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Reproductive morphology and sperm depletion in crayfish

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by

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Abstract

Females of the species Orconectes limosus have shown a preference for virgin males over those which had mated recently, when given the choice in prior studies. It was hypothesized that a depletion in sperm stores in the males could be an explanation for this preference. Using conventional methods such as microdissection, resin embedding, and light and electron microscopy, we attempted to show evidence of this depletion and the sperm's deposition within the female's spermatheca.

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Introduction

Females of many taxa are believed to bear more of an energetic burden when it comes to reproduction, and therefore have to be more selective in their mate choice than males (Trivers, 1971). In this project, the crayfish species, *Orconectes limosus* was used as a model organism, demonstrating how morphology and physiology can influence reproductive behavior. Earlier research has shown that female crayfish of this species show a marked preference for virgin males over those that have mated previously (Mellan & Warren 2011). This project had two related objectives. Our first objective was to carry out exploratory investigations of the internal and external reproductive biology of the crayfish. Our second objective was to test the hypothesis that males become sperm depleted after mating. We predicted that mated males will have vasa deferentia with less weight and that mated males will have a lower sperm count in sectioned vasa deferentia. This depletion of available sperm may make these males less desirable as mates and explain the observed female preference for males who have not yet expended their supply of genetic material.

In the following sections, we provide the background information on the external and internal reproductive anatomy of the crayfish. Then we explained the basics of sexual selection theory. Finally, we provide the evidence of sperm depletion in males of other species and taxa.

1.1 Crayfish reproductive morphology

Because morphology and behavior can each influence the evolution of the other, it is important to understand at least the basics of crayfish reproductive anatomy. In this section, we first review what is known about the reproductive anatomy of the decapods and their comparisons among the different families. Then, we explain the reproductive anatomy of

Cambarid crayfishes in detail. Thirdly, we review the sexual selection theory and sperm depletion. Finally, we explain the previous experiment from which our hypothesis was derived.

1.1.1 Comparison of sperm transfer and storage in Decapod species

Crayfishes belong to the order Decapoda of the subphylum Crustacea. In most Decapoda females, fertilization is apparently external and the spermatheca, if present, is not connected to the ovaries (McLay, 2011). The spermatophores are either simply attached to the ventral surface of the female or placed in a kind of chamber until they are ready to fertilize the eggs (McLay, 2011). The storage site of the sperm on the females is normally referred by the term "spermatheca" (Tavares and Secretan, 1993). Among the decapod species, the structures which are hypothesized to play a role in transfer and storage of sperm and seminal substances vary by a considerable amount (Bauer, 1986). In the nephropid lobster species *Homarus* and *Nephrops* which belong to the family Astacidea, there is an open invagination which leads to a spermatheca in which the seminal substances are stored (Herrick, 1895; Farmer, 1974a, b; Aiken and Waddy, 1980). The first pleopods of the males contain an apparent sperm channel which inserts into the spermathecal opening during copulation (Farmer, 1974a). Females of the Cambarid crayfish also have an open invagination and a median spermatheca (Andrews, 1905, 1906; Hobbs, 1974). However, the pleopods of the Cambarid male crayfish (which will be described in the next section) are more highly elaborated and complex than those of nephropids. Males of the other lobster species such as the Palinura do not have gonopods (Phillips et al., 1980). In most Palinura, a spermatophoric mass which consists of tubes with sperm is attached between the posterior walking legs of the females (Matthews, 1951). In anomurans, the first pleopods are either absent or slightly modified which might serve in sperm transfer (Bauer, 1986). They have

long penis-like extensions of the vas deferens which may be used in transmitting spermatophores (McLaughlin, 1980).

The genitalia of Astacid crayfishes are less intricate compared to the Cambarid crayfishes (Bauer, 1986). The first pleopods of the males are identical in that they are both cylindrical. However, the females do not have an open invagination and a spermatheca. Spermatophores are attached externally on the female cephalothorax in *Pacifastacus* crayfishes of the family Astacidae (Andrews, 1931). Male crayfishes of the Parastacid lack the first pair of pleopods. Females also do not have a spermatophoric mass depository. These indicate that Parastacid crayfishes of the family Astacidae have a simpler mode of sperm transmission and storage when compared to the Cambarid crayfishes (Bauer, 1986).

The Brachyuran crabs have paired spermathecae to store the spermatophoric masses (Bauer, 1986). There is a papilla extending from the end of the vas deferens which introduces seminal material into the base of the tubular first pleopods (Williamson, 1904). Williamson's studies indicated that the second pleopod may serve as a piston as it pushes the spermatophoric mass through the first gonopod into the spermatheca. In all brachyurans except the primitive ones such as the Dromiacea and Archaeobrachyura, the spermathecae and oviducts are not separated and the spermatheca is formed partially from the distal end of the oviducts (Hartnoll, 1986). This leads to the assumption that internal fertilization is possible in advanced brachyurans (Bauer, 1986).

1.1.2Reproductive anatomy of cambarid crayfish

On female cambarid crayfish, between the fourth and fifth pairs of walking legs is located the ring-shaped annulus ventralis, concealing beneath it the small chitinous pocket which is used for the storage of a male's deposited sperm (Andrews, 1905). The external structure and topology of this feature is species-specific, however all share in common an internal curved tube, the entrance to which is partially obscured from immediate view by the protruding knobs (Huner, 1994). The openings to the oviducts are located at the base of third pair of walking legs. When it comes time to lay her eggs, the female will extrude them into the waiting "glair", a substance which has been released from glands along the length of her abdomen and tail fan (Huner, 1994).

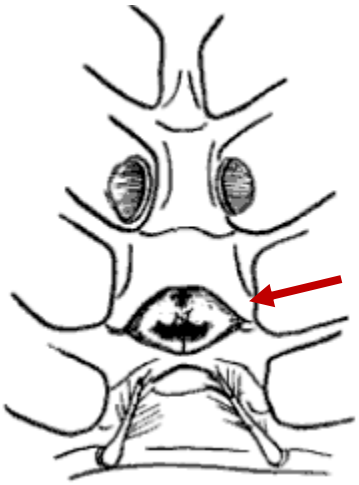

Figure 1 Ventral view of the female thorax (Andrews, 1906)

Males can be distinguished by the two pairs of gonopods which are the first two pairs of pleopods. These gonopods are longer and more robust than the rest of the pleopods. They are projecting forward between the bases of the legs. During mating, sperm are ejected from the gonopores and the gonopods are used to guide the sperm to the spermatheca of the females (Chidester, 1912). There are two morphological forms of male crayfish, and it is thought that form I males are sexually competent and form II males are not (Walls, 2009). They can also be distinguished by the first two pairs of pleopods. In form I males, the pleopods are bigger and ready to be used in mating (Walls, 2009). Sperm are ejaculated in the form of spermatophore which contain spermatozoa and may also contain other nutrients for females (Andrews, 1906).

1.2 Sexual selection theory & sperm depletion

Sexual selection is a form of competition where members of the same sex compete in respect to reproduction rather than competing for survival (Darwin, 1872). The theory for sexual selection is that males maximize fitness by mating with as many females as possible and females may achieve greater reproductive success by selecting males of high quality, irrespective of the number of mates (Bateman 1948; Trivers 1972; Thornhill and Alcock 1983; Arnold and Duvall 1994). Variation between individuals is the driving force of selection and reproductive success (Darwin, 1872). There are several mechanisms in which sexual selection takes place. They are parental investment, mate choice, inter-sexual selection and intra-sexual selection (Amendola et al., 2008).

Trivers (1971) defined parental investment as the cost endured by a parent to optimize the chances of offspring survival. Females have less abundant reproductive cells that are typically larger than male sex cells, and therefore, tend to have higher parental investment (Trivers, 1971). As a result, females have a higher energy cost in mating than males. Therefore, there would be selection on females to be more selective than males for mating choice: females have more to lose from each mating than do males.

In intra-sexual selection, one sex competes directly with members of the same sex to gain mates form the opposite sex (Krebs & Davies, 1993). While females decide for their mate choice, males compete with each other demonstrating their fitness in terms of their ability to produce viable, fertile offspring (Smith & Smith, 2011). Males may also fight to keep other males away from a specific location where a female is present (Moore et al., 2001). Therefore, male that wins different competitions would increase his number of female mates (Amendola et al., 2008).

In inter-sexual selection, members of one sex are attracted to members of the opposite sex based on different traits (Krebs & Davies, 1993). In apparent response to intersexual selection, males of many species have developed physical attributes as indicators of their competitive ability with respect to other males (Amendola et al., 2008). By avoiding competition, the males do not have to spend as much energy in getting females to mate. Physical attributes used in competitions and/or as indicators of dominance are also used to attract females which are known as ornaments (Berglund et al., 1996). These traits are inherited over generations since the victor of a battle is allowed to breed and are contained in the winner's genes (Darwin, 1871)

Whether or not a male mates with the female is often dependent on the female's choice of available males and male-male competition over available females (Kendall & Wolcott, 1999). Females should choose healthy males that have not recently mated because males with insufficient sperm resources may not provide enough resources needed to maximize the fertilization of the eggs (Kendall & Wolcott, 1999). However, males that mate with many females may mean that they have better genetic quality for the offsprings which allow them to be able to mate with more females. For example, females of *Drosophila melanogaster* and *Hyphessobrycon pulchripinnis* do not mate with recently mated males (Markow et al., 1978; Nakatsuru and Kramer, 1982). In some species, males may compete with each other to attract the females or to monopolize a female to maximize their fitness.

Females tend to have to invest more in their reproduction than males because they normally have fewer and larger gametes than males (Trivers, 1971). As a result, females may invest a larger energy cost in mating. Therefore, females should be selective in choosing their mate partners so that they do not mate only with males that have very low sperm resources to

provide for fertilization (Dewsbury, 1982). In a situation where a female mates with a depleted male, it is likely that the male will abandon her after mating (Amendola et al., 2008). Also, since females can only produce limited number of sex cells, their possibility of producing offspring decreases with each mating (Bateman 1948). Therefore, females need to make better choices in selecting mate partners to increase their chances of producing more viable offspring (Candolin, 1999).

The number of ejaculates males can deliver is limited because they need time to restore the depleted sperm after each mating (Dewsbury, 1982). It was believed that sperm are cheap to produce because they are small and numerous compared to eggs (Michalik and Rittschof, 2011). However, it has recently become clear that males also need to invest a lot of energy in mating for processes such as signaling, searching for mates and fights over territories or females (Andersson & Iwasa, 1996). Recent studies have shown that sperm production has significant costs (Dewsbury, 1982; Van Voorhies, 1992; Olsson, Madsen & Shine, 1997). This may result in limited sperm supplies and sperm resources in males.

Several studies have shown that males require time to restore their sperm reserves after each mating. An experiment examining reproduction in the blue crab showed evidence of sperm-store depletion and recovery by comparing the weights of the vasa deferentia in mated and unmated males (Kendall & Wolcott, 1999). Right after mating, the average vas deferens weight was half that of an unmated male's. By the ninth day after copulation, the weights of vasa deferentia were not distinguishable from those of unmated males, and by day twenty, had exceeded them. A study in the common toad, *Bufo bufo*, suggested that there is a change in sperm stores and ejaculate size after each mating (Hettyey et al., 2009). A further study in spiders showed that males of some species are unable to replenish sperm once it is used

(Michalik & Rittschof, 2011). Another factor that influences how male physiology affects mating interactions is that there is increasing evidence that males can and do manipulate their own ejaculates. For example, males with depleted resources reduce the overall size and the quantity of sperm that are transferred during mating (Rubolini, 2007; Galeotti, 2007).

1.3 Previous experiments and our hypotheses

Our project was designed not only to provide basic insight into reproductive morphology of the crayfish but also to test a specific hypothesis that had been generated by a previous student project in our laboratory. Mellan & Warren (2011) carried out an experiment to test the hypothesis that given the choice between a virgin and nonvirgin mate, individuals will both choose virgins more frequently and initiate mating faster with those virgins. The virgin males seemed to show no preference for either mating status in a female, but the females showed a marked preference for the virgin males, selecting them over the non-virgins nine times of eleven. In follow-up experiments, females were shown to have a similar preference, even if the non-virgin males had been given a week isolated from females to recover (Mathews, personal communications).

Prior research has suggested the role that depletion in sperm stores can play in the reproductive fitness of a male. With less sperm available with which to fertilize a female's eggs, and in some species, a smaller ejaculate to deliver them in, females may increase their own fitness by selecting mates that provide them with sufficient sperm to fertilize all their eggs (Galeotti et al, 2007).

Previous investigations of sperm deposition in crayfish have focused more on Astacid species as without the spermatheca, their spermatophore remains visible and exposed

(Dudenhausen and Talbot, 1979; Studitskij and Eljakova, 1972; Obradovic, 1989). A detailed study on the reproductive system of *Astacus leptodactylus* analyzed the spermatophore formation in vas deferens histochemically (Erkan et al, 2009). The testis and vasa deferentia were fixed with Bouin's solution and the tissues were serially dehydrated in an ethanol series and embedded in paraffin (Erkan et al, 2009). As our project focuses on the cambarid crayfish O. limosus, we had to use different methods, because we could not weigh nor observe the spermatophores directly. Instead, we dissected out the males' vasa deferentia and compared their mass. We hypothesized that, like in the blue crab experiment, a decrease in the weight of the vasa deferentia of our *O. limosus* would be indicative of a decrease in the male's remaining sperm store. Because previous data indicate that that females prefer virgin males over those that had mated a week ago (Mathews, personal communication), we predicted that the weight of the vasa deferentia of mated males will be lower than that of the virgin males.

Methods and Materials

Investigation of internal and external reproductive biology

We collected several crayfish species during the fall months of October and November for the purpose of anatomical exploration. Crayfish of the species *Procambarus* clarkii, an invasive species, were collected from Salisbury Pond in Worcester, Massachusetts, using traps baited with frozen chicken. Although not closely related to our species of interest, *O. limosus*, *P. clarkii* made for a good sample on which to test and hone our techniques, due to their abundance and larger size. *Orconectes limosus* and *O. quinebaugensis* specimens were collected via seining and hand collection at a site below the East Brimfield Dam in Sturbridge, MA along the Quinebaug River. All crayfish were transported back to and kept in the biology ProjectLab at WPI. Larger species, like the *P. clarkii*, were kept in individual tanks with air-stones to provide aeration, while smaller species like the *O. limosus* and *O. quinebaugensis* were kept in groups of eight to fifteen in larger, sex and species-separated containers, also outfitted with air-stones. Crayfish were fed on a diet of shrimp pellets twice-weekly. The water in the tanks was changed weekly, although the light cycles and water temperature varied with that of the room and the schedules of other students using the space.

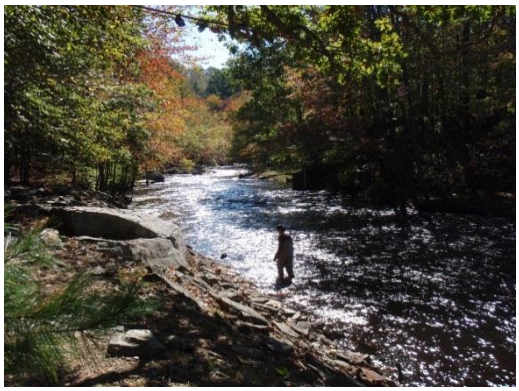

Figure 2 Collection below the Brimfield Dam

These were kept until mid-December, when the remaining population was paired off to mate, observed for mating behavior, and left overnight. We then sacrificed and fixed these crayfish via injections of 3-5mL of a Na cacodylate-buffered 2% glutaraldehyde solution through

the carapace in multiple locations. These specimens, once preserved, were kept in the refrigerator until they were later examined, and eventually disposed.

Objective 1. Finding and describing the spermatheca

Several females of the species *P. clarkii* and *O. quinebaugensis* were dissected to better understand the structure and function of the spermatheca, and whether it had any internal connections to the reproductive organs or nearby muscles. To begin, we found and identified the ovaries and then proceeded to trace along the path of the oviducts until they reached the gonopore located at the base of the third pair of walking legs, indicated by the red arrow in Figure 3

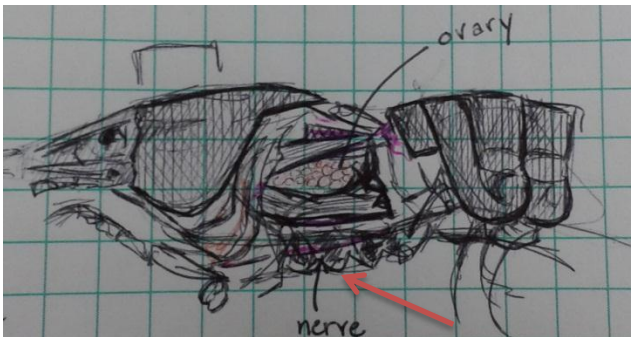

Figure 3 Lateral view of female crayfish anatomy. Drawing from observations made during dissections and not to scale.

To examine the spermatheca in some females, we made careful incisions along two of its sides to partially free it and allow for examination of any internal connections. In others, we approached the spermatheca dorsally, performing a general exploration of the region using watchmaker's forceps and minutiens pins. Once these examinations had been done, the spermathecae were completely removed from the female and examined primarily under a dissection microscope. For a clearer view of the structure, some were cleaned using a soft-bristled toothbrush to brush away any detritus accumulated in the wild. When cleaned and backlit under a dissecting scope, some spermathecae had darker regions along the inner curves, indicating something was inside. If the spermatheca seemed to contain something, we tried a number of methods to extract a sample, including but not limited to: centrifugation of the spermatheca to create a pellet, insertion of a transfer pipette tip into the invagination and

applying suction, and piercing the shell of the spermatheca using a syringe to capture the contents.

Objective 2. Visualizing male anatomy and gametes

As with the females of the species, we wanted to familiarize ourselves with the reproductive anatomy of the males so that we could better design our later experiment and interpret our results. To do so, we worked with males of the *P. clarkii* and *O. limosus* species. Again, a general tour of the gluteraldehyde-fixed reproductive system was made, following the vas deferens along their coiled length from the gonopores at the fifth walking leg to the testes. Samples of the vasa deferentia and testes were taken, and once examined beneath a light microscope, were set aside for resin-embedding and sectioning.

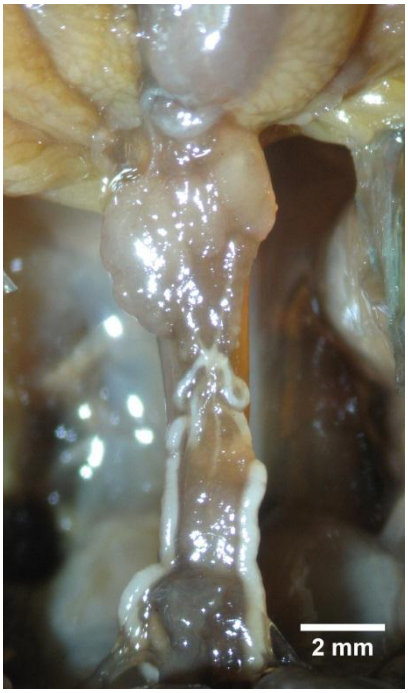

Figure 4 Gluteraldehyde-fixed male *O. limosus* with visible vasa deferentia

The samples were trimmed to a smaller size, less than 0.5cm in length, pinned into silicone molds, and then post-fixed with 1% osmium tetroxide in 100mM Na cacodylate buffer, pH 7.6, as for the fixation with glutaraldehyde. These were then dehydrated by soaking in a series of 70%, 95%, and three subsequent 100% ethanol baths to prepare for resin embedding. Samples were infiltrated with a set of ethanol and Spurr's low-viscosity resin mixtures, (2:1 then 1:2for an hour each), before being soaked in two batches of pure resin and allowed to cure for 24-36 hours in a 70C oven. Once cured, the resin surrounding the samples was trimmed, and embedded tissue was sectioned with glass knives on a Porter-Blum Mt-2 Ultramicrotome at multiple locations along the vas deferens. Sections 0.5 µm thick were mounted on glass

microscope slides, stained with 1% toluidine blue and examined beneath a light microscope at various objectives. Ultrathin sections (60-90 nm) were collected on 3mm 300-mesh nickel grids (Electron Microscopy Sciences, Hatfield, PA) and post-stained with Uranyl Acetate and Lead Citrate to enhance electron density. They were examined in a JEOL 100-CX transmission electron microscope at 80kV at magnifications from 2,000 to 10,000.

Evaluation of sperm depletion in mated males

The remaining goal of this project was investigate males for evidence of sperm depletion in males after mating. Specifically, we tested the hypothesis that males who had recently mated would have vasa deferentia of a smaller mass than virgin males. We also hypothesized that given more time after mating to do so, mated males would recover some of their available sperm stores, and similarly show an increase in the mass of the vasa deferentia.

To do so, crayfish of the species *O. limosus* were collected from the same site on the Quinebaug River, just below the East Brimfield Dam. As we wanted to compare the sperm capacities of mated and un-mated males, these were collected in the summer, before the mating season began in the fall. These were kept separated by gender and in individual tanks in a recirculating system at the WPI Life Sciences Center at Gateway Park. The light cycles and water temperatures of the system were arranged to mimic natural conditions, and the crayfish were fed on a regular diet of plant materials and detritus also collected from the Quinebaug River, as well as commercially-available shrimp pellets.

As the crayfish used for this experiment had been collected and separated by gender before the start of the year's mating season, we considered their initial mating status to be

virginal, meaning they had not yet mated for the current year. With this assumption, we assumed the males' sperm stores to be at their full possible capacity.

We had 23 virgin males and 24 virgin females available for the mating experiment. Each was measured for the length of its carapace, from the tip of its rostrum to the end of the cephalothorax. These measurements were used to sort them into one of three groups, distributing them so that the average carapace length for each group was within 0.1mm of the other two. Seven males were kept as the first, control group and remained unmated. The remaining sixteen males were placed into tanks with a female of the same relative carapace length and observed for the hour following. Once a pair had assumed a copulatory position, like the one shown as Figure 5 above, the pair was noted as having mated. Left overnight, we could assume that these pairs had mated at least once, if not multiple times, as when we returned the following day, they were all locked in the mating position. Those who were not observed mating were excluded from further examination.

Figure 5 *O. limosus* pair in the mating position

Of the sixteen males we allowed to mate, eight were preserved with the virgins on the first day afterwards, and the other eight were preserved one week later. The males were all fixed using an injection of approximately 5mL of a 2% glutaraldehyde solution and refrigerated until their dissection. The vasa deferentia of the males were removed and then weighed on a Mettler Toledo classic plus enclosed balance, accurate to 0.0001g. The length of each vas deferens extracted was also measured. As the vasa deferentia are a highly coiled structure within the

male's thorax and the gluteraldehyde works to link proteins, some samples retained some of these coils. Samples were laid out on a Sylgard-layered petri dish, carefully stretched as straight as possible, and pinned into place to be measured. Figure 6 below shows a spermatophore stretched and pinned into the Sylgard. This sample's wall snapped, exposing what appeared to be the spermatophore.

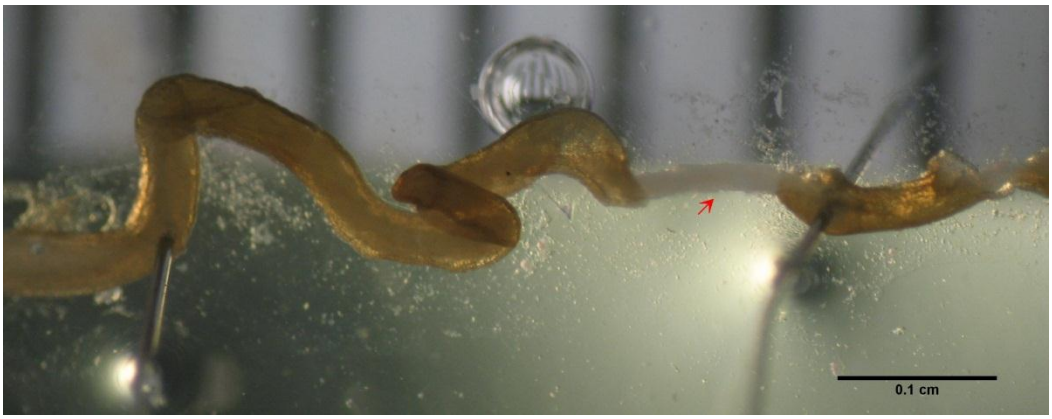

Figure 6 Pinned vas deferens showing exposed spermatophore at break (red arrow)

Samples that were to be sectioned were removed from their Sylgard plates, cut into smaller, 0.5-1.0cm sections, and re-pinned to silicone molds. As with the previous examination of the vasa deferentia, these were embedded in resin for microtome sectioning. These sections were examined beneath a light microscope to look for visible differences in the contents across the three groups. The denser vas deferens from one male of each group was used for this, taking sections at three locations along the length; near the gonopore, at approximately the midpoint, and near the gonads.

Analysis of quantitative data

The crayfish carapace lengths, and vasa deferentia lengths and weights were all entered into a worksheet in Microsoft Excel 2010 for easy comparison. As there was a variation in body size across our samples we normalized the vasa deferentia masses by dividing them by the

samples' lengths to return a 'density' value. Using the built-in functions, the mean density for each group of males, (virgins, one-day, one-week, and all mated males), was calculated and plotted as a bar graph, also showing the calculated standard error for each.

With such a small sample size, we could not assume our data would be normally distributed, so we used a Kruskal-Wallis test to compare the results across the three groups of virgins, mated males after one day, and mated males after one week. A comparison of virgin versus non-virgin data was also made using the same vas deferens data and the Mann-Whitney U-test. For the purpose of these evaluations, our null hypothesis was that there is no significant difference in the density of the vasa deferentia of a virgin or mated male, nor between mated males who have had different amounts of time post-copulation to recover their stores.

Results

Investigation of internal and external reproductive biology

Objective 1. Finding and describing the spermatheca

Through careful dissection of the region surrounding the spermatheca in the females, no apparent connections to other portions of the reproductive system (ovaries or oviducts) were found. There did not seem to be any muscular or vascular connections to the spermatheca either, with it only being held in place by tissue that appears to be thin, flexible cuticle.

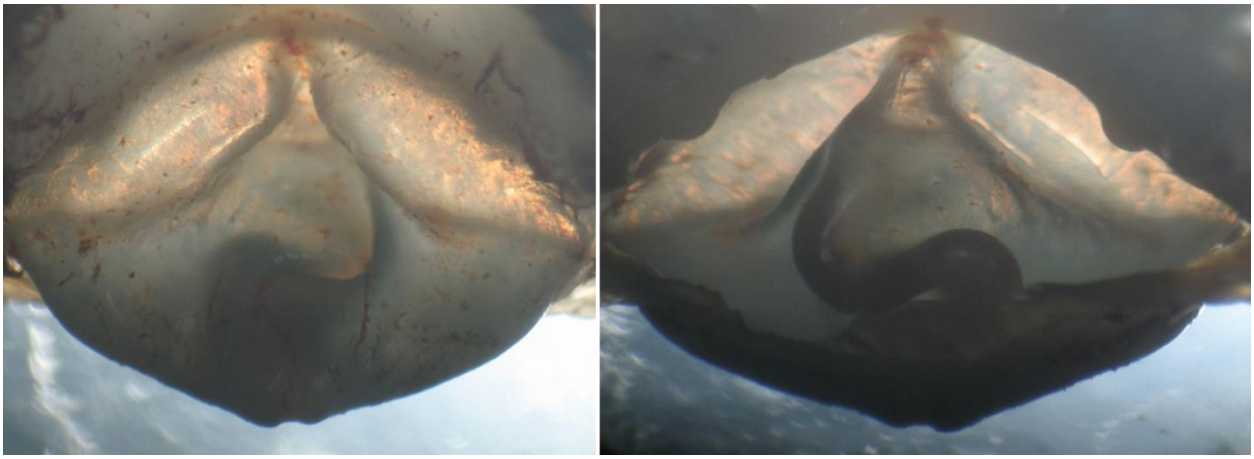

Figure 7 External (left) and internal (right) view of a cleaned *O. quinebaugensis* spermatheca

The spermatheca for the *P. clarkii* and *O. quinebaugensis* differed in their external appearance, but shared a similar structure, like that described by Andrews (1906b), sharing the suture on the external surface and the winding inner tube. It was observed to be a solid piece of the exoskeleton, lacking any pores or connections to softer tissue inside, as can be seen in Figure 7 above. Further evidence of its disconnect from other systems was found when examining the moult of a female *O. limosus* in the ProjectLab. This portion of cuticle was shed with the remainder of the exoskeleton and remained intact until it was later consumed by the female.

Objective 2. Visualizing male anatomy and gametes

From an *O. limosus* male, we sectioned both a segment from the testes and the vas deferens. From the sections of both of these organs, one can make out the individual sperm cells, stained a vibrant blue by the toluidine stain. Figure 8 shows a section of the testes of a recently-mated *O. limosus* male. Clustered together near the center of the frame are visible a number of sperm cells, similar to those found later in sections of the vasa deferentia.

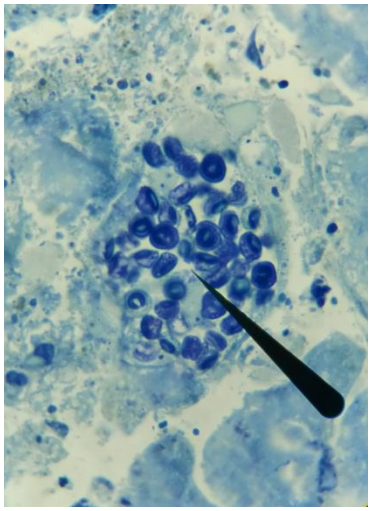

Figure 8 Section of *O. limosus* testes (100x

In the image of the vas deferens cross section, (Figure 9) one can make out the organ's wall around the edge of the frame. Within it the spermatophore, seen as the large acellular blue mass indicated by a red arrow, is clearly visible and abundant. Also visible are the sperm, present in the matrix outside the spermatophore, and suspended within it.

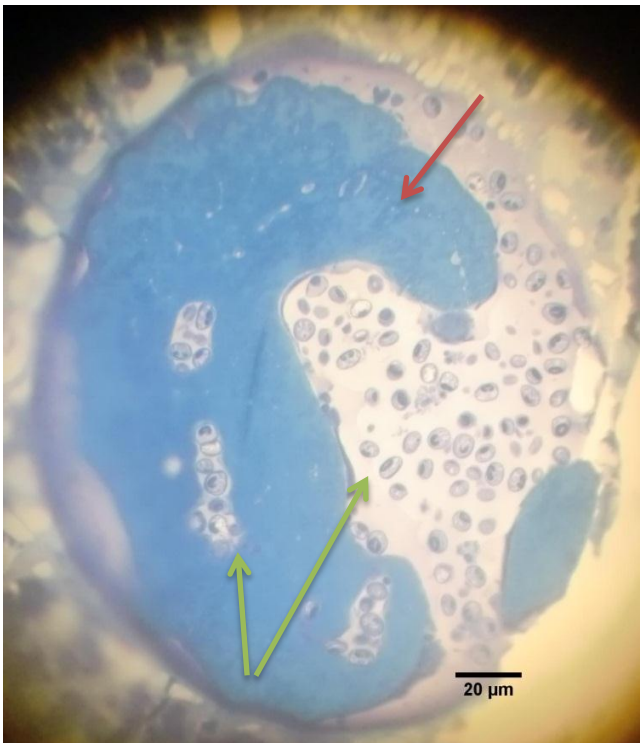

Figure 9 Cross-section of the vas deferens of a recently-mated *O. limosus*

Two sperm from a nearby section of the vas deferens were taken, one free-floating outside the spermatophore, and one from within it. Photographed under transmission electron microscopy, these can be seen in Figure 10 at left, with the acrosomal cap (red arrows) clearly visible.

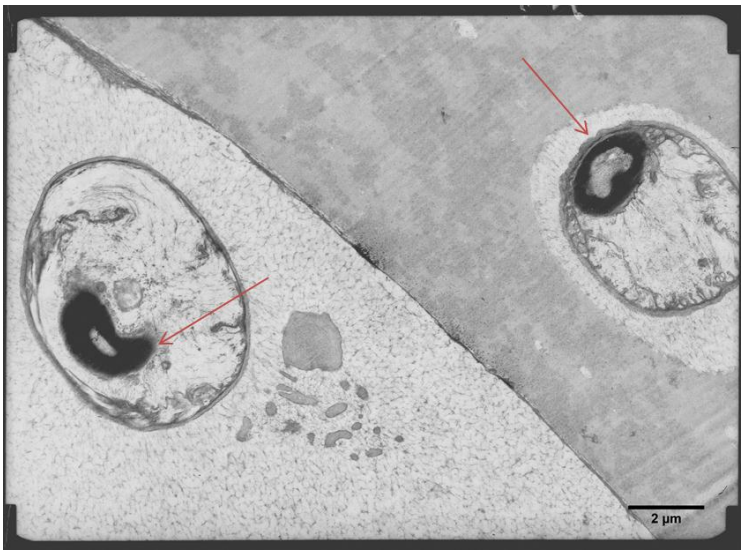

Figure 10 TEM of an *O. limosus* vas deferens, showing sperm outside spermatophore (left) and within the spermatophore (right)

Further TEM was performed, examining the structure of the wall of the vas deferens. The resulting image can be seen as Figure 11 below. The left potion of this image shows the wall of the vas deferens, containing a number of nucleated cells and vacuous spaces that are presumably secretory in purpose. Along the wall can be seen microvillar extensions pushing into the spermatophoric matrix at right. As these tubules did not seem to be surrounded by muscle, these extensions may play a role in urging the spermatophore along the length of the vas deferens.

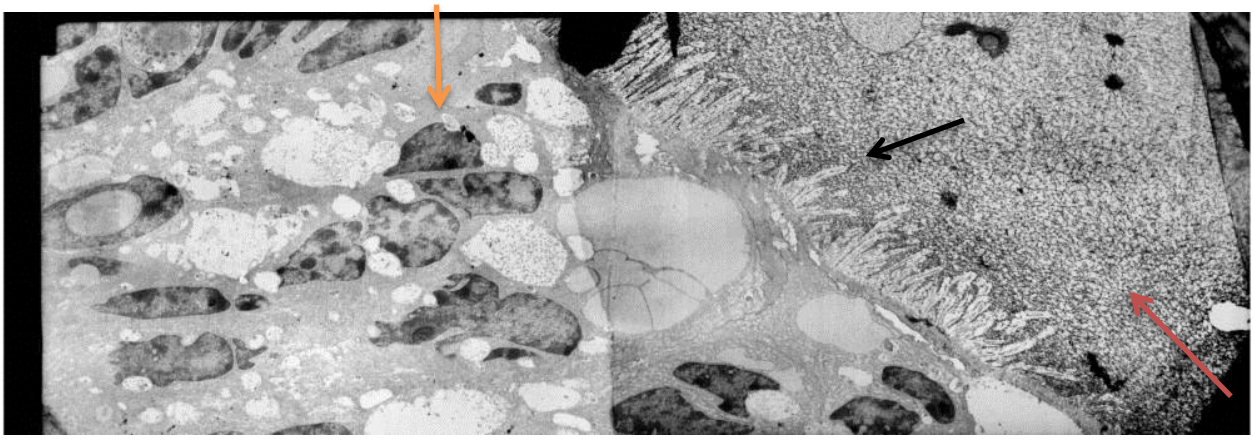

Figure 11 Composite of two TEM images of a vas deferens wall with arrows indicating cell nuclei in the wall (orange arrow), microvillar extensions (black arrow), and spermatophoric matrix (red arrow).

Evaluation of sperm depletion in mated males

Of the twenty-three males provided for this experiment, seven males remained virgins as laid out in the initial plan. From the eight males assigned to be a part of the one-day trial group, two had to be excluded from the data as we could not confirm if they had mated during the overnight period. The remaining eight from the one-week group were all observed in the mating position. Of these twenty-one males eligible for further study, logistic constraints required that only seventeen were dissected and had their vasa deferentia data recorded. These were selected blindly from the eligible samples, but provided a relatively even distribution across the three groups, with six virgins, five one-day males, and six one-week males. The normalized weight data can be seen below as Figure 12, with standard error bars included.

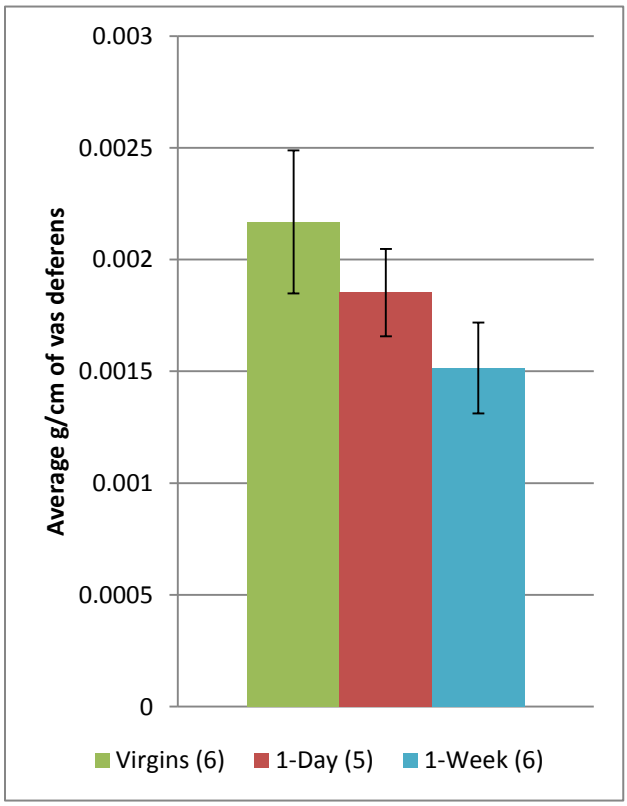

Figure 12 Graphs of mean vasa deferentia data across three groups: virgins (N=6), males fixed one day post-mating (N=5), and males fixed one week post-mating (N=6)

When the three-group data was input into a Kruskal-Wallis test, the difference in vas deferens density was not shown to be significant ($H=2.76$, $df=2$, $p=0.2516$). To determine if there could be a difference between the data for virgin males and those that had mated, the collected data was regrouped and put through a Mann-Whitney U test ($U=18$, $z=1.46$, $p=0.0721$). Neither of the p-values obtained with these methods fell below the 0.05 threshold commonly accepted for statistical significance.

To supplement the quantitative data gathered above, we returned to some of the techniques explored earlier in the project. By taking one of the vasa deferentia extracted for these measurements and embedding it in resin, we were able to look at thin cross-sections of each sample. Figure 13 below shows sections taken from the approximate midpoint of each sample, with the green arrow indicating the pocket of sperm, and the red the spermatophoric mass. In the virgin male (panel A), both are plentiful and well-defined. In the one-day sample (panel B), neither can be seen, and the one-week male (panel C) seems to be an intermediary, showing broken masses of spermatophore and a much smaller and less dense pocket of sperm in the center. These results are only from one male from each group, and therefore cannot be used as definitive examples, but merely examples of a possible trend.

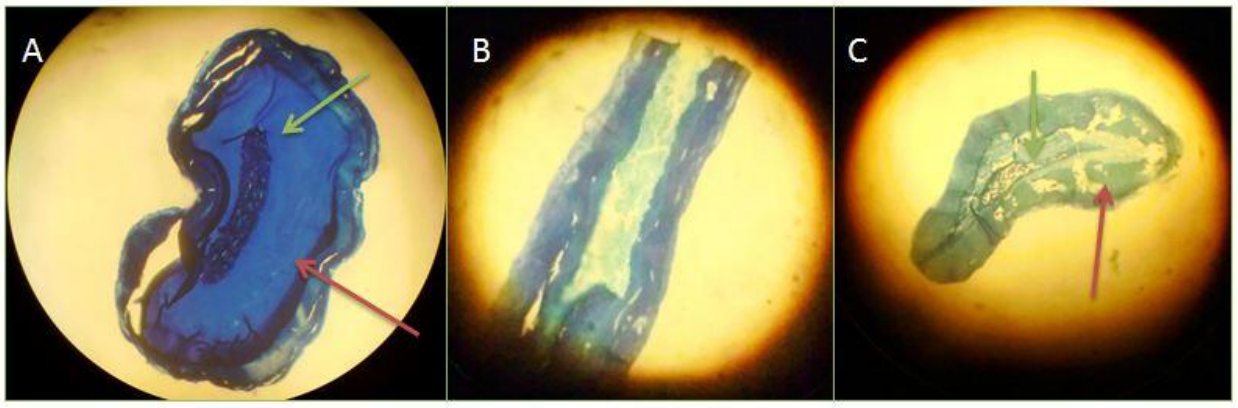

Figure 13 Sections of vas deferens from (A) virgin male, (B) 1-day mated, and (C) 1-week mated male *O. limosus*

Discussion

The goal of this project was to gain an understanding of the male and female crayfish reproductive systems and anatomy, and to then apply this knowledge to test a hypothesis explaining previously observed behaviors. Virgin females of the species *O. limosus* had shown a distinct preference for virgin males over mated males (Mellan & Warren 2011). Evidence of sperm depletion has been found in other crayfish and decapod species. We hypothesized that these previously-mated males had suffered a depletion in their stores of sperm as well, and that this difference in sperm quantity would be reflected in the weight of their vasa deferentia.

In our examinations of the spermatheca, we found this pouch to have no outlet, nor any connections to internal structures, other than those holding it in place. Sperm is known to be deposited within this closed pouch, and then used to subsequently fertilize the eggs laid by the wwhich we examined. As the spermatheca is an inflexible chitinous structure that lacked any visible orifices other than the externally-visible one, we assumed that some other mechanism must be employed to remove the sperm to fertilize the eggs externally. Female *O. virilis* secrete a substance called glair prior to releasing their eggs, which dissolves the deposited sperm plug. This distributes the sperm into the glair through which the eggs are passed to be affixed to the female's swimmerets (Clifford, 1991).

If male *O. limosus* deposit sperm plugs into the spermatheca of females after they've finished mating with them, these should work to inhibit other males from mating with her. As such, one would expect males to, when given the choice between virgin and mated females, to show a preference for the virgins. When this hypothesis was tested previously, the males showed no preference, choosing virgins just as frequently as nonvirgins (Mellan & Warren 2011). In *A. italicus*, males have been shown to be able to adjust their sperm expenditure as a

response to mating with a nonvirgin female (Galeotti 2007). It is possible that *O. limosus* have developed something similar to this, allowing for sperm competition. Further exploration could be done to examine the nature of the sperm plug and any other material deposited in the spermatheca during mating. If this can be examined, then these depositions could be compared across the primary and secondary males.

Although unable to extract anything of this type from the spermathecae of mated females, we were able to examine sperm cells and spermatophores as extracted from male specimens. Using some of the methods we used to observe these structures, we obtained a measure of vas deferens density that we believed would correlate with the amount of sperm available for future matings. Given a population of virgin *O. limosus*, we allowed some males to mate overnight with a female, preserving them along with the virgin samples either one day or one week after mating. The vasa deferentia were weighed and measured, providing us with data which we could compare statistically across groups.

The quantitative data obtained from the mating experiment was not enough on its own to support our initial hypothesis. While the virgins did show the highest average vas deferens density, the difference between this value and that of the two mated male groups was not large enough to confirm that this was not merely by chance. There was an unexpected trend in the data we gathered that indicated the males who had six days more to recover their sperm stores had, on average, a lower mass per length of vas deferens than the males preserved directly after mating. This could be the fault of a small sample size, but this does give some hint that the males are not so quick to recover their stores as some other decapods. It is possible that their depletion model more closely resembles that of the astacid crayfish, *A. italicus.* These were

observed to be depositing smaller quantities of sperm with successive matings, suggesting also a limited supply and possibly a slow regeneration period (Rubolini et al. 2007).

Our quantitative data suggested that there may not be a significant depletion of sperm store occurring after a male crayfish mates. From the sections of vasa deferentia that we examined, there did seem to be at least a visual difference in the contents of the vas deferens. In the section of the virgin male's vas deferens, a large mass of spermatophore surrounding a dense pocket of sperm in the center was visible. The male fixed one day after mating lacked either of these, seeming to have collapsed and split. The one-week sample showed a small quantity of both spermatophore and sperm near the center. This could be possibly interpreted as evidence of an initial depletion after mating, and the recovery of sperm stores after some time without mating has passed. However, these observations must be kept in perspective, as these sections were only taken from the vas deferens of one male from each group.

It is possible that there is no significant difference in the vas deferens mass and sperm quantity between virgin and mated males. However, there is also the possibility that by expanding upon what was done, more robust results could be found. A sample size of $N<5$ in each group is considered "too small" for the Kruskal-Wallis test for it to return a firm conclusion, and with five or six to a group, our data is on the edge of that acceptable range (McDonald 2009). By expanding the sample groups in size, or by including in future a group that mates multiple times or is given more time to recover after mating, one might be able to obtain more robust results and clear up possible ambiguities in our own.

It is also possible, that although the weight of the vas deferens is a decent measure of sperm capacity in the blue crab, it is not the most reliable measure for crayfish. An alternative

method that could be explored would be performing sperm counts at various slices along the length of the vasa deferentia, although this would be a time and labor-intensive process.

Although some of our findings did not fully support our hypothesis about sperm depletion in mated *O. limosus*, the techniques we employed might be of use to future studies in the species or other crayfish and decapod species. Future work could focus more on the nature of the spermatophore, its makeup, deposition, and eventual dissolution, as well as the existence of a sperm plug. Additional research could examine what cues females use to differentiate virgin males from mated ones, whether they be pheromonal, behavioral, or a physical change, as this still remains somewhat unknown.

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