

The effect of operational sex ratio on fertilization success and clutch size in Japanese medaka

*(Oryzias latipes)*

By

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**Abstract**

The operational sex ratio is the number of fertilizable females to mature males in a population at a particular time. Variation in this ratio is often associated with change in behaviour during mating, including differences in male tactics. In Japanese medaka (*Oryzias latipes*), a species of freshwater fish found throughout Japan, there are two types of male alternative mating tactics: sneaking in which small males attempt to achieve some fertilization success by joining a spawning pair and releasing sperm, and interference where there is a disruption to a reproductive event. In addition, females adjust their clutch sizes in response to male behaviour. The operational sex ratio in this species varies across latitudes and this variation is linked to differences in mating behaviour and morphology. The objective of this work is to determine whether clutch size and proportion of fertilized eggs varies with differing operational sex ratio. To measure these responses, I collected eggs from Japanese medaka under four experimental operational sex ratios. I determined fertilization success and clutch size for females, as well as female growth rate over a 4-month period. There was no significant difference in fertilized eggs or clutch size among all four treatments. This can be attributed to fertilization assurance in higher operational sex ratios.

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## Introduction

Mating system structure is influenced by both phylogenetic and ecological factors (Emlen and Oring 1977). Mating systems are typically characterized based on the number of mates acquired by the non-limiting or most abundant sex of any given species (Emlen and Oring 1977; Andersson 1994). To better understand mating systems, it is important to determine how sexual selection shapes differential success among individuals in a population. Typically, the intensity of sexual selection is higher for males than females due to the differences in reproductive investment between the two sexes (Emlen 1977; Gopurenko 2007). This idea was first proposed by Darwin (1871), defined as a selective pressure that results in the evolution of characteristics that allow an individual to be successful in terms of mating by adopting certain behaviours or distinguishable features. For selective pressure to result in evolutionary change there must be a positive correlation between mating success and reproductive success such that it results in an increase in fitness for the individual (Jones et al. 2004).

Many studies claim that the strength of sexual selection can be shaped by environmental factors or constraints (Emlen and Oring, 1977; Cogliati et al. 2014). While mating systems are often considered as “fixed” properties of a species (Cogliati 2014), temporal or spatial variation across populations can alter the relative advantage of a particular mating behaviour or morphology. This can ultimately lead to a change in mating system structure (Emlen and Oring 1977).

The intensity of sexual selection is relatively low in monogamous groups but increases as reproductive success becomes skewed in polygamous groups. This relationship can be explained by variation in the operational sex ratio (OSR), the number of fertile males to fertilizable

females, which ultimately influences mating system structure (Emlen 1976). When measuring the effects of OSR, the greater the degree of imbalance in the number of individuals of each sex, the greater the expected variance in reproductive success. For example, if the ratio of all sexually active males in a population to receptive females is 8:1, the distribution of reproductive success is expected to be more strongly skewed than in a male to female ratio of 1:1, as in the biased sex ratio not all males will have the opportunity to mate and variance in reproductive success among males is expected to be high (Emlen and Oring 1977).

High variance in reproductive success leads to the evolution of alternative male mating strategies and tactics. Alternative reproductive tactics (ARTs) develop from fluctuations in environmental conditions, population densities and relative size of a rival (Emlen 2008). ARTs are a type of phenotypically plastic trait often referred to as a threshold trait, such that the expression of a certain tactic depends on the conditions an animal faces in its surrounding environment (Emlen 2008). ARTs include mutually exclusive tactics; they are not expressed at the same time in the same individual, but each individual holds the genetic potential to adopt either tactic in a particular condition (Emlen 2008). Males that compete aggressively for females may exhibit alternative tactics due to a shift in competitive modes associated with changes in the OSR and density of populations (Grant et al. 2000; Weir 2013). Sneaking is a type of ART commonly displayed in arthropods and some chordate groups (Emlen 2008). This tactic is often performed by small males who seek fertilizations while attempting to avoid conflict with larger males. A sneaker male uses stealthy movements and inconspicuous behaviour to fertilize eggs in the presence of another male. Typically, sneaker males do not fertilize as many eggs as males adopting more conventional tactics, but they obtain some reproductive success (Kokko 2008).

Fish with external fertilization release sperm into the water, making it difficult to monopolize access to females. This reproductive mode is favourable for the evolution of alternative mating tactics (Taborsky 2008). When ratios are biased, sneaking can be observed in which an unsuccessful male fish may join a spawning pair to achieve some reproductive success (Taborsky 2008). In OSRs that are strongly male biased it may be difficult for a male to guard a specific female for reproduction and variance is predicted to become lower as mate guarding and monopolization is not possible (Emlen and Oring 1977; Weir et al. 2011). In OSRs that are female biased, variance in reproductive success is predicted to be smaller as males have access to an abundance of females, and sneakers are expected to be less successful.

Japanese medaka (*Oryzias latipes*) exhibit male alternative mating tactics. This species is a tropical, freshwater fish found around the Japanese archipelago (Fujimoto et al. 2015). When mature, medakas copulate daily at dawn and can produce a clutch of approximately 25 eggs per day in quality habitats (Grant & Foam 2002). Sexual selective pressure on the medaka has resulted in sexual dimorphism; males tend to have large anal fins which are used to hold the female during copulation, but females have larger bodies at maturity. Male anal fin size and behaviour varies with latitude across their natural range, and this variation can be attributed to variation in OSR (Fujimoto et al. 2015). The occurrence of alternative mating tactics in the medaka may also result in sneaking behaviours from sexually active males seeking fertilizations (Weir 2013). Mating in Japanese medaka involves a tight coupling of males and females, but sneak matings and fertilization success by more than one male during a single mating event occur frequently under some conditions (Grant et al. 1995; Weir 2013). In addition, males that do not initiate copulation can disrupt matings, resulting in a decrease in fertilization success (Grant et al. 1995; Weir 2013).

Sneaking is more likely to occur in OSRs that are male-biased (Grant et al. 1995; Weir 2013), as males are less likely to secure a female and those who do not mate may use this approach to pass on their genes to the next generation. Sneaking in medaka may occur as a result of changes in competitive mode among males when OSR changes; as OSR becomes increasingly male-biased, contests for mates may switch from interference competition, which is characterized by aggression, to scramble competition observed as the searching and securing of mates (Grant et al. 2000). Under scramble competition conditions, sneaking may be more prevalent, and thus the variance in reproductive success among males may decrease.

In highly male biased OSRs, there is more interference than in OSRs closer to unity (Grant et al. 1995; Weir 2013). Interference is a type of scramble competition associated with a disruption in reproductive events, where a male will insert himself between a spawning pair to prevent successful coupling (Weir 2013). In addition, interference can result in a reduction in egg fertilization, as well as an increase in female refusal behaviour (Weir 2013). In medaka, there is an association between clutch size and male courtship rate (Weir and Grant 2010), such that clutch size decreases as males decrease courtship rate due to sperm depletion. Thus, an adjustment of clutch size may also be observed when sex ratios are highly male biased, and interference occurs more frequently.

To assess the effects that OSR has on clutch size and fertilization success in female Japanese medaka, I measured both clutch size and fertilization success across different OSRs. I predict that because of a breakdown of aggressive defense at very highly male-biased OSR, variability in mating success will reach a peak and then subsequently decline as interference begins to occur. When OSR is female-biased, the distribution of male success should be

relatively even because females are abundant and most males should get the opportunity to mate. I am also interested in whether or not growth rate varies across operational sex ratio as well as if the relationship between body size and clutch size varies among operational sex ratios; these were assessed secondarily to my main experimental objectives.



## Methods

### 2.1: Study Species and Experimental Design

Two hundred and eighty-eight juvenile Japanese medaka (*Oryzias latipes*) were obtained from Aquatic Research Organisms in New Hampshire, U.S.A. Fish were housed in freshwater at a salinity of 0.25ppt and temperature ranging from 26-29°C. These parameters were used to replicate a medaka's natural aquatic environment. Lights above the tanks simulated daytime with a 14-hour daylight period, with lights on at 7h45.

#### 2.1.1: Tagging, measuring and fin clips

Fish were anaesthetized using 0.15g/L MS222 (Tricaine S) buffered with 0.3g/L Sodium Bicarbonate in 1L of water. The fish were tagged by placing Visual Implant Elastomer tags under the first layer of the skin in two of four different locations on each fish, each having its own unique combination of tag colours (pink, blue, black, orange, green or yellow). A fish was placed on a damp paper towel and a paint brush was used to spread out the anal fins.

Photographs of the fish were taken at 60x magnification through a dissecting microscope. A small ruler was placed next to the fish while photographs were being taken for size reference. Standard length measurements were taken using Vernier calipers. A small portion of the caudal fin was cut and then placed in a labeled tube in 95% ethanol for later DNA extraction using a GenElute™ Mammalian Genomic DNA Miniprep Kits. Fish were chosen for each OSR at random, but the mean and variance among tanks was approximately the same. The average male size ranged from 18-25mm and average female size ranged from 18-24mm.

### 2.1.2: Tank Set-up

Two hundred and eighty-eight fish were then placed in 24 10 gallon tanks (50cmx25cmx30cm) in groups of 12 and at four different operational sex ratios: 4:8, 6:6, 8:4 and 10:2. Fish were sexed based on morphological traits, being classified as male by the presence of a large, parallelogram-shaped anal fin or as female based on the presence of a smaller triangular anal fin and with a large, rounded abdomen. Fish were fed twice a day: in the morning with frozen adult *Artemia* and in the afternoon with live *Artemia* nauplii.

## 2.2 Data Collection

### 2.2.1 Egg Collection

For a two-month period, eggs were collected from females on a daily basis. Each OSR treatment was sampled each day. Females with eggs on their ventral side were identified and collected in small plastic containers, ensuring a sufficient amount of water in the dish for the female to remain submerged. A small paint brush was used to brush along each female's ventral side to remove the eggs. The eggs were placed into a petri dish and immersed in rearing solution (1.0g NaCl, 0.03g KCl, 0.04g CaCl<sub>2</sub>· 2H<sub>2</sub>O, 0.163g MgSO<sub>4</sub>· 7H<sub>2</sub>O, 10mL 0.01% methylene blue and distilled water to 1L). Eggs that were adhered together with filaments were separated using a metal spatula to ensure that eggs were completely separated and submerged in the rearing solution. Each female's identification was recorded before she was placed back into her original tank. Another female with eggs was then identified and this process was repeated for every female for approximately four tanks a day. Twelve tanks were sampled twice while the other twelve tanks were sampled three times. The number of times a tank could be sampled was restricted by time allowed for the experiment.

### *2.2.2 Counting Fertilized Eggs*

Successful egg fertilization was assessed 24 hours after the eggs were first placed in rearing solution. Fertilized eggs were identifiable by the formation of a perivitelline space between the outer and inner egg membranes. The egg itself has a yellowish hue due to the presence of yolk. When observing the difference between fertilized and unfertilized eggs, the blue dye in the rearing solution (methylene blue) will enter unfertilized eggs, resulting in a blue colour. The number of fertilized and unfertilized eggs for each female was recorded. Embryos remained in rearing solution for 9 days, after which they were stored in a 1ml tube with 95% ethanol for later DNA analysis.

### **2.3 Data Analysis**

Data were analyzed using Graphpad Prism™. Relationships between operational sex ratio and fertilized eggs, unfertilized eggs, and total clutch size were tested using a one-way ANOVA. To determine if operational sex ratio impacted female growth rate, the relationship between growth rate of females and operational sex ratio was assessed using a one-way ANOVA. Linear regression was used to determine whether the relationship between clutch size and body size varied across OSRs.

## Results

### 3.1: Measurements

Proportion of fertilized eggs, body size, clutch size and growth rate for each operational sex ratio are summarized in Table 1.

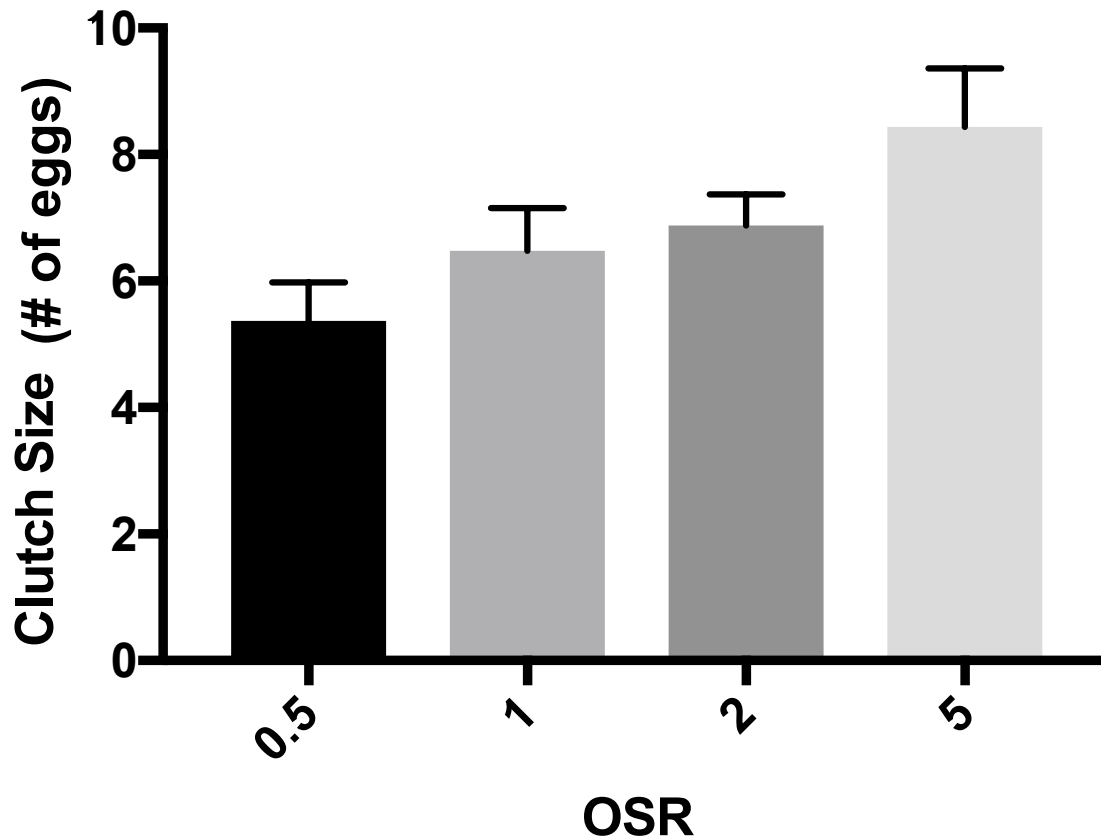
**Table 1:** Proportion of fertilized eggs (%) with standard error (SE), mean body size (cm) with standard error (SE), the average clutch size (number of eggs) with standard error (SE) and the average growth rate (mm/3 months) with standard error (SE) for four different operational sex ratios.

<b>Operational Sex Ratio</b>	<b>Proportion of Fertilized Eggs (%)</b>	<b>Initial Body Size of Females</b>	<b>Clutch Size (number of eggs)</b>	<b>Growth Rate (mm/3 months)</b>
4:8 (0.5)	95.3 ±2.13	21.99cm ± 0.370cm	5.44 ±1.00	1.87 ±0.172
6:6 (1)	92.9 ±2.80	21.08cm ± 0.346cm	5.50 ±0.701	2.38 ±0.233
8:4 (2)	97.8 ±1.22	22.12cm ± 0.263cm	7.50 ±1.21	1.92 ±0.247
10:2 (5)	100 ±0	22.76cm ± 0.254cm	7.20 ±1.85	2.09 ±0.660

### 3.2 Effects of Operational Sex Ratio

#### 3.2.1 Operational sex ratio vs clutch size

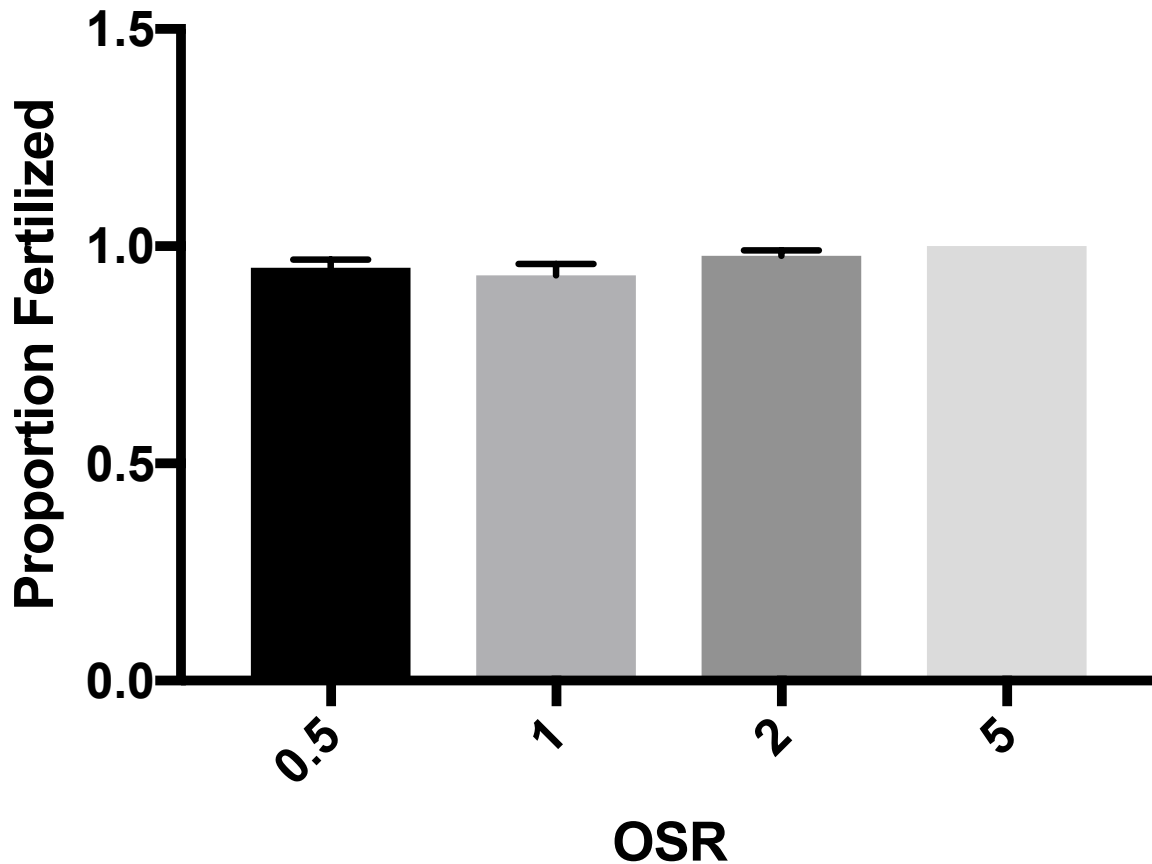
The relationship between clutch size and OSR was not significant (one-way ANOVA:  $F_{3,20}=0.40$ ,  $P=0.76$ ; Figure 1).



**Figure 1:** Relationship between operational sex ratio and clutch size. Data are averages and error bars represent standard error.

### 3.2.2 Operational sex ratio vs proportion of fertilized eggs

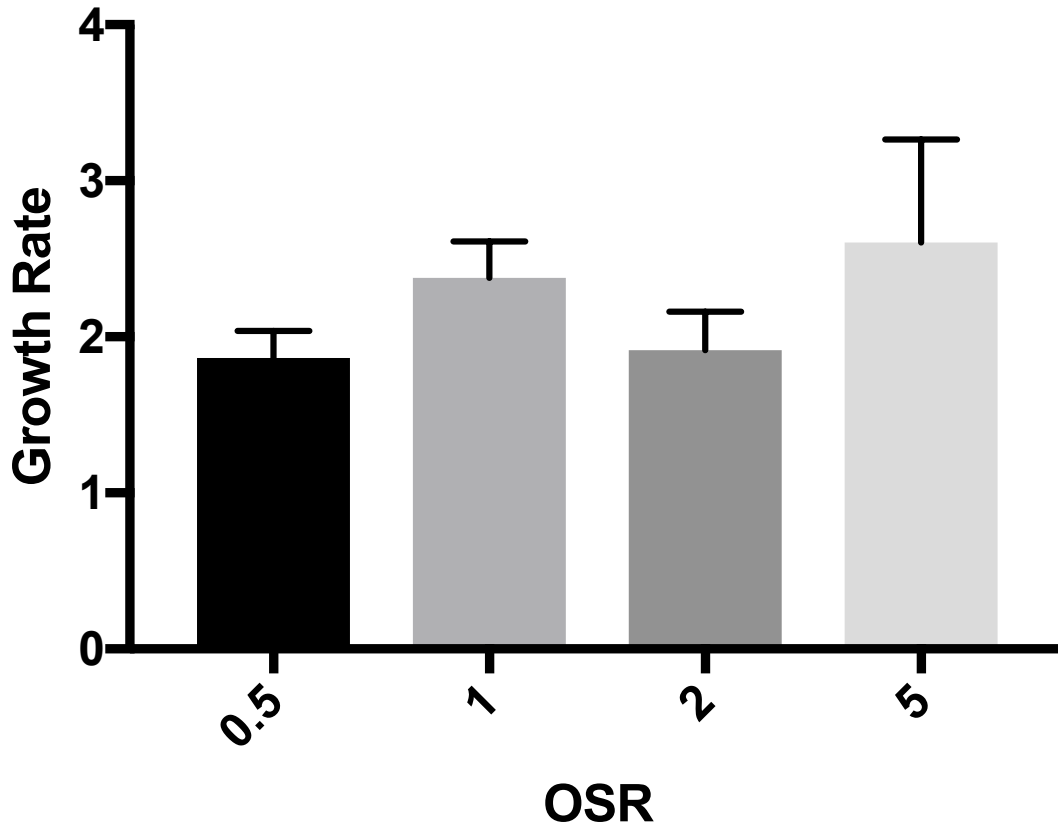
The relationship between OSR and proportion of fertilized eggs was not significant (one-way ANOVA:  $F_{3,20} = 1.49$ ,  $p=0.22$ ; Figure 2).



**Figure 2:** Relationship between operational sex ratio and proportion of fertilized eggs. Bars represent averages; error bars are standard error. Operational sex ratio of 5 had a fertilization success rate of 100%, therefore there is no error bar.

### 3.2.3 Operational sex ratio vs growth Rate

There was not a significant relationship between growth rate (mm/3 months) and OSR (one-way ANOVA:  $F_{3,20} = 2.67$ ,  $p = 0.6177$ ; Figure 3)



**Figure 3:** Relationship between operational sex ratio and growth rate. Bars represent averages; error bars are standard error

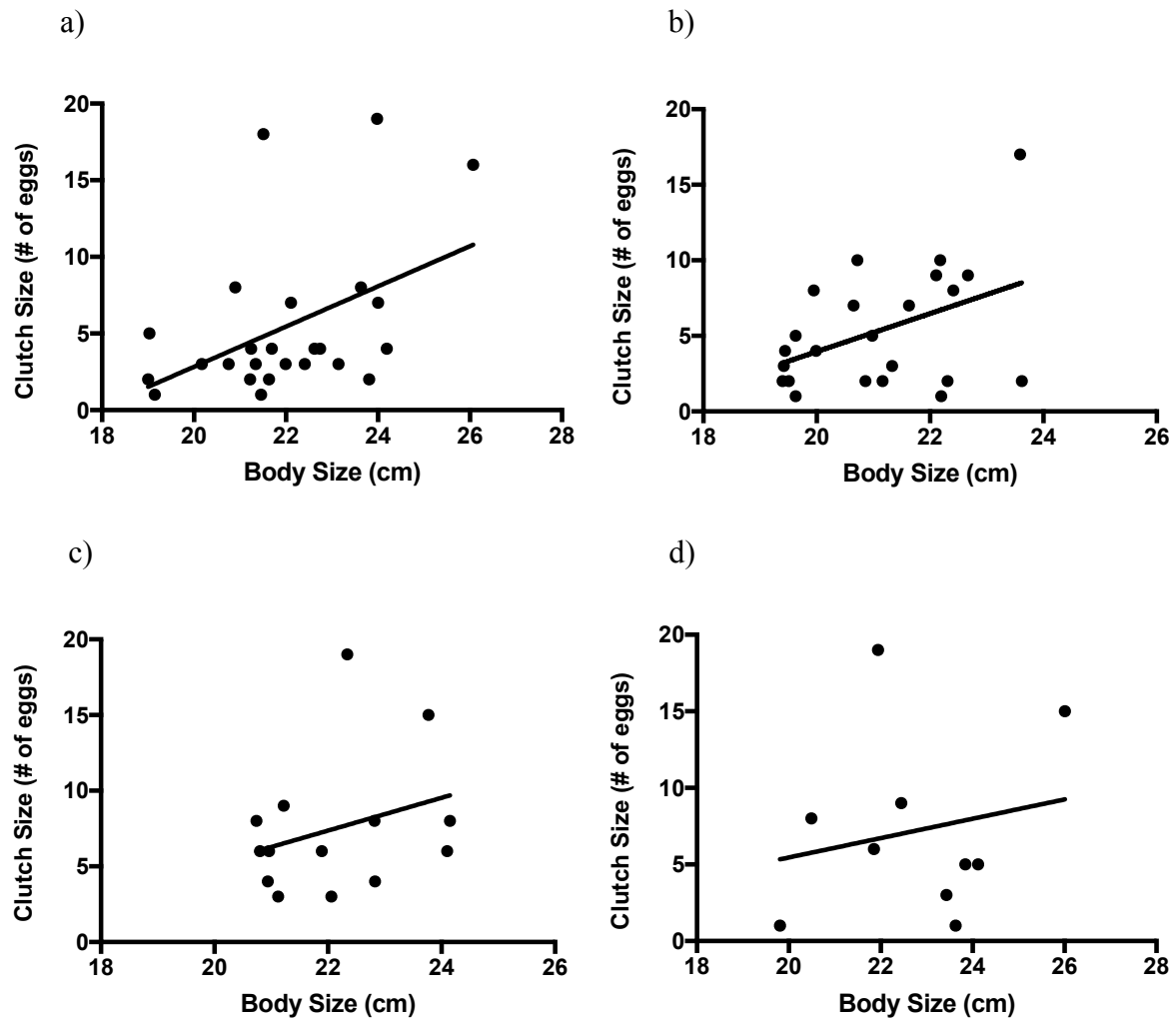
### 3.2.4 Female body size vs clutch size

There were significant relationships between body size and clutch size for the lower OSRs (0.5 and 1), while they were not significant for the male-biased OSRs (Table 2, Figure 4). Outlying data points in OSRs 0.5, 1, 2 and 5 are real data and were not removed for this reason.

**Table 2:** Regression analysis on female body size versus four different operational sex ratio treatments. The slope, F-statistic and p-value are summarized for each operational sex ratio.

	OSR			
	0.5 (4:8)	1 (6:6)	2 (8:4)	5 (10:2)
Slope	1.31± 0.54	1.31± 0.54	1.08± 1.0	0.63± 1.11
F statistic	F <sub>1,23</sub> =5.938	F <sub>1,21</sub> =4.766	F <sub>1,12</sub> =1.169	F <sub>1,8</sub> =0.3246
p-value	p=0.0230	p=0.030	p=0.31	p=0.58





**Figure 4:**

Relationship between clutch size and body size for OSR of: a) 4:8; b) 6:6; c) 8:4; d) 10:2.

## Discussion

The proportion of fertilized eggs and clutch size did not differ across operational sex ratios. However, there was a positive trend in clutch size with increasingly male-biased sex ratios. Relationships among body size and clutch size for four different operational sex ratios differed across OSRs; the 0.5 and 1 operational sex ratios were significantly positive, while the operational sex ratios of 2 and 5 were not. Overall, there were observed positive trends in each operational sex ratio, indicating larger females typically produced larger clutch sizes.

The relationship between proportion of fertilized eggs and operational sex ratio did not match my predictions. I predicted that at a high male density, disruption during mating would result in a decrease in spawning duration, egg fertilization and clutch size (Weir 2013; Klemme et al. 2007). However, the observed absence of this relationship from this study could be attributed to the alternative hypothesis that increasing the number of males may also increase fertilization assurance. This could be because the larger number of males in a sex ratio, the more eager they are to mate, resulting in increased fertilization. Vahl et al. (2013) found no indication of fertilization success correlated with sex ratio, much like I observed in this experiment. Similarly, the absence of any observed decrease in the proportion of fertilized eggs in the highly male biased operational sex ratio observed in this experiment could be because female encounter rate is lower, high-quality males may not fully express their competitive potential (Dreiss et al. 2010). The constant harassment from males may force females to mate with more males, therefore giving them a higher reproductive success because more sperm would be available.

Frequent copulation during mating may be another type of fertilization assurance in extremely high male biased ratios in medakas, ensuring fertilized eggs regardless of the operational sex ratio because of an abundance of sperm. Often, a strongly male biased OSR leads to stronger sexual selection over generations (Dearborn 2001). Typically, as the operational sex ratio becomes skewed towards males, polygyny should occur, where individual males can monopolize more than one female and where some males may gain more success than others (Emlen and Oring 1977). More males mean more competitors; this could result in more available sperm for fertilization, thus increasing fertilization success.

Successful reproduction depends on a series of complex courting events such as male encounter, mate choice and mating (Vahl et al. 2013). Unfortunately, spawnings were not directly observed during this experiment. The results with respect to clutch size and the proportion of fertilized eggs may be due to lack of interference, which was typically expected to occur at extremely male biased sex ratios. If interference was occurring as predicted, it is likely that interference could result in a decrease in fertilization success and clutch size in the female medaka (Weir and Grant 2010). This indicates that females are more likely to decrease their clutch size in response to extremely highly male biased ratios where interference is presumed to be occurring more. Similarly, females respond to male signals of fertility by reducing clutch size (Weir and Grant 2010).

Based on previous results in this experiment that male growth increased with OSR, I examined female growth rate. While not significant across treatments, female growth rate was highest in the 10:2 operational sex ratio. The findings are consistent with other studies showing

that intensity of selection acting on female body size was higher in male biased operational sex ratios (Fitze & Gaillard 2008). This could be because females are being subjected to more sperm in the 10:2 operational sex ratio, therefore a faster increase in body size may be beneficial to compensate for the cost of breeding (Fitze & Gaillard 2008).

Larger females can have more eggs (Darwin 1874). This is likely a result of selection that may have resulted from the onset of size-dependent costs of increased male aggression for females in the highly male-biased operational sex ratio (Fitze & Gaillard 2008; Head & Brooks 2006). This is a trend commonly seen over generations, however this experiment was only done over a single generation, and thus differences in growth rate are attributable only to phenotypic plasticity. The increased size may also be due to the larger observed clutch sizes in females in male biased operational sex ratios. These extra eggs may have been an extra source of nutrients for the fish to feed on, leading to larger female size in higher operational sex ratios.

The relationship between female body size and clutch size was significantly positive in the 4:8 and 6:6 operational sex ratios, however it was not significant within the 8:4 and 10:2 operational sex ratios. Body size is often subject to variation depending on nutrition, temperature, environmental conditions and genes (Gürtler et al. 2017). Typically, larger females have higher fecundity (Marshall et al. 2013; Monroe 2015) because in theory larger females have more space and capacity to produce eggs (Darwin 1874). Marshall et al. (2013) predicted that female body size is more sensitive to environmental change than male body size. This may result in lack of a significant relationship between body size and clutch size in the higher male biased operational sex ratios. The male biased environment may affect only the fecundity rate of

females. Outlying data points in all four OSRs could be influential to the significant relationship found in OSRs of 4:8 and 6:6. These points were left in the analysis because they are real data collected from the experiment and therefore should not be excluded.

Operational sex ratio and the effects on fertilization success and mating system structure show differing trends throughout literature (Weir 2013; Klemme et al. 2007; Vahl et al. 2013). The results obtained from this study suggest that operational sex ratio does not significantly affect clutch size or the proportion of fertilized eggs. Fertilization assurance is most likely the reason for these results; the more sperm in the environment, the more likely eggs will become fertilized and the larger the clutch sizes will be. Studies on growth rate of females in operational sex ratios are scarce, and though some studies suggest that females grow in more highly-male biased ratios faster than female-biased, the reason is unclear (Fitze & Gaillard 2008). Operational sex ratio does show interesting effects on clutch size, proportion of fertilized eggs, growth rate and relationship between clutch size and body size. Though some relationships were not significant and did not match predictions, the number of males in a sex ratio does alter some aspects of female reproductive success. The current literature appears to have mixed results (Weir 2013; Klemme et al. 2007; Vahl et al. 2013), and thus it would be beneficial to repeat similar experiments to determine if interference occurs at the rate at which we had predicted and observe any further effects of operational sex ratio on not only the medaka but other species as well.

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