The Effects of Sexual Selection on Mate Choice in Freshwater Crayfish

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Abstract

Mate choice is though to be affected by selection for characteristics that enhance the probability of producing abundant and viable offspring. Samples of *O. limosus* were collected from the Sturbridge, MA in the Quinebaug River and studied in the lab for different mate choice responses. Recent experimentation suggested that dominance can have an effect on mating success. In the absence of any competitors submissive and dominant males had similar mating success, however when both males are present the submissive male experienced a significantly reduced mating success and a reduced ability to initiate mating. This work tests the hypothesis that in a given three-male dominance hierarchy, the dominant male will mate with a single added female more frequently and will initiate mating faster than the intermediate and submissive males, and also tests the hypothesis that given the choice between a virgin and nonvirgin mate, an individual will both choose more frequently and initiate mating faster with a virgin.

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1. Introduction

The study of sexual selection and behavior are of vital importance to ecology. Knowledge of the role of dominance and mating status in sexual selection further our understanding of the evolutionary biology and behavioral ecology of a species. The ecology and physiology of a particular species can influence specific sexual selection which may in turn lead to different behavioral patterns (Aquiloni & Gherardi, 2008). Our goal is to generate new understanding of the roles of ecology, physiology, and sexual selection in driving behavioral evolution using the model crayfish species *Orconectes limosus*.

In this paper, we give a general overview of sexual selection and behavioral ecology, and discuss what is known about the effects of dominance hierarchies and mating status on mate choice. Finally, we test our hypotheses that in a three-male hierarchy there will be a clear dominant, intermediate, and submissive male and that the dominant male will mate with a single added female more frequently and will initiate mating faster than the intermediate and submissive males. We also chose to explore whether mating status provides any advantage for mate selection. For this test, we hypothesized that given the choice between a virgin and nonvirgin mate, individuals will choose the virgin more frequently and initiate mating with the virgin faster. Given these predictions we constructed experimental designs to test them, and explain our rationale for these suggestions in the context of the larger research field.

1.1 Sexual Selection and Behavior Background

Natural selection theory postulates that reproductive success is the greatest force driving interactions among members of the same species. It is therefore important to understand how these interactions determine which members have access to potential mates. Both males and females of many species demonstrate selectivity in mate choice depending on a variety of factors. Parental investment theory states that any expenditure of time, energy, and risks by a parent on offspring may reduce the chance that a parent will have more offspring in the future (Coleman, 1985; Trivers, 1972).

Parental investment theory is based on physiological differences between males and females. Males almost always have sufficient supplies of gametes to fertilize multiple females, and their contribution to the next generation therefore depends on the number of sexual partners that they have. As a result, the time and energetic costs required of a male involves the creation of sperm and the logistics involved in copulating with a female. A male may also be available to mate at any time during the mating season, since he produces much more sperm relative to the female's egg production. This male-biased gamete production is evident in the male coho salmon, which release up to 100 billion sperm on only 3,500 eggs. Males also tend to invest less time in offspring development after fertilization than females (Alcock, 2001).

In contrast, females generally make a larger parental investment per offspring than males. The mass of an egg, and therefore the energy required to produce one, is much greater than that of a single sperm. Females must take additional time and secure additional resources in order to create and egg, and the female generally spends additional time caring for the offspring after fertilization. Since females invest much more time in offspring development than males, there may be a smaller pool of sexually receptive females than males in a given population. Inequalities in parental investment lead to a male-biased operational sex ratio, which in turn leads to male competition for mates and careful selection by females. This type of sexual selection is known as intrasexual selection, since the outcome of competition among male members results in access to females and subsequent mating success (Alcock, 2001).

Male competition for mates causes variation in potential reproductive rates among males in a population. Selection should therefore favor males who compete aggressively with rivals and copulate at every opportunity and females who avoid the costs of additional mating. This type of sexual selection is known as intersexual selection, since the outcome of male-male- agonistic interactions results in access to female mates and subsequent mating success.

However, this is not the situation in all species. In some cases, the operational sex ratio is female-biased, leading to female competition for mates and careful selection by males (Moore et al, 2001). One study focused on six female swarms of empid flies, *Rhamphomyia longicauda*, along the banks of the Credit River in Ontario, Canada for the entire swarming season of the flies. The results revealed a sex role reversal in *R. longicauda*. The male fly leaves the swarm to collect insect prey as a mating inducement. Upon returning to the nest multiple females may advertise themselves as sexually receptive in a role-revered dance, resulting in careful mate selection by male members of this species (Gwynne and Bussière, 2002).

Because sex roles differ between males and females, both may affect each other's chances of reproduction. Sexual selection theory states that certain traits or behaviors can be explained by competition among members of the same sex for a limited number of mates. This may account for the evolution of traits which increase the individual reproductive success by enabling individuals to acquire mates in competition with others, such as fighting and nest building behaviors or colorful plumage. Competition for mates can be a powerful force for evolutionary change, as these traits and behaviors may result in a change in genetic makeup of the species. A recent study collected and analyzed 124 colonies of *Cardiocondyla obscurior* ants from Bahia, Brazil. It revealed that this evolutionary force is demonstrated in male *C. obscurior* ants, which have evolved large powerful jaws to eliminate rivals in order to gain access to the several dozen virgins that emerge from the nest (Heinze, 2005). If the ultimate significance of fighting behavior is to acquire mates, then rank in a dominance hierarchy should correlate strongly with male copulatory success, as the alpha male southern elephant seal demonstrates by achieving a higher reproductive success along with social success (Alcock, 2001).

1.2 Effects of Dominance on Mating Interactions

Sexual selection theory accounts for the evolution of survival-decreasing traits, which still increase individual reproductive success by enabling individuals to acquire mates in competition with others (Alcock, 2001). This theory could possibly explain unique survival-decreasing traits exhibited by certain species such as fighting, nest building, or flamboyant coloration. As such the cost of these sexual behaviors may be offset by the added reproductive success to the individual. The individual may increase their reproductive success through these behaviors, which are driven by sexual selection. It has been seen in many taxa that the establishment and inclusion of dominance play a role in the social mating of many species.

Dominance has also been found to bestow advantages on males during competition for mates (Wong & Candolin, 2005; Trivers, 1972). These advantages can include the fact that females of many taxa prefer successful males of antagonistic encounters. Given that dominance is at least partially hereditable and bestows advantages for reproductive success to at least one sex, then intra-sexual and/or inter-sexual selection are likely to act on dominance.

Most species of clawed decapod crustaceans, such as our model species *O. limosus*, exhibit what is called a dominance hierarchy where individual males exert superiority, or dominance, over other males through physical interactions or chemical signaling. Assuming formation of these hierarchies causes considerable predation risk or energetic costs to those participating in them, then it would stand to reason that participation in such activities bestows certain reproductive benefits upon those individuals. Chemical signals are a common mechanism by which information is conveyed in decapods, such as male sexual maturity and genetic quality. However, chemical signals are not always the only factors used by the female during sexual selection. There may be social eavesdropping, or third party observation, by prospective females on male-male interactions and their possible display of dominance (Aquiloni et al, 2008). It is this male-male competition that allows for females to detect and observe possible mates, the honest display of genetic quality between competing males, as well as to allow for the continuation of alternative mating strategies (Wong & Candolin, 2005).

It is generally accepted that females should select for dominant males due to the benefits that they can provide. However, it has also been shown that females in multiple taxa do not prefer dominant males over subordinate males (reviewed in Qvarnstrom & Forsgren, 1998). Females selecting for dominant males may incur both benefits and costs as a result of this choice (Qvarnstrom & Forsgren, 1998). Direct benefits have been noted to include high quality resources such as territory or shelter. It has been seen in many species that dominant males are able to secure and maintain high quality resources, which would be considered a benefit to females. However, this same benefit may become a net cost in polygynous species as multiple females must share these resources. Benefits have also been noted to include the disclosure of the male's disease state and general physical condition. A dominant male is considered to be of higher physical quality and lower disease state, as these would be taxing on the male and most likely render him unable to maintain its dominant status. Additionally, in certain species, competition between males for mating rights to a female may be so intrusive the males may cause physical injury or even death to the female. By selecting for the dominant male, females can most likely lower the risk of injury, as the male's status will deter interference from competitors.

However, costs have also been identified with female selection of dominant males. One example is sperm depletion and the decreased likelihood of fertilization by a dominant male, should those males perform multiple matings before replenishing their sperm stores. In addition, it has also been found that the sperm ejaculate size in *Austropotamobius italicus* decreases as male body size increases (Rubolini et al, 2007). Consequently, females may benefit from a preference for a non-dominant male, should dominance have a direct correlation to body size. Other costs have been investigated; however, further exploration and experimentation is necessary to ascertain their priority in the role of female selection of dominant males. Additionally, female choice may be made irrelevant, should dominant males be able to coerce a female into a forced mating. (Qvarnstrom & Forsgren, 1998)

1.3 Effects of Mating Status on Sexual Selection

It has been observed in multiple species of crayfish that both male and female behavior is directed by the mating status of females (Durgin et al, 2008; Galeotti et al, 2007; though only observed in males by Aquiloni & Gherardi 2008). Males of *O. quinebaugensis* show a preference for virgin females over nonvirgins, while virgin females showed an attraction to male signals and mated females did not (Durgin et al, 2008).

When females of a species have the ability to store sperm and fertilize eggs with sperm from multiple donors, the males may be subjected to sperm competition (Diesel, 1991). Storing sperm allows a female to fertilize her eggs with genetic material from multiple donors, thereby increasing the chance of offspring survival and success (Foerster et al, 2003). Males may also increase their sperm expenditure to increase their chances of fertilizing multiple eggs, or may remove sperm from the previous mating if the female's morphology allows it. For example, in the crayfish species *A. italicus*, males feed on previously deposited sperm before releasing their own (Galeotti et al, 2007). However, in the crayfish species *O. limosus*, females store sperm in an internal spermatheca, which likely prevents sperm removal (Hamr, 2002). Another way males avoid sperm competition is by mating with females not previously inseminated in a given reproductive season, or virgin females. While this may not guarantee their sperm is used during fertilization as a second mating with a different male may occur at a later time; it does provide that male a better probability at that given time. One example of virgin selection has been observed in the guppy species *Poecilia reticulate*, where males are able to distinguish between mated and virgin females using olfactory cues, and subsequently followed, attempted copulation, and copulated more often with virgin females. The reason for the selection of virgins by this species of guppies is not entirely understood, however it is believed that by being able to identify and court virgin females, males are able to copulate more successfully than if they court "choosier" mated females (Guevara-Fiore et al, 2009). Similar behavior has also been documented in the flour beetle species *Tribolium castaneum*, where males initiate contact more frequently and also copulate more often with virgin females than with mated females, when provided with equal access to both types (Lewis & Iannini, 1995). For these reasons, virgin females should be the preferred mate choice for males among sperm-storing species or those that wait long periods of time between mating and fertilization.

Virgin selection may not be as crucial in organisms that do not store sperm, as previously deposited sperm can be accessed and removed if desired (Galeotti et al, 2007). However, virgin selection may be crucial in those organisms that have fast reproductive cycles as they will give birth often. Therefore, the chance of their mating multiple times with different males is decreased significantly, possibly warranting the energetic cost for males to identify females and decrease the chances of sperm competition.

Additionally, it has be identified that females should prefer virgin males as sperm depletion caused by multiple matings significantly decreases the probability of successful fertilization in future mating occurrences, as well as decreases male ejaculatory size. It has also been found in the crayfish species *A. italicus* that the amount of sperm per ejaculate decreases as male size increases (Rubolini et al, 2007). Given these findings, it would be advantageous for females to invest the energetic costs associated with being able to identify and mate with smaller virgin males.

1.4 Previous Experiments and Current Hypotheses

Recent experimentation and analysis has explored the relationship between dominance hierarchy status and mating tactics (unpublished data). In one study, two males were allowed to interact in an experimentation tank in order to establish a dominance hierarchy, and once this hierarchy was established the males were separated into smaller containers and each was presented with a receptive female. This work showed that there was no difference in mating frequency or the time it took to achieve mating position between the dominant and submissive male. Thus, there appeared to be no effect on an individual male's ability to achieve mating after recent male-male agonistic interactions. There is no evidence from this experiment that a male's perception of his own dominance status or immediate social history affects his tendency to attempt mating.

Additional recent experimentation and analysis has confirmed that ongoing social interactions between males affect the outcome of male-female mating interactions (unpublished data). As in the previous experiment, two males were allowed to interact in an experimentation tank in order to establish a dominance hierarchy. However, in this experiment the males were then isolated in separate smaller containers and a female was placed alone in the experimentation tank. Both males were then reintroduced to the experimentation tank and allowed to interact with the female. This work showed that dominant males mate with the female more frequently than submissive males, and that submissive males took significantly longer to initiate mating than dominant males. Therefore, the presence of another male appeared to reduce the mating success of submissive males.

Taken together, these experiments suggest that dominance can have an effect on mating success. In the absence of any competitors submissive and dominant males had similar mating success, however when both males are present the submissive male experienced a significantly reduced mating success and a reduced ability to initiate mating.

Our experimentation continues to explore the relationship between dominance and mating success by investigating whether the dominant male will have the same mating advantage that they had in the 2-male experiment if a third male was added to the dominance hierarchy. We hypothesized that in this three-male hierarchy there will be a clear dominant, intermediate, and submissive male and that the dominant male will mate with a single added female more frequently and will initiate mating faster than the intermediate and submissive males. We also chose to explore whether mating status provides any advantage for mate selection. For this test, we hypothesized that given the choice between a virgin and nonvirgin mate, individuals will choose the virgin more frequently and initiate mating with the virgin faster.

2. Methods

2.1 Collection, Observation, Measurement, General Care, and Labeling

A total of 220 individuals of the species *O. limosus*, 84 females and 136 males, were collected by hand or dip net from the East River Dam, a U.S. Army Corps of Engineers site on the Quinebaug River (Figure 1). Collection occurred from July to early September just before the beginning of the mating season, which occurs from late September to early November (Mathews, personal communication). The crayfish were transported live in water filled buckets to the WPI biological laboratory located in Gateway Park. Once back in the laboratory males and females were separated into different tanks.

Figure 1: Collections site located at the East River Dam, a U.S. Army Corps of Engineers site on the Quinebaug River

All crayfish spent approximately 2 months in a recirculating system in Gateway Park with up to two crayfish of the same sex per tank, in which the light cycle and temperature were regulated to mimic natural conditions (Figure 2). The 2 month delay from initial capture to actual experimentation was due to the fact that the crayfish were collected before the mating season had started. This increased the probability that all crayfish had not yet mated for the season. Individuals that were not selected for a certain batch of experimentation remained in the recirculating system. Crayfish in the aquatic system were fed a compacted shrimp pellet diet of 1-2 pellets per crayfish three times a week.

Figure 2: Recirculating aquatic system located in WPI Biological Labs at Gateway Park

Those individuals selected for experimental use were transferred in a water filled bucket to a greenhouse in Salisbury Labs for up to a week and a half. They were individually isolated in a 15.30 x 30.50 centimeter plastic holding tank filled with approximately 3000mL of water and fed with a continuous air supply through an electric air pump and air stone. Water was changed on a weekly basis and between batches and these crayfish were fed the same shrimp-pellet diet of 1-2 pellets three times a week. The greenhouse allowed for natural day night cycles.

Morphological observations such as missing legs, chelae, and antennae were recorded for each crayfish along with a measurement from the tip of the rostrum to the end of the carapace using calipers, recorded to the nearest 0.1 mm (Figure 3 panel b). After measurement and observations were taken, each crayfish was assigned a number. A small piece of electrical tape about 1.5 x 1.5 centimeters was prepared for each crayfish with its unique number written in Sharpie marker (Figure 3 panel a). This tag was cut from the role of tape and a small amount of Krazy Glue brand superglue was used to adhere it to the carapace of the crayfish. Only crayfish with all walking legs and chelae of the same size (assessed visually) were considered for experimental selection. The measurements and individual numbers were recorded in Microsoft Excel 2007, and subsequently sorted by size. Replicates were chosen from this size-sorted list so that every participant's length was within 1-2mm.

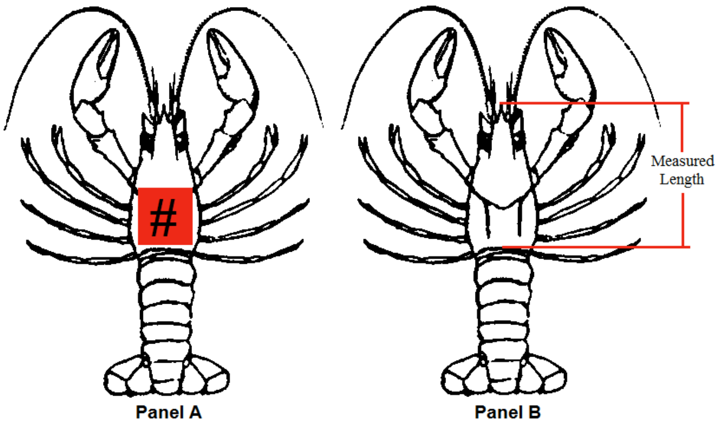

Figure 3: Panel a shows tag placement and panel b shows measured length from the tip of the rostrum to the end of the carapace

Experimentation took place in the greenhouse in Salisbury Labs. Upon selection for experimentation, a batch of approximately 60 crayfish separated by gender was transported from Gateway Park to Salisbury Labs. Two to three replacement crayfish were kept on reserve in same physical specifications for substitution if a selected crayfish died. Upon completion of experimentation, these crayfish were separated by gender and transported in water filled buckets from Salisbury Labs back to Gateway Park. They were placed in empty tanks when available, and if two crayfish were placed in the same tank we made note that they could not be used in the same experimental replicate in the future.

2.2 Male mating tactics are affected by recent male-male agonistic interactions

Three male crayfish of similar size (within 2mm in length) were placed in a 90 x 60 centimeter plastic aquarium filled with 7000mL of water with a video camera positioned approximately 140 centimeters above it. Four tanks were recorded at a time in the field view of the camera. The males were allowed to interact within the aquarium for 30 minutes in order to establish dominant and submissive roles. After the 30-minute period, a female of a similar size (within 2mm in length of the males) was added to the tank and allowed to interact with the three males for five hours (Figure 4). The entire five and a half hour experiment was recorded by the video camera and replicated 23 times.

Figure 4: Dominance hierarchy experimental setup of three virgin males and a single virgin female

Video was scored for the first half hour prior to the addition of the female. During this time period all instances in which two or more males came into direct contact were considered interactions. All interactions were scored based on which male fled and which male stood its ground. In each particular interaction, if one or more males fled from the interaction we considered them the ‘loser’ of the interaction while the male who stood his ground was considered the ‘winner’ of the interaction. If two males stood their ground and one fled, the two who stood their ground would be considered winners and the one who retreated would be considered the loser. Any subsequent interaction between these two males was recorded as a separate interaction, such that both of them won the interaction between them and the third males, and after the third male retreated the one who stood his ground again would be considered the winner and the other male would be considered the loser. If one male stood his ground and two fled, the one would be considered the winner and the two that fled would be considered losers.

The total number of individual interactions and the relative percentages of wins and losses were quantified for each male, without considering the identity of the other males involved in those interactions. Once these values were calculated, the dominant male was identified as the individual with the highest winning percentage of the three males. The submissive male was identified as the individual with the lowest winning percentage, and the intermediate male was identified as the individual with an intermediary winning percentage.

In most cases there was a clear dominant, intermediate, and submissive male. However, in cases where two males had similar winning percentages, we considered the specific cases in which those two males interacted. If one male won significantly more frequently (greater than 65% of the time) against the other male, this male was considered dominant. If the two males won equally against each other they were considered codominant.

Figure 5: Crayfish mating position (Penney, 2009).

Video was also scored for the five hours following the addition of the female. During this time all instances of male and female contact were documented. For each mating attempt, we recorded the time that it took the male to get the female in the proper mating position (Figure 5), duration of the mating, and the identities of the participants.

2.3 Mating status affects the outcome of mate choice, such that virgins will be selected for preferentially over nonvirgins

We assumed that all crayfish collected from the river had not yet mated for the season and we considered them all virgins. Crayfish of opposite sex were not allowed to interact prior to experimentation in order to ensure their virgin status. We considered nonvirgins as those crayfish that successfully mated once in the dominance hierarchy experiment, and ensured that these nonvirgins had never had prior contact with the other individuals in the experiment.

Two size-matched crayfish of the same gender but opposite mating status (virgin and nonvirgin) were secured to opposite corners of the same side in a 90 x 60 centimeter plastic aquarium with 7000mL of water. The crayfish were secured to the corner using nonflavored and nonwaxed floss adhered to their label with Krazi Glue brand super-glue and then taped to their corner of the tank. The floss was secured to the back so as to not hinder natural movement. The length of the floss was measured to allow adequate movement but without the two individuals being able to physically interact. A third size matched crayfish of virgin status and opposite gender, called the chooser, was then placed within the aquarium in the middle of the opposite 90cm wall facing the wall (Figure 6).

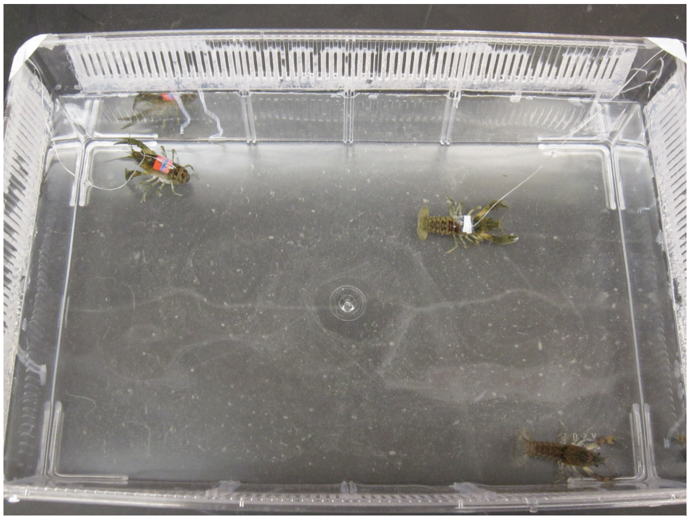

Figure 6: Mating status experimental setup of two tethered crayfish of the same gender and opposite mating status and one free-moving virgin crayfish of the opposite gender, known as the chooser

The behavior of the chooser was then observed in person until either the female assumed the mating pose (Figure 5), or the 30-minute time limit had expired. If mating had occurred then the time until mating was recorded and the selected individual also noted. This experiment was replicated 26 times, yielding a sample size of 13 for each gender as the chooser.

2.4 Data analysis

Data from both experiments were analyzed using Microsoft Excel 2007. For our dominance experiment, the number of times each category of male (dominant, intermediate, and submissive) mated with the female was determined. The relative percentages of total mating occurrences were also calculated for each category of male. The average time to initiate mating was calculated for dominant and intermediate groups. There was only one occurrence of submissive male mating, and therefore this value is not included in the time to initiate mating analysis and was only included in the comparison of the numbers of mating occurrences.

In order to determine whether the number of mating occurrences was statistically significant for each category of male, a Chi-Square test with two degrees of freedom was completed, comparing actual mating frequencies from each group (dominant, intermediate, and submissive) and to expected values obtained by dividing the total number of mating occurrences by three.

In order to determine whether the duration of mating and time until the female assumed the mating pose were significantly different between dominant and intermediate males, a Mann-Whitney U test with two degrees of freedom was performed and z and p values were obtained for each group.

For the mating status experiment, for each sex we calculated the number of trials in which choosers selected virgins and non-virgins. In order to determine if the number of virgin and nonvirgin selections was statistically significant for both male and female choosers, a Chi-

Square test with two degrees of freedom was completed. The p value was calculated using observed frequencies from each group (virgin selection and nonvirgin selection) and compared to expected values obtained by dividing the total number of selections by two for each choosing sex.

The average time elapsed until a mate was chosen was also calculated. In order to determine whether the elapsed time was significantly different between virgin and nonvirgin choices, a Mann-Whitney U test with two degrees of freedom was performed and z and p values were obtained for each group. For all analysis of variance statistical tests, we assessed statistical significance as $p \leq 0.05$ indicating a 5% or lower probability that the observed data could occur by chance.

3. Results

3.1 Male mating tactics are affected by recent male-male agonistic interactions

This experiment had a total 23 replicates with 6 replicates being excluded from the dataset, because either none of the males mated with the female (5 replicates) or one of the crayfish died during experimentation (1 replicate). Among the 18 replicates in which mating did occur, the dominance relationship between two males could not be assessed with certainty in only one replicate because neither male showed submissive behavior towards the other. Among the remaining 18 replicates, the dominant male mated with the single female in 6 replicates, the intermediate in 11 replicates, and the submissive in 1 replicate ($\chi^2 = 8.33$, Df = 2, p = 0.0155; Figure 7).

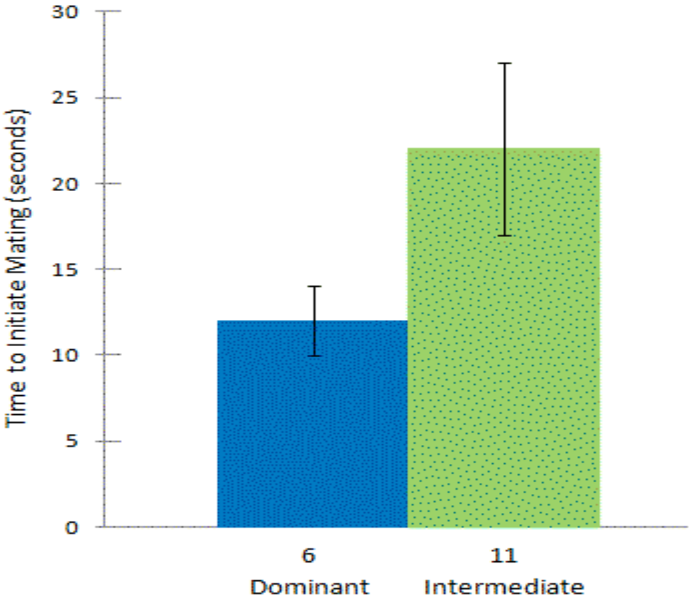

Figure 7: Average time to initiate mating and total frequency of mating occurrences. This data reflects replicates in which the dominant male mated with the female, and in replicates in which the intermediate male mated with the female. Bars show standard error and numbers below bars indicate mating occurrence.

Furthermore, in this experiment, intermediate males took significantly longer to initiate mating compared with dominant males in the same experiment (z-score = 1.193, p = 0.116; Figure 7). Because 11 of the 18 pairs were still copulating after 5 hours, we were logistically unable to determine an average mating duration.

Interestingly, we witnessed several occasions in which two males assumed the mating pose, with one male assuming the typical female posture (Figure 5). In the 11 replicates in which this behavior was observed, the dominant male assumed the mating pose in 1 replicate, the intermediate male in 6 replicates, and the submissive male in 4 replicates ($\chi^2 = 3.471$, Df = 2, p = 0.018; Figure 8). Male-female mating interactions occurred in all 11 of these replicates, however only 5 of the males who displayed this behavior were successful in mating with the single female and all of these males were intermediate in dominance status.

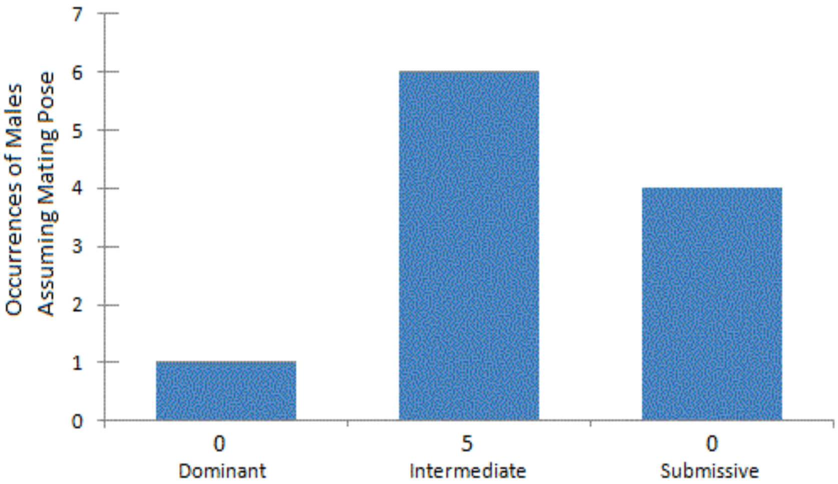

Figure 8: Male-male interactions in which one male assumed the mating pose. Occurrences of males assuming the mating pose while interacting with another male, separated by dominance status determined in the first 30 minutes of experimentation. Numbers below bars indicate male-female mating occurrences.

Thus, in this experiment, subordinate males had lower mating success than intermediate or dominant males, mating in only 1 of 18 (5%) replicates. Intermediate males had the highest mating success, mating in 11 of 18 (61%) replicates, while dominant males mated in 6 of 18 (33%) replicates. Additionally, dominant males were able to initiate mating in a shorter period of time than intermediate males in the 6 replicates in which the dominant male was successful in mating. Furthermore, males that assumed the mating pose while interacting with another male seem to be equal in mating success to males who did not display this behavior, since 5 of the 11 (45%) males who displayed this behavior were successful in mating. In recent experimentation, it was shown that the presence of another male appeared to reduce the mating success of submissive males. Interestingly, this experiment suggests that the presence of a third male reduces the dominant male's advantage in mating success.

3.2 Mating status affects the outcome of mate choice, such that virgins will be selected for preferentially over nonvirgins

This experiment had a total 13 replicates in which a male virgin was allowed to choose between a virgin and nonvirgin female, and 1 replicate was excluded from the dataset because no mating occurred. Among the 12 replicates in which the male was successfully choose between a virgin and nonvirgin female, in 6 replicates the virgin was chosen and in 6 replicates the nonvirgin was chosen ($\chi^2 = 0$, Df = 1, p = 1.00; Figure 9). This experiment also had a total of 13 replicates in which a female virgin was allowed to choose between a virgin and nonvirgin male, and 2 replicates were excluded from the dataset because no mating occurred. Among the 11 replicates in which the female successfully choose between a virgin and nonvirgin male, in 9 replicates the virgin was chosen and in 2 replicates the nonvirgin was chosen ($X^2 = 4.455$, Df = 1, p = 0.0348; Figure 9).

Furthermore, in this experiment there was no significant difference in the time it took males to initiate mating between virgins and nonvirgins (z-score = 0.480, p = 0.315; Figure 9). However, females chose nonvirgins significantly faster than virgins (z-score = 0.943, p = 0.173; Figure 9).

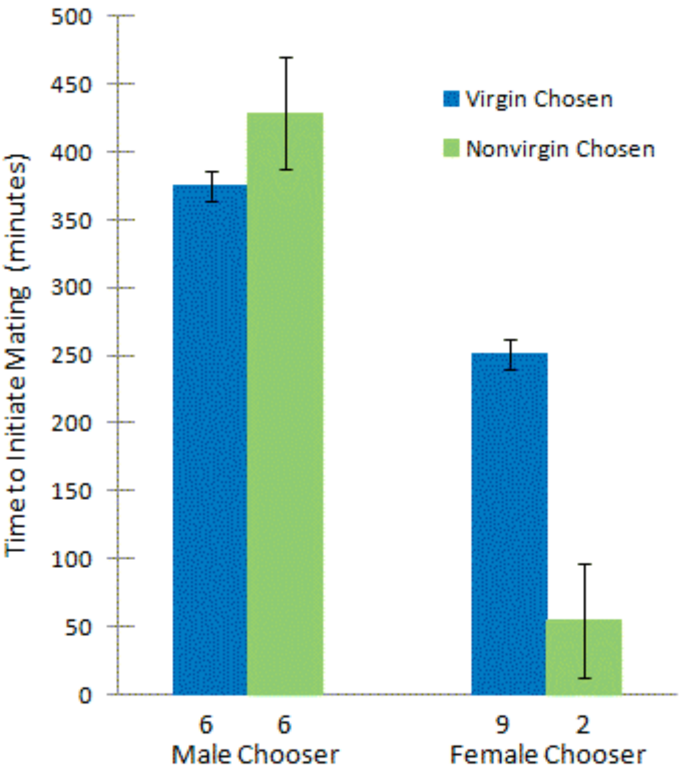

Figure 9: Average time to initiate mating and total frequency of mating occurrences in replicates in which a male was allowed to choose between a virgin and a nonvirgin male, and in replicates in which a female was allowed to choose between a virgin and a nonvirgin male. Bars show standard error and numbers below bars indicate mating occurrences.

Thus, this experiment showed that males do not preferentially choose mates based on mating status, choosing virgin females in 6 of 12 (50%) replicates and nonvirgin females in 6 of 12 (50%) replicates. However, females do show preference for virgin males, choosing virgin males in 9 of 11 (82%) replicates and nonvirgin males in 2 of 11 (18%) replicates. Additionally, males initiated mating with virgin and nonvirgin females in approximately the same amount of time, while females selected for nonvirgin males significantly faster than virgin males.

4. Discussion

Social structures are often shaped by agonistic interactions and the subsequent formation of dominance hierarchies, such as in the crayfish species *O. limosus.* However, the role of these dominance hierarchies in crayfish mating behavior remains unclear. If these interactions are under selection, then dominant males should have an advantage in gaining mating access to a receptive female through increased competitiveness with other males.

The goal of our experiments was to determine whether long-term social experiences between size-matched males affects the likelihood of mating with receptive size matched females. We placed three size matched males into an experimentation tank and allowed them to interact for 30 minutes, then added a size-matched female to the experimentation tank and video recorded the interactions for an additional 5 hours. We hypothesized that in this three-male hierarchy the dominant male will mate with a single added female more frequently and will initiate mating faster than the intermediate and submissive males.

Contrary to our hypothesis the results of our experiment showed that the intermediate male mated more often with the female than the dominant male. This was unexpected, as previous experiments using *O. limosus* have shown that in the absence of any competitors submissive and dominant males had similar mating success, however when both males are present the submissive male experienced a significantly reduced mating success and a reduced ability to initiate mating (unpublished data). This raises the question of why in our experiment the dominant male was found to have mated with the female noticeably less than the intermediate male, given the previous data. As both experimental designs are very similar in nature and the only significant variable is the addition of the third male, we infer that the addition of the third male was enough to alter social interactions.

This behavior coincides with mating behavior observed in other species, such as the freshwater prawn *Macrobrachium rosenbergii* (Ra'anan & Sagi, 1985), that exhibit a reproductive dominance hierarchy. It has been seen that while the dominant male is distracted exerting his dominance over other males alternative mating strategies, such as "sneak copulation" (Ra'anan & Sagi, 1985), may allow for lesser males to copulate with the prized female regardless of their submissive status. Therefore, it is possible that we are observing an alternative strategy exhibited by the intermediate males that is allowing them to mate more successfully than the dominant males.

In many taxa the mating status of an individual has an effect on the likelihood of that individual being chosen as a mate, especially in species in which the female is able to store sperm from multiple male partners, as is the case in the crayfish species *O. limosus*. However, the role of mating status in mate choice behavior remains unclear. If the advantage of mating with a virgin partner correlates with an increase in individual reproductive success, virgins should be preferentially chosen over nonvirgins.

The goal of our experiment was to determine whether mating status has an effect on the likelihood of being chosen for mating. We tethered two size-matched crayfish of the same sex but opposite mating statuses to either side of an experimentation tank, and then added a third size-matched virgin crayfish of the opposite sex to the experimentation tank. We hypothesized that given the choice between a virgin and nonvirgin mate, individuals will choose the virgin more frequently and initiate mating with the virgin faster.

Our results suggest that males have no preference for either mating status. Males also initiated mating with virgins and nonvirgins in approximately the same amount of time. It seems that although females of this species are able to store sperm from multiple partners, males have no preference for either mating status. The theory of sperm competition suggests that males should prefer virgin females, since nonvirgin females contain sperm from previous copulations and this decreases the probability of individual male fertilization success. In a similar species of freshwater crayfish, *A. italicus*, males have evolved the ability to increase ejaculate expenditure when the risk of sperm expenditure is high, or when the female has recently mated (Galeotti, 2007). This evidence supports the theory of sperm competition, although our results provide no insight into whether males have any mating status preference. Further research should continue to explore the relationship between sperm competition and male mate choice.

However, females did show a preference for male virgins. Interestingly, females chose nonvirgins significantly faster than virgins. One possible explanation for this preference is that virgin males are likely to have a higher sperm count than males who have recently copulated, resulting in a higher probability of reproductive success. Although males generally have a larger supply of gametes available at a given time than females do, this supply is diminished after copulation and time is required for spermatogenesis. Therefore a male that has recently mated may have a reduced sperm supply, which would decrease both his reproductive success and constrain the fertilization success of any female partners occurring after the first copulation. This phenomenon is known as sperm limitation, and explains why females would prefer virgin males over nonvirgin males (Sato & Goshima, 2007).

Thus recent experimentation combined with our results has furthered our understanding of the relationship between dominance hierarchy status and mating tactics. These preliminary experiments suggested that dominance can have an effect on mating success. While a previous experiment had shown that in the absence of any competitors, submissive and dominant males had similar mating success, a second experiment showed that when two males are present, the submissive male experienced a significantly reduced mating success and a reduced ability to initiate mating (unpublished data). Our experiment expands on the inferences from those previous datasets, and involved the addition of a third male. Our data suggest that the presence of two other males significantly reduces the mating success of the dominant male. Our other experiment suggests that males do not preferentially choose mating status and will initiate mating with virgin and nonvirgin females in approximately the same amount of time. Interestingly, females do show preference for virgin males, although they selected for nonvirgin males significantly faster.

While our data revealed interesting insights into the role of social experience on mate choice in *O. limosus*, further research should continue to examine the relationship between dominance hierarchy status and mating tactics. It is currently unknown whether the presence of additional individuals would have any effect on the mating success of the dominant male. Research should also continue to examine the relationship between mate preference and mating status, specifically the relationship between sperm competition and male mate choice. Additional data are needed to substantiate our findings, since we were working with such a small sample size.

5. References

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