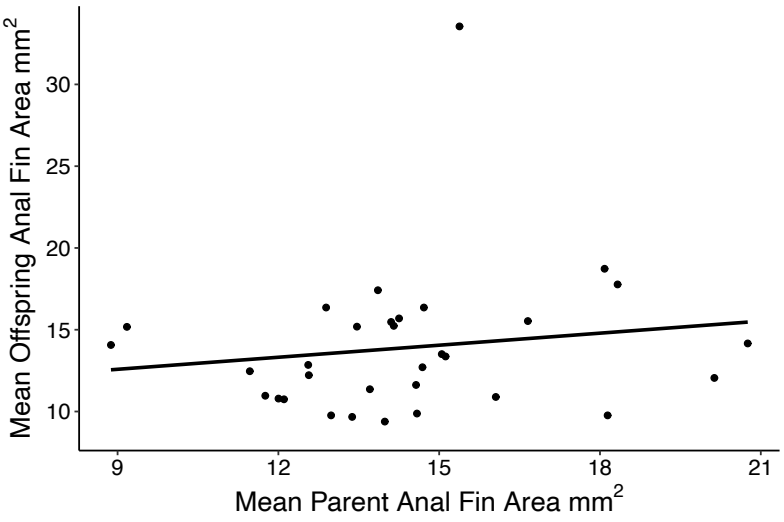
Figure 3.1.2: Heritability of anal fin area by sex and tank, analyzed with linear regressions. A) Mean male anal fin area per tank with respect to parental male anal fin area (R^2=0.0332, p=0.1728, h^2=0.3295). B) Mean female anal fin area per tank with respect to parental female anal fin area (R^2=-0.0201, p=0.5083, h^2=0.1004). C) Mean offspring anal fin area per tank with the mean parental anal fin area per tank (R^2=-0.0104, p=0.4161, h^2=0.2455).


Male Offspring Anal Fin Area mm2
30
20
10
15
20
25
30
Male Anal Fin Area mm2

A)


Female Offspring Anal Fin Area mm2
14
12
10
8
4
6
8
10
12
14
Female Anal Fin Area mm2

B)


Mean Offspring Anal Fin Area mm2
30
25
20
15
10
9
12
15
18
21
Mean Parent Anal Fin Area mm2

C)

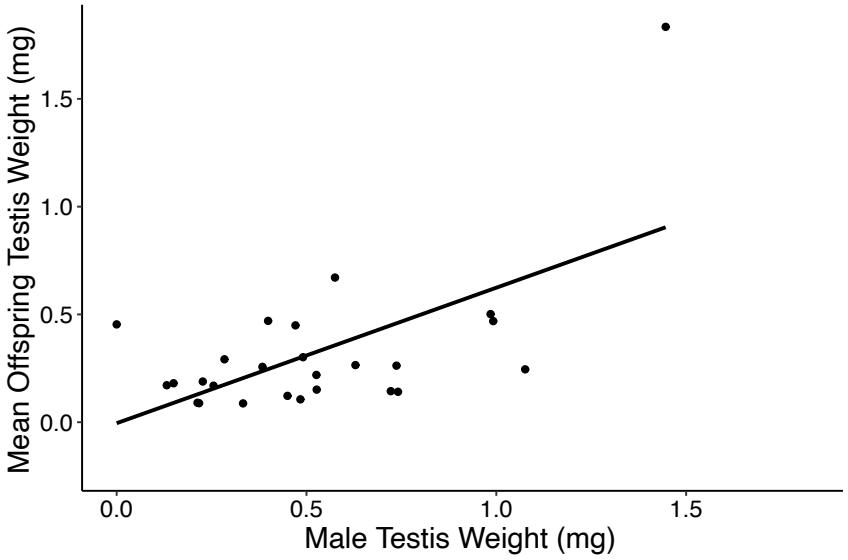


Figure 3.1.3: Heritability of testis weight modeled with a correlation plot and analyzed with a linear regression. The correlation coefficient was significant, and the heritability factor was high (R^2=0.3446, p=0.001, h^2=0.6286).

Table 3.1.1: Summary of the heritability of medaka body length (mm), anal fin area (mm^2), and testis weight (mg). Linear regressions were used to predict the offspring traits from the parental traits. Each factor was separated by tank. Bolded rows are those with the highest heritability factors for that trait. Listed degrees of freedom, t-value, p-value, the adjusted correlation coefficient squared, and the narrow sense of heritability.

Offspring Trait	Parent Trait	*df*	t	p-value	Correlation	Heritability
Male Body Length	Male	32	1.5318	0.1354	0.0234	0.2916
Female Body Length	Female	31	2.3349	0.0262	0.1019	0.3925
Mean Body Length	**Parent**	**35**	**2.3342**	**0.0254**	**0.1100**	**0.5430**
Male Anal Fin	**Male**	**27**	**1.4004**	**0.1728**	0.0332	**0.3295**
Female Anal Fin	Female	27	0.6704	0.5083	-0.0201	0.1004
Mean Anal Fin	Parent	30	0.8246	0.4161	-0.0104	0.2455
Testis Weight	**Male**	**24**	**3.7608**	**0.000962**	0.3446	**0.6286**

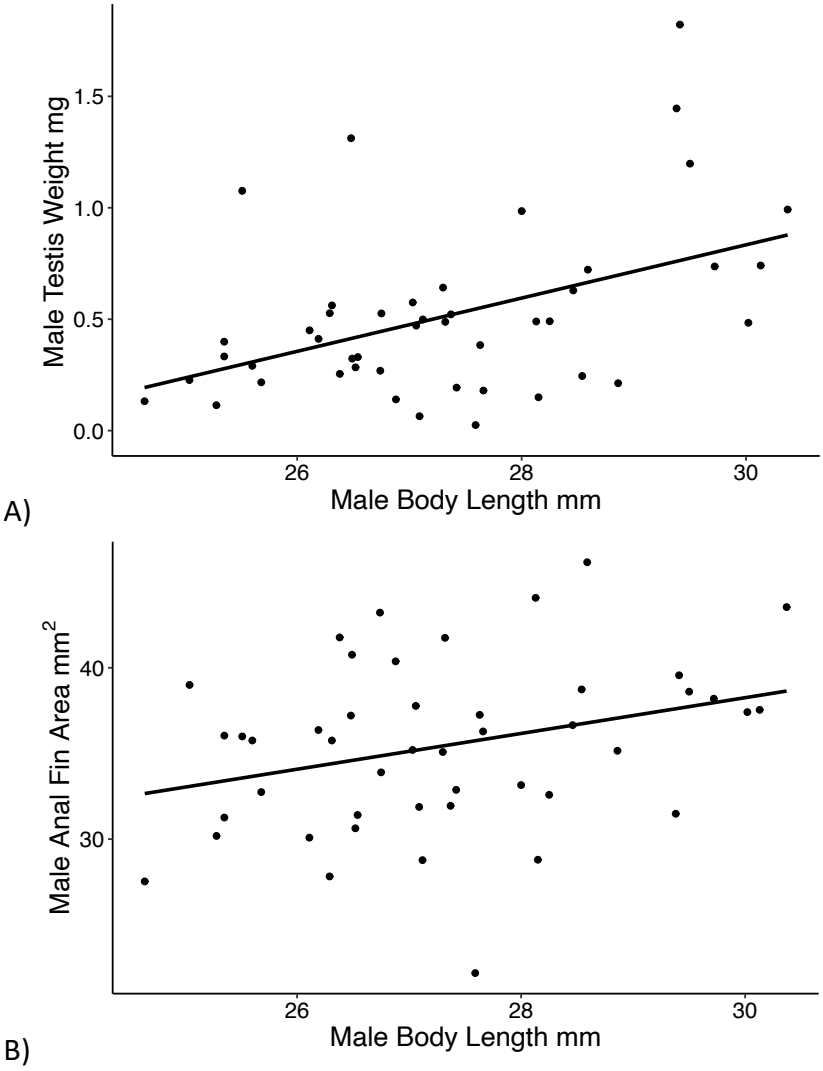


Figure 3.1.4: The influence of male body length (mm) on testis weight (mg) and male anal fin area (mm^2) analyzed with a correlation test. A) There was a significant relationship between testis weight and body length (R^2=0.1904, p=0.001); and B) no relationship between body length and anal fin area (R^2=-0.0212, p=0.799).

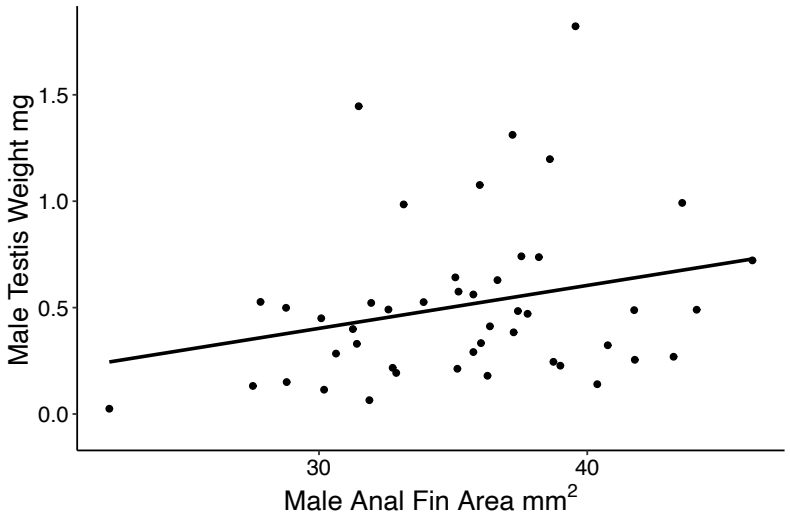


Figure 3.1.5: The correlation between testis weight (mg) and anal fin area (mm^2) in male medaka. There was no significant relationship between the two factors (r=0.044, p=0.7711).

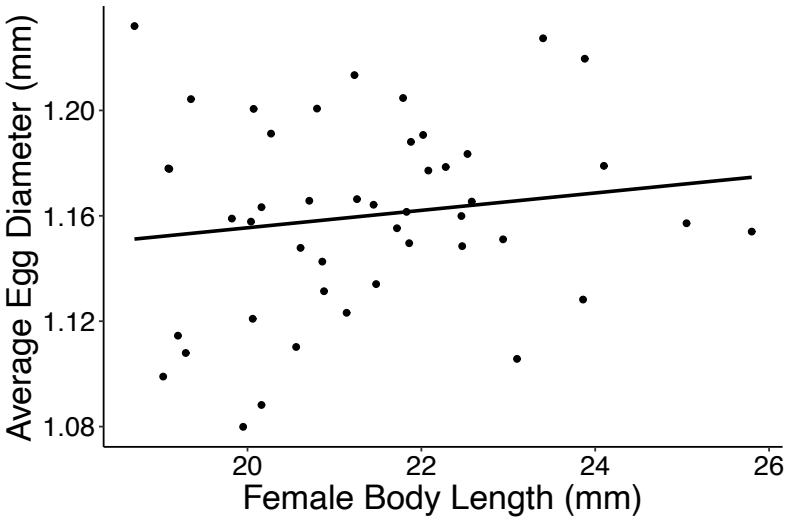
3.2 Female fecundity

An average clutch size and average egg diameter was calculated for each female from data collected over the course of the experiment. It was found that larger females did not produce larger clutches or bigger eggs (R^2=0.000, p=0.9981 and R^2=-0.0085, p=0.4182, respectively; Figure 3.2.1 a and b). Larger clutches also do not produce smaller eggs or vice versa (R^2=0.0634, p=0.0504, Figure 3.2.1 c).

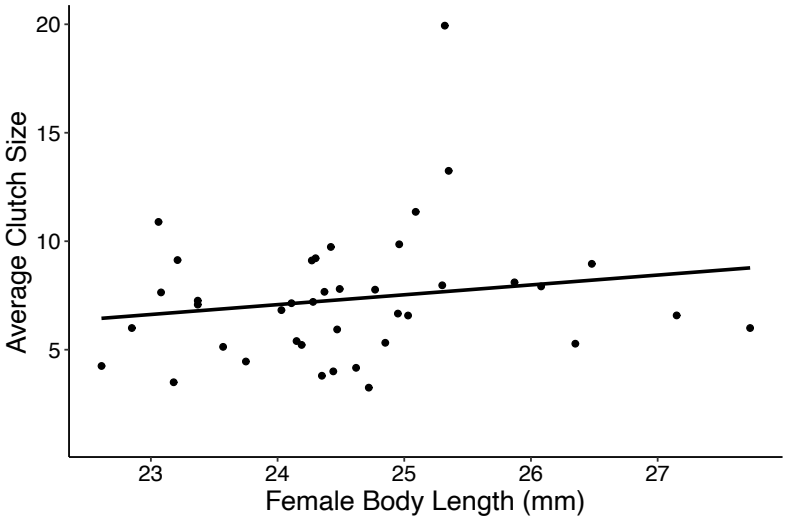
Table 3.2.1: Relationship between female body length (mm), egg diameter (mm), and clutch size. Egg diameter and clutch size were averaged for each female. Listed is the degrees of freedom (*df*), t-value, p-value, and the adjusted correlation coefficient squared (R^2).

Independent Variable	Dependent Trait	*df*	t	p-value	Correlation
Female Body Length	Egg Diameter	38	0.8185	0.4182	-0.0085
Female Body Length	Clutch Size	44	0.0150	0.9981	0.0000
Clutch Size	Egg Diameter	44	-2.0120	0.0504	0.0634

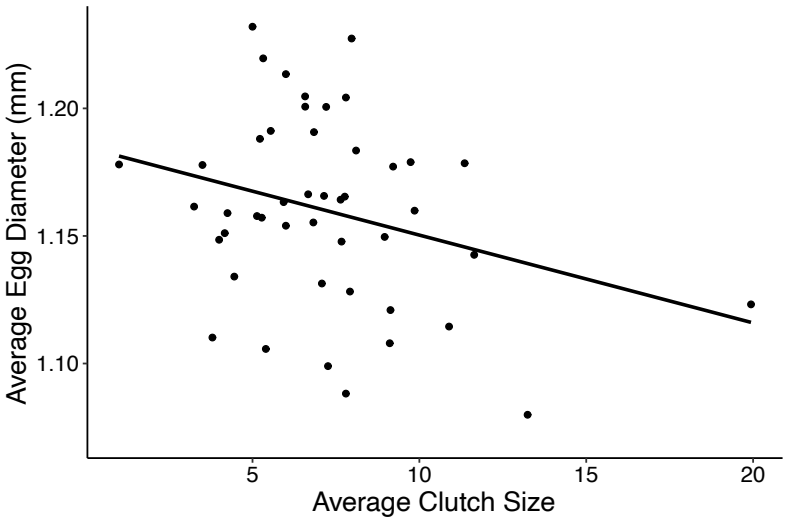
Figure 3.2.1: The relationship between female body length (mm), the mean clutch size, and the mean egg diameter. A) The influence of female body length on mean egg diameter (R^2=-0.0085, p=0.4182). B) Influence of female body length on mean clutch size (R^2=0.0000, p=0.9981). C) Mean egg diameter with respect to mean clutch size (R^2=0.0634, p=0.05036).


Average Egg Diameter (mm)
1.20
1.16
1.12
1.08
20
22
24
26
Female Body Length (mm)

A)


Average Clutch Size
20
15
10
5
23
24
25
26
27
Female Body Length (mm)

B)


Average Egg Diameter (mm)
1.20
1.15
1.10
5
10
15
20
Average Clutch Size

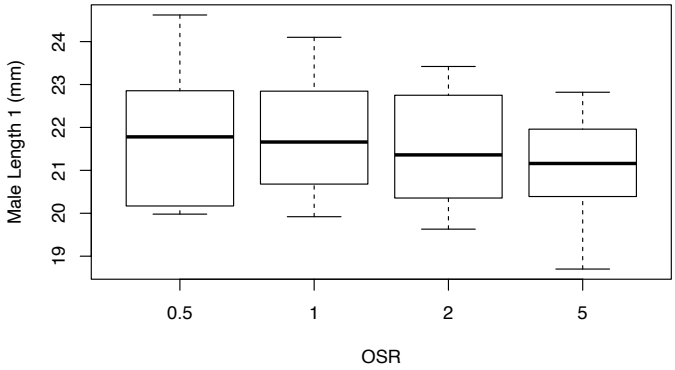
C)

3.3 Influence of OSR on parental traits

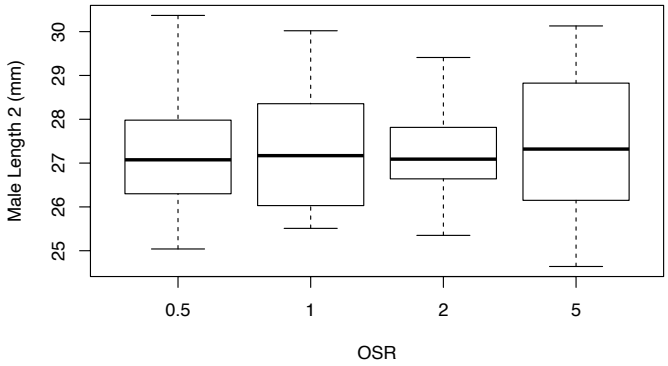
The first trait analyzed for influence of OSR was standard body length, for both the males and females of the parental generation (Figure 3.3.1). There was a very weak influence of OSR on the body length for males and females; the intercept held the most weight for the prediction (Table 3.3.1).

The influence of OSR on parental anal fin area was also very weak for females, but stronger for males (Table 3.3.1). Female anal fins were best predicted by Length (Table 3.3.1). OSR and length together had the greatest influence on male anal fin area, but length alone also predicted anal fin area (Table 3.3.1). Post-hoc testing showed that there was a significant difference between OSR 2 and 5 for male anal fin area (Figure 3.3.2 a). The final parental trait analyzed for OSR influence was testis weight, which also had a weak influence of OSR and was best predicted with OSR and length + OSR x length, but length also predicted OSR (Figure 3.3.3, Table 3.3.1).

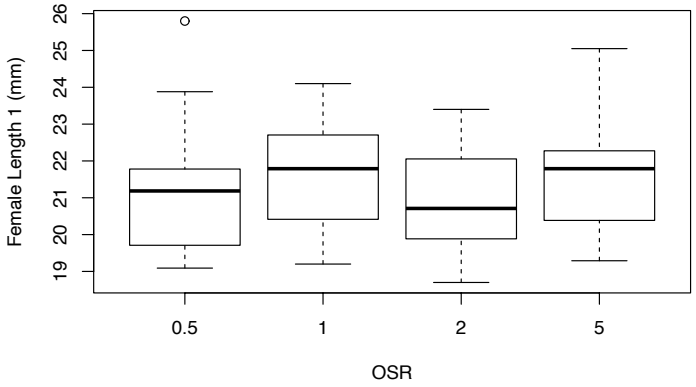
Figure 3.3.1: The influence of OSR on standard body length for the parental fish. General linear models determined that there was a weak influence of OSR on body length in both males and females throughout the experiment. A) Male length per OSR at the beginning of the experiment. B) Male length per OSR at the time of euthanization. C) Female length at the beginning of the experiment per OSR. D) Female length per OSR at the time of euthanization.


Male Length 1 (mm)
19
20
21
22
23
24
0.5
1
2
5
OSR

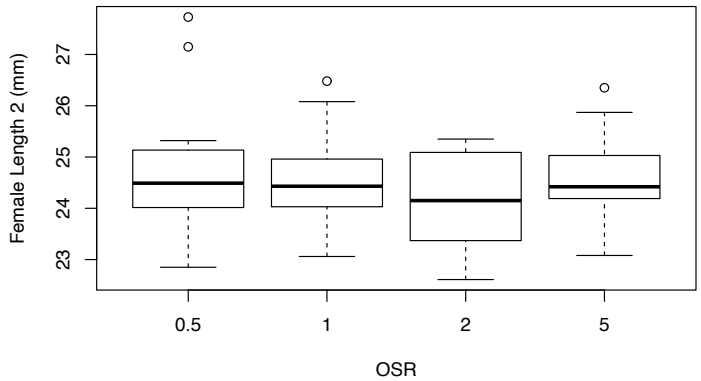
A)


Male Length 2 (mm)
25
26
27
28
29
30
0.5
1
2
5
OSR

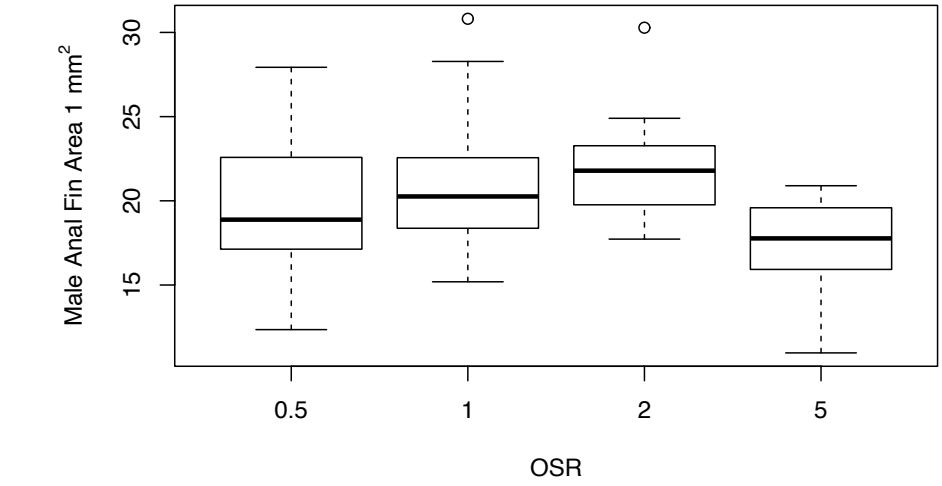
B)


Female Length 1 (mm)
19
20
21
22
23
24
25
26
0.5
1
2
5
OSR

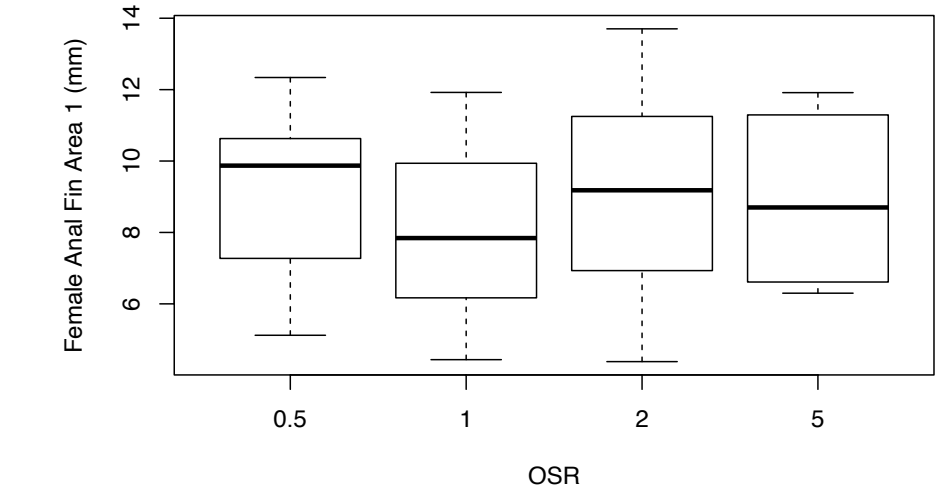
C)


Female Length 2 (mm)
23
24
25
26
27
0.5
1
2
5
OSR

D)



A)



B)

Figure 3.3.2: Male and female anal fin areas by OSR at the beginning of the experiment represented with a five-number summary. A) Male anal fin area had a moderate influence of OSR. B) Female anal fin area had a weak influence of OSR.

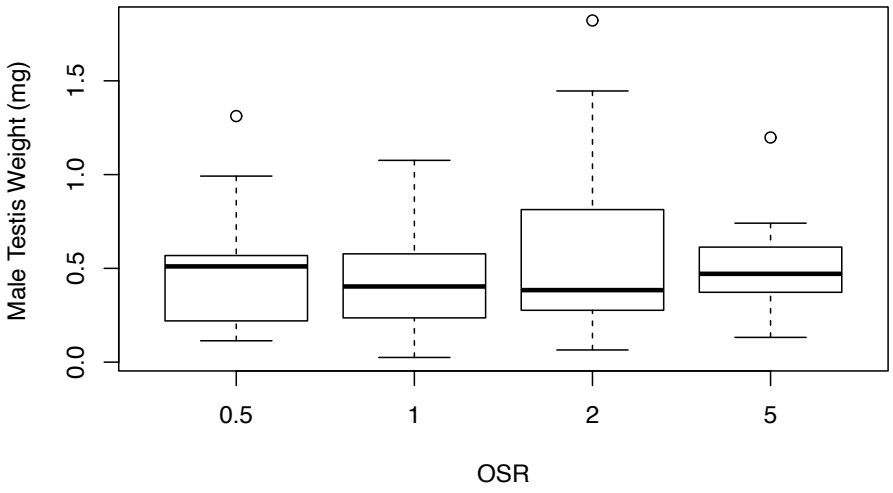


Figure 3.3.3: Influence of OSR on testis weight (mg) represented with a five-number summary. Horizontal black lines represent the median. There was a weak influence of OSR on the testis weight, the most weight was designated to OSR when body length was also factored into the general linear model.

Table 3.3.1: Influence of OSR on male and female body lengths (mm), anal fin areas (mm^2), and male testis weight (mg) determined using general linear models. Degrees of freedom, AICc values, ΔAICc values, and weight for various predictors of each trait. Bolded rows represent data that best fits the model.

Trait	Predictor	*df*	AICc	ΔAICc	Weight
Male Length 1	**(Intercept)**	**2**	**161.5**	**0.00**	**0.913**
	OSR	5	166.2	4.71	0.087
Male Length 2	**(Intercept)**	**2**	**168.7**	**0.00**	**0.970**
	OSR	5	175.6	6.93	0.030
Female Length 1	**(Intercept)**	**2**	**180.1**	**0.00**	**0.940**
	OSR	5	185.6	5.49	0.060
Male Anal Fin Area	**OSR + Length**	**6**	**259.2**	**0.00**	**0.517**
	Length	**3**	**259.6**	**0.36**	**0.431**
	OSR	5	265.0	5.76	0.029
	(Intercept)	2	266.8	7.52	0.012
	OSR + Length + OSRXLength	9	266.9	7.65	0.011
Female Anal Fin Area	**Length**	**3**	**210.9**	**0.00**	**0.784**
	(Intercept)	2	214.5	3.63	0.128
	OSR + Length	6	215.9	4.98	0.065
	OSR + Length + OSRXLength	9	218.7	7.84	0.016
	OSR	5	220.3	9.43	0.007
Testis Weight	**OSR + Length + OSRXLength**	**9**	**35.6**	**0.00**	**0.645**
	Length	**3**	**36.9**	**1.32**	**0.334**
	OSR + Length	6	43.0	7.37	0.016
	(Intercept)	2	45.4	9.78	0.005
	OSR	5	51.2	15.65	0.000

4.0 Discussion

4.1 Heritability of body length and testis

Standard body length in medaka is monomorphic, which is why there was a stronger relationship across generations with data averaged between the sexes (Shima & Mitani, 2004). A significant linear regression and narrow sense of heritability factor of 0.543 indicates that additive genetic variation is playing a role in the variation in this trait; however, it cannot be concluded that other factors are not at play. Although it is a monomorphic trait, when given the choice between small or large males, females always choose the larger males; males also choose larger females (Grant, 1995; Howard et al., 1998). This indicates an influence of sexual selection in the development of this variation across generations in experimental populations.

A strong correlation between male body length and testis weight was found, indicating that larger males could have a reproductive advantage against smaller males. This information, in conjunction with the high heritability factor for testis weight implies that larger males will produce offspring that have both a larger body size and larger testis, creating a possible reason as to why females choose to mate with larger males. Previous studies have shown that larger male medaka have a higher fertility rate after multiple matings, which could indicate that gonad size is correlated to spermatogenesis, as is true for various other fishes (Howard et al., 1998; Stockley, Gage, Parker, & Møller, 1997).

Standard body length was not best predicted by OSR, but body length was standardized across OSRs at the beginning of the experiment, so this was expected. A previous study also found a weak influence of OSR on male body length, but a close

relationship across generations, supporting the claim that the trait is influenced, in part, by heritability (Allen, 2019).

4.2 Heritability of anal fins

Anal fins are a sexually dimorphic trait, with the fins being highly important in male reproductive strategies, and reproductive function being unknown in females (Koseki et al., 2000). Therefore, the weak influence of OSR on female anal fin area and the low correlation between parental females and female offspring was expected. On the other hand, there was a stronger influence of OSR on the male parental anal fins at the beginning of the experiment, which has been found in previous experimental populations (Allen, 2019). The same study was using fish that had been randomly assigned to various operational sex ratios, so the strong influence suggested plasticity of the trait (Allen, 2019). The linear regression performed across generations was not significant, indicating that the parental male anal fin areas were not predicting the male offspring anal fin areas. By the end of the experiment, there was only a weak influence of OSR on male anal fin areas, suggesting that a lack of social environment caused the trait to standardize across OSRs.

Although there was a heritability factor of approximately 0.3, it cannot be said that additive effects of alleles are contributing to the variation in this trait, and there must be other factors at play that are influencing male anal fin size (Visscher et al., 2006). Other factors could include sex hormones, the influence of the female parent on the male offspring, or plasticity. Testis size may be, at least in part, influenced by sex hormones; therefore, a correlation test between the testis weight and anal fin area was run to determine if this was influencing variation for the anal fins as well (Yoshikawa et al.,

2020). There was no relationship between testis weight and anal fin area, suggesting that sex hormones are not influencing anal fin area. Although male anal fins are reproductively important, females chose their mates based on body size, not anal fin size (Koseki et al., 2000). When testing whether male body length could predict male anal fin areas, there was no significant regression. Alongside other evidence, the lack of narrow sense heritability supports the argument that male anal fin area could be plastic.

4.3 Female fecundity

Other studies on female medaka have suggested that larger females produce larger clutches (Teather et al., 2000). However, I did not find a correlation between female body length and her average clutch size or average egg diameter. The origin of the literature discrepancy is unknown but could be attributed to the differences in measuring female body size (weight versus length). Some bony fish species have strong female fecundity, such as salmonids (de Eyto et al., 2015; Thorpe et al., 1984). The variation across species could be due to the differences in their life-histories. Salmonids only reproduce once a year, so producing an abundance of eggs during that period is essential to an individual's fitness (de Eyto et al., 2015). Medaka reproduce almost every day in the wild during their breeding season and every day in the laboratory (Koseki et al., 2000). Both reproductive strategies result in a sufficient amount of offspring being produced; the amount of time available to produce these offspring is what causes the extreme variation in clutch sizes.

A typical trade-off in egg-producing species is the idea that larger clutches will have smaller eggs and vise versa (Brown & Shine, 2009; Stearns, 1989). Large clutches of offspring increases the probability that some individuals will survive, even if they are small while smaller clutches have increased survival rates because the offspring are often

larger. A previous study on medaka demonstrated this trade-off, but I did not observe a significant regression of clutch size predicting egg diameter (Leaf et al., 2011). This could be due to the small size of the eggs, making any intraclutch egg size variation within narrow margins.

4.4 Conclusion

Further research on the relationship between females and their eggs would benefit medaka life-history research by reducing the doubt that surrounds some of the current data. For example, doing a larger scale study on whether clutch size predicts egg volume, or looking into intraclutch egg size variation and its implication in female reproductive strategies. Evidence of plasticity in male anal fin areas is mounting, but complex genetic variation should not be ruled out until studies support that notion. Although other factors are most certainly contributing to phenotypic variation in Japanese medaka, by showing that select sexual traits are inherited, we provide evidence that additive genetic variation is playing a role in the variation of standard body length and testis weight between and among experimental populations of Japanese medaka.

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