The relationship between sexually selected morphological and behavioural traits in male Japanese medaka (*Oryzias latipes*) and their variation across social settings.

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Abstract

Sexual selection can lead to the development of secondary sex traits and mating behaviours. Morphological and behavioural traits may increase individual reproductive success, however, under varying sexual selection types and intensities the relative importance of morphology and behaviour on reproductive success will vary. This study examines the relationship between the morphology of a sexually selected trait (anal fin size) and three mating behaviours of male Japanese medaka (*Oryzias latipes*), and how their frequency varies across four different social settings. To determine if male mating behaviours varied across social settings, I manipulated the intensity of sexual selection in the laboratory by subjecting the medaka to four different operational sex ratios (defined as the ratio of sexually mature males to fertilizable females). Anal fin size, and aggression, following and courtship behaviours were quantified for all treatments. Based on previous literature, I predicted a positive relationship between mating behaviours and anal fin size in male biased operational sex ratios, as well as plasticity in the frequency of mating behaviours performed across varying social settings. I found a significant relationship between standard length (mm) and anal fin surface area (mm^2) in male Japanese medaka. In highly male biased operational sex ratios, there was a positive relationship between anal fin size and aggressive behaviour, and male medakas show plasticity in their mating behaviours across varying social settings (measured by OSR), as they react to varying intensities of sexual selection pressures.

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1. Introduction

1.1 Background

Sexual selection is a type of selection associated with increasing an individual's reproductive success and can lead to changes in morphological and behavioural traits that increase an individual's chances at mating. This process can occur in two ways: intersexually, where typically females choose their mates, with males often using elaborate courtship displays or ornaments to attract females, or intrasexually, where males fight directly with each other to gain access to a sexually mature female (Andersson, 1994).

Sexual selection can lead to the development of secondary sex characteristics (Réale et. al., 2009), which are often elaborate morphological traits including extremely long tails in some bird species or large antlers in ungulates (Andersson, 1994). This can happen through both behavioural and morphological plasticity, as well as through inter-generational evolutionary processes. These characteristics have a mating-related function yet are not part of the reproductive system. The function of these traits is to attract a female as a signal of mate quality, or to act as weapons during male-male combat for mates (Qvarnström & Forsgren, 1998). By either making the male more attractive to the female or increasing success during male-male fighting and aggression, the size and quality of secondary sexual characteristics is often positively correlated with an individual's reproductive success.

In addition to morphological characteristics, certain behaviours can increase an individual's reproductive success (Sasaki & Yamahira, 2016). Aggressive behaviours such as fighting and displays of strength allow males to compete with each other for females. Aggressive males are typically successful in mating systems where intrasexual conflict is high and females are a limiting resource (Emlen & Oring, 1977). Courtship displays are a set of behaviours in which individuals of one sex use to attract the attention of a potential mate. (Andersson, 1994). These behaviours are often repeated until the female accepts or rejects the male as a mate. Males who perform the most high-quality courtship displays are typically the most successful in mating systems where intersexual selection is strong (Sasaki & Yamahira, 2016).

Under many circumstances, there is a relationship between morphological characteristics and mating behaviours. For example, in promiscuous mating systems, the largest and most aggressive males normally secure the most matings, and therefore have increased reproductive success (Howard et. al., 1998). These males are able to monopolize access to reproductive females, as they possess both the morphological traits, such as larger combat ornaments or body mass, and the behavioural traits, such as aggression, to deter other males or fend them off physically. A combination of morphological and behavioural traits can allow a dominant male in the population to monopolize access to females (Emlen & Oring, 1977). This phenomenon typically occurs when mature males outnumber mature females during the mating season, as many males are competing for access to fewer females.

As the ratio of fertilizable females and sexually mature males (defined as the operational sex ratio) changes, the type of sexual selection and the intensity of selection pressure may also shift (Emlen & Oring, 1977). By using a range of different operational sex ratios (OSRs) in the laboratory, we can manipulate the intensity of sexual selection pressures and create different social situations across experimental populations. When operational sex ratios differ across populations, an associated shift in selection pressure can have an effect on mating behaviours, as well as the relative importance of morphological secondary sexual characteristics (Sasaki & Yamahira, 2016). Due to the variance in sexual selection pressures, populations experiencing different operational sex ratios may evolve differences in behavioural and morphological traits associated with reproduction, as reproductive success is highly dependent on morphological and behavioural traits (Fujimoto et. al., 2014).

1.2 Study Species

Japanese medakas (*Orzyias latipes*) are an excellent model for behavioural studies. They can be raised under laboratory conditions, and have easily observable and well defined mating behaviours (Howard et al., 1998; Ono, Y. & Uewmatsu, 1957). In optimal conditions, Medakas spawn daily at first light (Fujimoto et. al., 2014). By creating an optimal laboratory environment and manipulating the photoperiod, it is very simple to observe spawnings and mating behaviours of a population. Male medakas also exhibit three well defined mating behaviours (aggression, following and courtship) (Ono & Uewmatsu, 1957) which vary in frequency depending on the type and intensity of sexual selection pressures present within a given population (Fujimoto et al., 2014; Grant, et. al., 2000; Grant et. al., 1995; Howard et al., 1998).

In addition, the medaka anal fin is sexually dimorphic, with the males having significantly larger fins than the females. The anal fin plays an important role in fertilization success (Koseki et. al., 2000). During copulation, medakas will align in a horizontal, parallel position, and males will wrap their dorsal and anal fins around the female (Ono & Uewmatsu, 1957). Larger anal fins aid in positioning the female and ensuring sperm does not scatter throughout the water column, increasing fertilization rate and overall reproductive success (Koseki et al., 2000). Variation in anal fin size occurs across populations with varying operational sex ratios in the wild, which suggests that there is selective pressure on this trait associated with competition for mates (Fujimoto et al., 2014). Although body size of the Japanese medaka is not a sexually dimorphic trait, previous research suggests that the largest males have increased mating success (Howard et al., 1998). Large body size is favoured by both competition and mate choice, which allows these males to monopolize the majority of matings and increase reproductive success (Grant et al., 1995; Howard et al., 1998). By examining both male anal fin size and body size, we can gain insight to which selective pressures may be present in a population.

Similar to variation in anal fin size, variation in mating behaviour also occurs across different operational sex ratios (Fujimoto et al., 2014; Grant, et. al., 2000; Grant et. al., 1995; Howard et al., 1998). When competing for females, males will often chase, nip and confront other males in the population (Magnuson, 1962). Successful males are typically the largest and most aggressive under male biased operational sex ratios that allow individual males to control access to mates (Fujimoto et al., 2014; Magnuson, 1962). However, when the operational sex ratio is highly male biased, male aggression tends to decrease (Grant et. al., 2000; Howard et al., 1998). This pattern is also seen in male courtship behaviour, which consists of a male approaching and following a female, and then performing "quick circles", where they circle quickly below the head of the female (Grant et. al., 2000; Ono & Uewmatsu, 1957). An increase in following, a type of harassment behaviour, is typically seen in highly male biased operational sex ratios. This behaviour is typically used as a form of mate guarding (Weir, 2013), or to coerce a female into spawning (den Hollander & Gwynne, 2009). An increase in following coupled with a decrease in both aggression and courtship behaviours generally characterizes a shift from direct interference competition, where males use aggressive behaviour to gain access to mates, to scramble competition, where alternative mating tactics such as sneaking are used when aggression becomes energetically unfavourable (Weir, 2013).

The variation of both morphology and behaviour of the Japanese medaka when under varying intensities and types of sexual selection pressures allow for the investigation of how operational sex ratio may influence morphology and behaviour, and sexual selection pressures within a population.

1.3 Study Objectives

While it is known that the operational sex ratio influences sexual selection pressures (Emlen & Oring, 1977), it is not clear whether the operational sex ratio is the true driver of morphological and behavioural plasticity in male Japanese medakas. It is also unclear whether a relationship exists between the anal fin size and specific mating behaviours of male medakas. This study aims to answer the following questions: (1) Is there a relationship between anal fin size and specific mating behaviours in male medakas? (2) Do these mating behaviours vary in frequency in varying social settings, measured by the operational sex ratio? To address these questions, I measured the anal fin surface area (mm^2) of individually marked, sexually mature male medakas from a single population that show variation in anal fin size. The fish were divided among treatments with different operational sex ratios and I measured individual male aggression, courtship and following behaviours over a two-month period. I predict that the males with the largest anal fins will be the most aggressive when the operational sex ratio is male biased. This prediction is based on a study by Fujimoto et. al. (2014), who determined that a Southern wild population in Japan was on average more aggressive and had a larger average anal fin size than that of a Northern Japanese population. The Southern population was also significantly male biased (approximately 2:1 males:females), which differed again from the Northern population that had an even ratio (Fujimoto et al., 2014). I also predict that as the operational sex ratio becomes increasingly male biased the frequency of aggressive and courtship behaviours of males will decrease, and the frequency of following behaviours will increase, as this pattern characterizes a shift from interference competition to scramble competition. Previous studies have demonstrated that as male bias increases significantly, acting aggressively becomes energetically unfavourable for the males and they must switch to alternative mating tactics to maintain reproductive success within the population (Jirotkul, 1999a; Mills & Reynolds, 2003; Weir, 2013).

2. Materials and Methods

2.1 Animal Marking and Identification

Two hundred and eighty-eight Japanese medaka (*Oryzias latipes*) were obtained from Aquatic Research Organisms (New Hampshire, USA). Upon reaching sexual maturity, fish were anesthetized using 0.15g/L MS222 buffered with 0.3g/L sodium bicarbonate. Each fish was then tagged for individual identification in two of four possible locations (left or right side, anterior or posterior to the dorsal fin) using Visual Implant Elastomer Tags (Northwest Marine Technology). Standard length in millimeters (defined as the length from the tip of the snout to the posterior end of the last vertebra) was measured using Vernier calipers and fish were placed under a dissection microscope at 15× magnification and photographed for future anal fin measurements.

2.2 Experimental Design

Four operational sex ratio (OSR) treatments were used for this experiment (male:female ratios of 0.5, 1, 2, 5). There were six replicates of each OSR treatment, for a total of twenty-four ten-gallon tanks (50cm x 25cmx 30cm). Each freshwater tank was kept at temperatures between 24°C and 27°C and equipped with an undergravel filter and air stone for water filtration and aeration. Artificial plants were also added to provide habitat enrichment for the fish. Each replicate block of four OSRs were separated onto six different shelves, and the position of tanks containing fish at a particular OSR was randomized within each replicate block. Twelve individually marked medakas from stock tanks were placed into each experimental tank at the appropriate operational sex ratio, maintaining the mean and variance as constant as possible among tanks. To simulate a natural environment, the fish were kept on a photoperiod that mimicked daily conditions during spring and summer (14h light: 10 hours dark), with lights on at 0745h. The fish were fed twice daily, with frozen adult brine shrimp (*Artemia* spp.) in the morning, and brine shrimp naupilii (*Artemia* spp) in the evening.

2.3 Behavioural Observations

All behavioural observations began between 0745h and 0815h, as medakas spawn at or shortly after first light. One replicate block of four OSR tanks was observed during each trial. I observed the replicate block from tanks left to right, as OSR treatments within treatment blocks were randomized. Prior to observation, I removed the artificial plants from each tank to improve visibility and allowed the fish five minutes to acclimate to this environment before I began observations. I observed each male medaka for two minutes, recording every aggression, following and courtship behaviour. The order of which I observed each individual fish was randomized for each treatment tank within the replicate block. I defined an aggressive behaviour as every time a male chased after or attempted to nip at the fins of another male. A following behaviour was recorded each time a male approached a female from underneath and followed her (Ono & Uewmatsu, 1957). I defined courtship behaviour as a quick circle underneath a female's head (Ono & Uewmatsu, 1957). Each of the three behaviours was quantified by counting the frequency of occurrence in each two-minute period. This observational procedure was repeated three times in random order of replicate blocks, to give a total of six minutes of observation time for each of 168 male medakas.

2.4 Fin Measurements

I used ImageJ to measure the area of each male's anal fin (mm^2) in the photographs taken during the tagging procedure. Firstly, I calibrated the ImageJ scale by using the ruler photographed with each fish under the dissecting microscope at 15x. I then used the zoom and freehand drawing tool to outline each male's anal fin. I used the measure tool to analyze the area of the fin I outlined with the freehand tools, which calculated the total surface area (mm^2) for each male anal fin by taking into account the scale I had calibrated when first opening the program.

2.5 Statistical Analysis

All statistical analyses were performed using the program RStudio, an interface of the program R (R Foundation for Statistical Computing, 2016). To determine if there was a significant relationship between anal fin surface area and standard length in male medakas, I conducted a linear regression analysis. Similarly, I conducted regression analyses to assess the relationships between anal fin size and each mating behaviour recorded during the observational experiment (aggression, following and courtship). To determine if variation in mating behaviours was associated with different operational sex ratios, generalized linear models were used to assess the relationship between each mating behaviour and operational sex ratio.

3. Results

3.1 Relationship Between Anal Fin Size and Mating Behaviours

A statistically significant relationship was found between standard length (mm) and anal fin surface area (mm^2) in the 168 male medakas ($F_{1,165}$= 199, R^2=0.54, p-value <0.001). Because of this association, I used the anal fin surface area relative to standard length to assess the relationship between anal fin size and mating behaviour. There was a significant relationship between total aggressive behaviour and relative anal fin size in male biased OSRs (OSR 2: $F_{1,46}$=6.46, R^2=0.1041, p-value<0.01; OSR 5: $F_{1,58}$=6.186, R^2=0.8207, p-value<0.01; Figure 2A). By contrast, there were no statistically significant relationships between aggression and relative anal fin size was deemed significant for OSR 0.5 or OSR 1, or for following and courtship in any of the four OSRs (Figure 2B, 2C).

3.2 Male Mating Behaviour Across Varying Social Settings

The average rate of male following behaviour varied significantly across OSRs (p-value<0.001; Figure 3C). There was no significant variation in both the average aggression and courtship rates of male medakas across varying operational sex ratios. Trends of increasing aggression and decreasing courtship were observed as the operational sex ratio became increasingly male biased (Figures 3A, 3B).

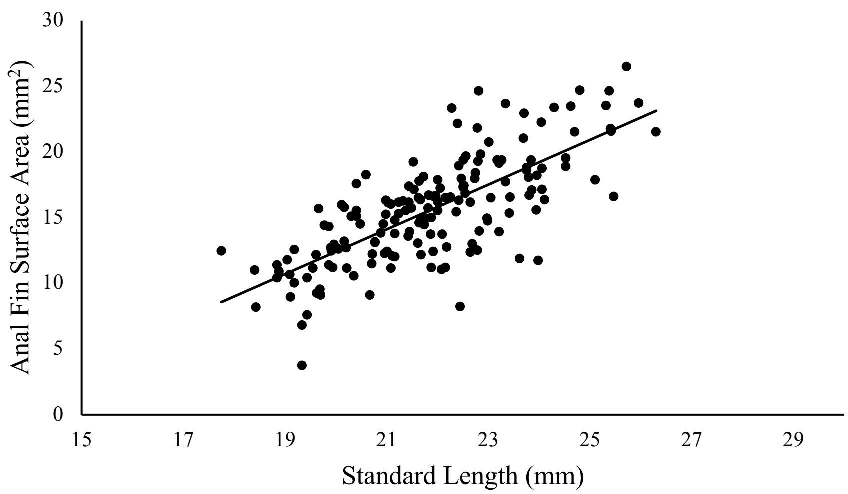

Figure 1. The relationship between standard length (mm) and anal fin surface area (mm^2) of 168 male Japanese medakas (*Oryzias latipes*).

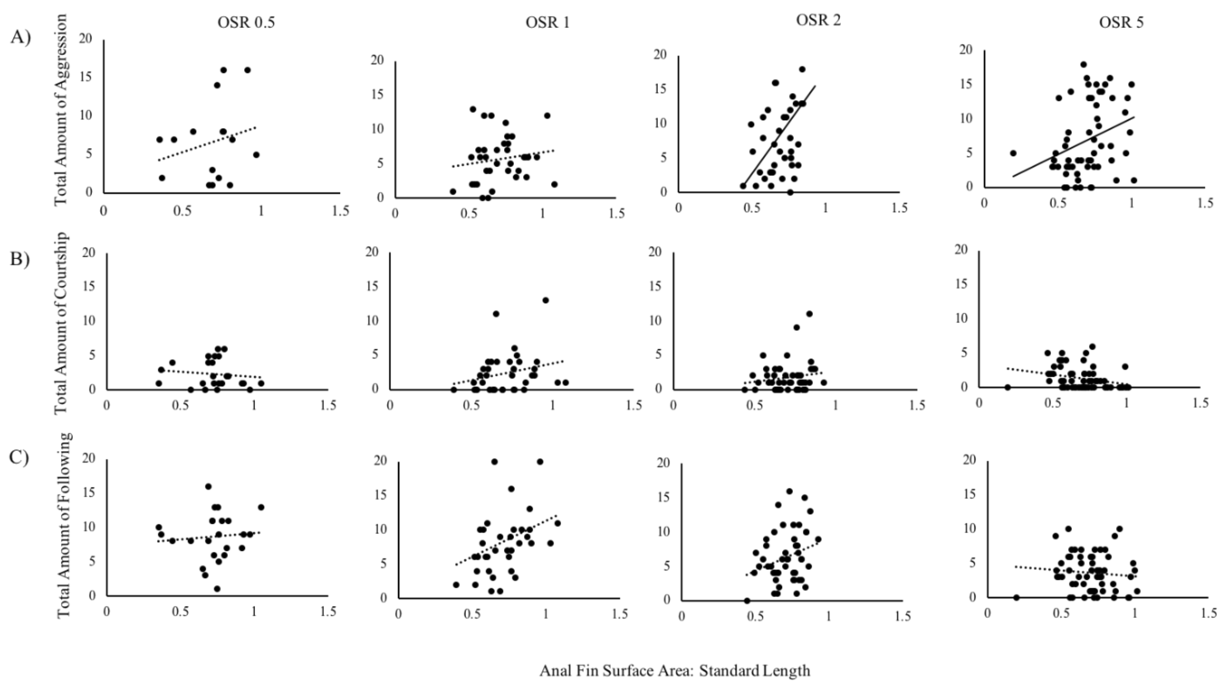

Figure 2. The relationship between A) aggression, B) courtship, C) following, and anal fin surface area (mm^2) in 168 male Japanese medakas, across four different operational sex ratio treatments (OSRs 05, 1, 2, 5). Behavioural data are total counts for each male. Solid lines represent statistically significant relationships, while dotted lines indicate non-significant relationships.

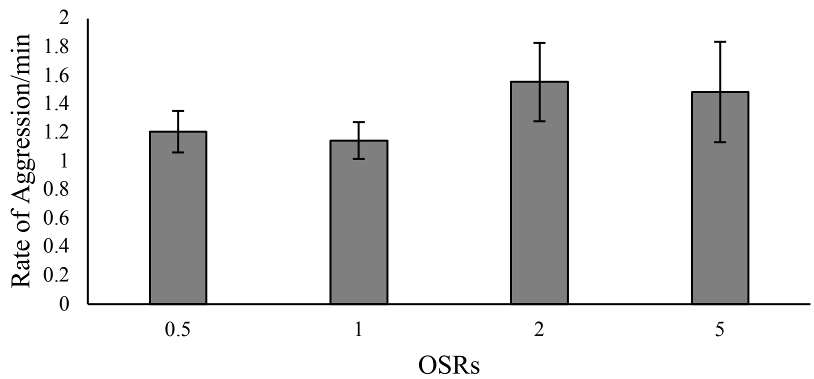

Figure 3. A) The average rate of aggressive behaviours performed per minute by 168 male medakas across four varying operational sex ratios. Error bars represent standard error.

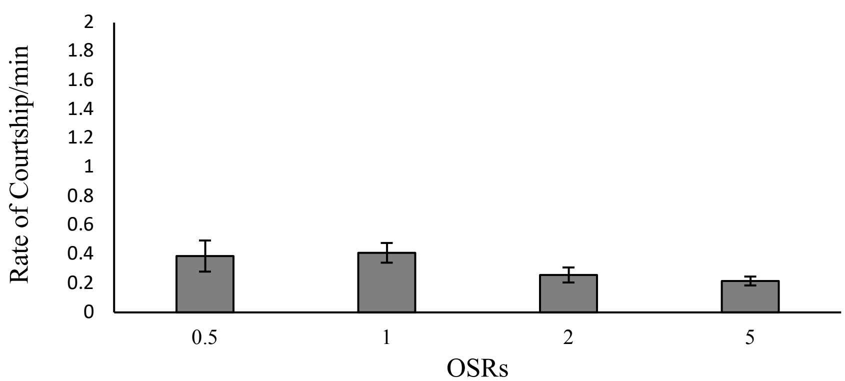

Figure 3. B) The average rate of courtship behaviours performed per minute by 168 male medakas across four varying operational sex ratios. Error bars represent standard error.

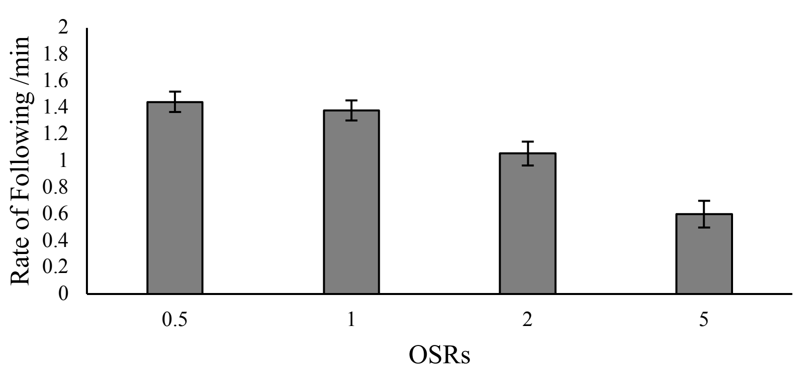

Figure 3. C) The average rate of following behaviours performed per minute by 168 male medakas across four varying operational sex ratios. Error bars represent standard error.

4. Discussion

4.1 Relationship Between Anal Fin Size and Behaviour

4.1.1 Relationship between body size and anal fin size

My data reflect a significantly positive correlation between male body size and anal fin surface area. This result reflects previous observations of an allometric relationship between overall body size and male ornament size (Clutton-Brock, T. H., Albon, 1980). Overall, anal fin surface area was positively related to standard length for the 168 male Japanese medakas used in this study. This relationship is statistically significant, and was predicted based on current literature on male medakas (Fujimoto et al., 2014; Howard et al., 1998), as well as other studies that suggest that the size of secondary sexual traits scale with body size (Andersson, 1994; Clutton-Brock & Albon, 1980). An example of this relationship is commonly found within species of Cerividae, where the males who stood tallest also possessed the largest antlers and were able to fight their competitors off more frequently, and with more force. The advantage of large body size and ornament size in turn led to increased reproductive success. Differences in the slopes of these relationships can offer insight into the evolution of different breeding systems, and the intensity of sexual selection on secondary sexual traits (Clutton-Brock & Albon, 1980).

Although body size of the Japanese medaka is sexually monomorphic (Howard et al., 1998), the anal fin is sexually dimorphic, where the males have significantly larger anal fins than females. This sexual dimorphism suggests that the male anal fin plays a role in reproduction and is an evolved secondary sexual trait (Koseki et al., 2000). During spawning, the male wraps the anal and dorsal fins around the female (Ono & Uewmatsu, 1957). It is hypothesized that this holds the female in oviposition and prevents sperm from scattering throughout the water column. The importance of the male anal fin has been demonstrated by Koseki et. al. (2000), where the size of male anal fins was manipulated to study potential differences of fertilization rate. Koseki et al. (2000) found anal fin size was positively related to fertilization success, as males who experienced removal of half the anal fin had significantly lower fertilization rates than males who underwent only partial removal or no removal treatments. Increased fertilization success occurred in fish with large anal fins (Koseki et al., 2000), and the largest males in a population monopolized matings (Howard et al., 1998). Large body size and anal fin surface area in combination contribute to male Japanese medaka reproductive success, both for securing mates through competition, and by increasing the fertilization rate of the female's eggs.

In medakas, the largest males also have a greater mating advantage, as large body size is associated with both intrasexual and intersexual selective pressures (Howard et al., 1998). Larger males are able to outcompete smaller individuals, and females show preference for the largest males in the population. Because large body size is a trait favoured by both mate choice and competition, the largest male medakas monopolize the majority of matings, and therefore are expected to have the highest reproductive success (Grant et al., 1995; Howard et al., 1998).

4.1.2 Relationship between mating behaviours and anal fin size

A relationship between body size and aggressive behaviour in males is often seen in species where intrasexual selection pressures are intense (Andersson, 1994). This relationship between morphology and behaviour in males often leads to increased reproductive success; in species with male-male competition, the largest or most aggressive males will outcompete smaller, less aggressive individuals (Andersson, 1994). This is commonly seen in elephant seals, in which the largest males exhibit the most aggressive behaviours, outcompete other males, and secure a harem of females, all of which lead to greater reproductive success for the dominant individual (Le Boeuf & Reiter, 1988).

A significant relationship between anal fin size and aggressive behaviour of the male medaka was observed in only the male biased operational sex ratio populations (OSRs 2 & 5). This could be due to increased intrasexual selection pressures within those populations, leading to more competition among males (Grant et. al., 2000; Mills & Reynolds, 2003). Previous research suggests that male medakas in male biased sex ratios exhibit more aggressive behaviours than males in even ratio populations. These males were also larger than those from populations with even sex ratios, which suggests a link between body size and aggressive behaviour in medakas (Fujimoto et al., 2014). From this information, it can be concluded it is most likely that a relationship between aggression and anal fin size would be significant in male biased operational sex ratios, which is what my results depicted.

No relationship was found between anal fin surface area and courtship or following behaviours. This result reflected my predictions, as links between male morphology and behaviour are most frequently linked to advantages when engaging in aggressive behaviour when intrasexual selection pressure is intense. As discussed in section 4.2, the males in highly male biased operational sex ratios were likely not engaged in scramble competition, where sneaking and sperm competition play an important role in reproductive success. A large anal fin would also benefit males in scramble competition, as they could use it to prevent sneakers from fertilizing eggs. A relationship between anal fin size and following behaviour rather than aggression may also be seen in scramble competition, because following is associated with mate guarding.

4.2 Variation of Mating Behaviours Across Social Settings

4.2.1 Aggression

An increase of the average rate of aggressive behaviour was observed as the operational sex ratio became increasingly male biased, but this trend was not statistically significant. Initially, I predicted the average rate of aggression to first increase as operational sex ratio became slightly male biased, but then decrease once operational sex ratio became highly skewed because defense of mates against a large number of competitors is not economical (Grant et. al., 2000; Weir, 2013). This prediction was based on previous behavioural studies using Japanese medaka that suggest that male-male competition and aggressive behaviour increase as a population becomes more male biased, due to increased intensity of intrasexual selection pressure. However, once this ratio is extremely skewed towards males, aggressive behaviour will decrease, as competing against several other males becomes energetically unfavourable (Grant, 2000). This pattern is consistent in other fish species as well, such as guppies and European bitterlings (Jirotkul, 1999a, Mills & Reynolds, 2003). A decrease in aggressive behaviour suggests a shift in the population from interference competition, characterized by males directly competing and excluding other males, to scramble competition, characterized by indirect competition, such as sperm competition.

Other studies have predicted a continuous increase in aggression as the sex ratio becomes increasingly male biased (Weir et. al., 2011). This continuous increase in aggression may be due, in part, to the variation in male density across the four operational sex ratio treatments. As male density increases, the number of male-male interactions will also increase (Jirotkul, 1999b), regardless of sexual selection intensity. Because the density of males in male biased operational sex treatments (OSRs 2 & 5) is much larger than that of males in female biased or equal operational sex ratio treatments (OSRs 0.5 & 1), it would be expected that aggression rates would be higher in populations of higher male densities, due to more frequent encounters between males within male biased populations.

The intensity of male bias in the operational sex ratio treatments could have also influenced my results with regards to aggressive behaviours. An operational sex ratio of ten males to two females used for OSR 5 might not have been skewed enough to cause a shift from interference competition to scramble competition, particularly because individual females may be defensible if they do not mate synchronously (Grant et al., 1995). As mentioned previously, individuals switch from interference competition to scramble competition when aggressive behaviour becomes energetically unfavourable, and eventually this can lead to a more even distribution of reproductive success (Jirotkul, 1999a; Weir, 2013). My results suggest that the male medakas in OSR 5 continued using male-male aggression as a reproductive strategy, as the intrasexual selection intensity may not have been strong enough to result in a shift from interference to scramble competition.

4.2.2 Courtship

Although not statistically significant, the average rate of courtship behaviour decreased as the operational sex ratio became increasingly male biased. This overall trend matched my initial prediction, as previous studies suggest that courtship frequency decreases in highly male biased sex ratios (Weir, 2013). This decrease in courtship rate is often associated with a decrease in aggression as males react to their competitive environment, rather than attempt to court females. This relationship between courtship and aggression was not seen in my data. Courtship behaviours are much less important in scramble competition, as it involves alternative reproductive strategies such as sneaking rather than traditional mating behaviours such as courtship and female mate choice. Although there was a trend of decreasing courtship rates, I cannot conclude that scramble competition was used in the highly male biased sex ratio (OSR 5), because the decrease in courtship rates were not associated with a decrease in aggression rates, and my data showed only insignificant trends for these two behaviours.

4.2.3 Following

The rate of males following females decreased as the OSR became increasingly male biased. The variation in the average rate of following behaviour did not match my initial prediction, however there was a significant difference in following rates among operational sex ratio treatments. Following is a type of harassment behaviour performed by male medakas and is generally used as to coerce a female into spawning (den Hollander & Gwynne, 2009), or as a form of mate guarding when alternative reproductive tactics such as sneaking are common within a population (Weir, 2013).

In regards to scramble competition, an increase in following behaviour is correlated with a decrease of both aggression and courtship behaviours (Weir, 2013). Because scramble competition relies on alternative reproductive tactics (e.g. sneaking in medakas) or indirect male-male interference such as sperm competition, it is expected that following would increase as the operational sex ratio becomes increasingly male biased, with the highest following rates being observed in highly skewed ratios (OSR 5). In my study, the opposite of this was observed, where individuals in male biased operational sex ratio treatments (OSRs 2 & 5) showed significantly lower rates of following than those in female biased or equal operational sex ratios (OSRs 0.5 & 1). This result indicates that males in OSRs 2 and 5 were not significantly using following behaviour as a way to gain or guard mates, unlike males in OSRs 0.5 and 1.

4.2.4 Summary

Overall, my results suggest that male individuals in OSRs 2 and 5 were engaged in male-male aggression, not scramble competition, as their following frequency decreased significantly compared to the other two operational sex ratio treatments. There was no need to increase following frequency as a form of mate guarding, as indirect interference was most likely not being used by the males in the population.

4.3 Conclusions

As the sex ratio of a population becomes more male biased, intrasexual selection pressures become more intense, which may lead to more competition for mates among males (Andersson, 1994). Therefore, a stronger relationship between morphological traits, such as body or ornament size, and aggressive behaviour in male individuals may be seen in populations with male biased sex ratios. This relationship may not be as strong in populations engaged in scramble competition, as aggression is not a favourable behaviour to gain mates (Weir, 2013). Overall, my results suggest there is a positive correlation between male medaka body size and anal fin size, as well as a significant relationship between anal fin size and aggression, when intrasexual selection pressures are most intense (OSRs 2 & 5). Non-significant trends suggest that male medakas show plasticity in their mating behaviours in different social settings (measured by operational sex ratios). Reproductive success is influenced by both morphology and behaviour in the male Japanese medaka, as well as the intensity of sexual selection pressures.

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