**Brood size and offspring sex ratio of the intertidal amphipod, *Corophium volutator*
(Pallas): apparent effects of a novel microsporidian.**

by

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ABSTRACT

I investigated the effects of a potentially sex-distorting parasite on host offspring sex ratios. The host-parasite relationship examined is that of the intertidal amphipod *Corophium volutator* and a novel microsporidian. Ovigerous females were collected from the field and resulting offspring were reared under environmentally controlled conditions. Using molecular screening techniques, microsporidian infection was determined. Further, I investigated the effects of this microsporidian on host brood size, namely the brood volume and the total number of embryos within a clutch, using ovigerous females collected in the field. I also tested whether prevalence of the microsporidian was variable among male and female *C. volutator* collected from the same population at the Bay of Fundy.

I found evidence suggesting that the microsporidian in question is a vertically transmitted sex ratio distorter. Additionally, I found that the parasite was correlated with smaller brood volumes, and a non-significant trend towards fewer embryos. *C. volutator* is an important prey species in the Bay of Fundy. Further work on factors influencing local population crashes and recolonization is warranted.

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GENERAL INTRODUCTION

Microsporidia are obligate intracellular parasites; there are more than 1200 known species of microsporidia in 143 genera (Mathis, 2000). Species from nearly all invertebrate phyla and all five classes of vertebrates are hosts to one or more species of microsporidia; as such, microsporidia can indeed be considered ubiquitous (Mathis, 2000). Microsporidia are the causative agents of several economically important diseases, including diseases affecting silkworms, honey bees, fish and various mammals (Becnel and Andreadis, 1999; Shaw and Kent, 1999; Canning, 1986). Additionally, microsporidia have been used as biological control agents, most successfully in grasshoppers (Canning, 1993). More recently, microsporidia have been identified as opportunistic pathogens of humans, primarily in immunocompromised individuals (Mathis, 2000).

As we have learned more about the phylum Microspora, there has been reclassification of the relationship this phylum has with other eukaryotes (Keeling et al., 2000), as well as renaming and reclassification of species (Agnew et al., 2003). Microsporidian cells lack several components considered standard in eukaryotic cells, including mitochondria, centrioles, peroxisomes, 80S ribosomes and stacked Golgi apparatus (Agnew et al., 2003). The 70S ribosomes that microsporidian cells contain are more representative of prokaryotes than eukaryotes (Isihara and Hayashi, 1968). However, microsporidia do contain a true nucleus. As such, microsporidia were considered to be ancient eukaryotes. More recently, molecular relics of mitochondria have been found in microsporidia, in the form of heat shock proteins. Therefore, it is likely that ancestors of microsporidia possessed mitochondria (Agnew et al., 2003). Molecular phylogenetics based on these heat shock proteins have determined that

microsporidia are most closely related to fungi. Additional evidence for this relationship is the microsporidian α and β -tubulins, which are highly similar to those possessed by fungi (Keeling and McFadden, 1998). According to this molecular data, microsporidia do not belong at the base of the phylogenetic tree, clustering with prokaryotes, but in the crown of the eukaryotes (Hirt et al., 1999).

Prior to the accumulation of molecular phylogenetic work, microsporidia were classified to species according to ultrastructural characteristics. Microsporidian spores are uniquely identifiable by the presence of a coiled **polar filament**¹. This filament is inverted and attached to the anterior wall of the spore. The number of coils that comprise the filament varies between species. When germination is triggered by appropriate environmental conditions, the polar filament inverts and extends, becoming a tube for the transport of the parasite's **sporoplasm** and nucleus into the host cell's cytoplasm. The extrusion of the polar filament is explosive, and when completely extruded, the polar filament can extend up to 12 times the length of the spore. In addition to the polar filament, spore size and shape are also characteristics used to classify microsporidia (Agnew et al., 2003). Species of microsporidia can contain a single nucleus or nuclei in diplokaryotic arrangements (Keeling and McFadden, 1998). The life cycle of microsporidia can also be used to classify species. Generally, there are two phases in the microsporidian life cycle. The first phase is proliferative, known as merogony. During merogony, division by binary or multiple fission occurs. The second phase is sporulation, which involves two steps. Sporogony is the first step in sporulation and consists of the **sporont** dividing into two or more **sporoblasts**. The sporoblasts then mature into spores during spore morphogenesis (Canning, 1993).

The route of microsporidian infection varies, but the most common is by ingestion of spores from the host's environment. Gut cells are the site of spore proliferation, resulting in the rupture of host cells. Free spores in the gut lumen are passed into the environment with the host's feces for **horizontal transmission** to other hosts. The mechanism of horizontal transmission suggested above is one of the most simple, and is associated with chronic negative effects on the host's fecundity and/or life span. Some microsporidia species spread from the host gut to other tissues by intrahost transmission. The spores produced for intrahost transmission are generally smaller, with shorter polar tubes and thinner spore walls. An additional mechanism for intrahost transmission is the dissemination of spores into host hemocoel, where the spores can be circulated throughout the host. The most pathogenic microsporidia exploit the inner tissues of the host for spore production, causing widespread pathology and host death. The host is essentially transformed into a sack containing millions of spores which, as the host decomposes, are released into the environment to infect other hosts (Agnew et al., 2003).

A less pathogenic form of intrahost transmission targets the host's offspring for infection. Circulating microsporidian spores infect reproductive tissue and the infection passes to host offspring. This **vertical transmission** ensures parasite transmission when horizontal transmission is inefficient due to low host density, or extreme environmental conditions (Agnew et al., 2003).

Historically, microsporidia species were thought to be strongly host-restricted and a microsporidian was considered a new species if it occurred in a new host. However, there is increasing evidence that some microsporidia infect several hosts and a parasite's morphology, including spore size, and even mode of transmission, can vary between

¹ Operational definitions can be found in Appendix 1 for terms bolded in the text

hosts (Canning, 1993). This **phenotypic plasticity** is not unique to microsporidia (e.g. Caswell, 1983; Bull, 1987). Between microsporidia species, and even between hosts, pathogenicity varies greatly. Infections may be systemic, specific to one type of host tissue, or even specific to one cell type within a host tissue. There are eight genera of microsporidia known to infect crustaceans (Canning, 1993). This study concerned the characterization and investigation of the effects of a novel microsporidian on its host, *Corophium volutator* (referred to hereafter as *C. volutator*, mudshrimp and/or amphipod(s)). There are several important aspects of *C. volutator*'s natural history and biology that are relevant to this study.

C. volutator is an intertidal amphipod. This species has a global distribution with populations found throughout the British Isles, from Norway to the Adriatic Sea, and along the North American coast line (Wilson et al., 1997). In Canada, *C. volutator* populations are restricted mainly to the Bay of Fundy, where they are commonly known as mudshrimp. The largest individuals are 10mm in size, and populations contain as many as ten thousand individuals per square meter (Gratto et al., 1983). *C. volutator* occupation prevents the gradual erosion of the mudflat, as individuals form permanent U-shaped burrows (Brower et al., 2000). However, the true ecological importance of this amphipod is as a prey species. Migratory shorebirds, including the semi-palmated sandpiper, *Calidris pusilla*, stop at the Bay of Fundy during annual migration to feed on *C. volutator* (Matthews, 1992). Mudshrimp are vulnerable to shorebird predation largely because of shallow burrows and the crawling behaviour of mate-searching males (Jensen and Kristenson, 1990; Schneider, 1994). In addition to the intense periods of shorebird predation, fish also prey on *C. volutator* (Wilson, 1989). Much research has been focused

on *C. volutator* population dynamics and life history. In a review of the literature, it can be seen that *C. volutator* is often referred to as a keystone species (e.g. Lui, 1998; Percy, 1999; McCurdy, 2000). There has even been a Keystone Species Working Group, active since 1987 and part of the Bay of Fundy Ecosystem Partnership. However, careful consideration of the literature indicates that the application of the term “keystone species” to *C. volutator* is inappropriate. The concept of keystone species is attributed to Paine, from his submissions to *American Naturalist* (Paine, 1966, 1969). Although this term has become widely used, Paine did not intend for “keystone” to be a defined concept; instead, Paine considered the term a metaphor for a predator with a large impact on a community (Paine, 1995). Keystone, however, has been used in the literature as an adjective for nearly everything, including parasites, pathogens, herbivores, roles, processes and communities (Hurlbert, 1997). Overall, the term keystone is broadly applied, poorly defined and non-specific in meaning (Mills et al., 1993). A well-operationalized definition of keystone species is a species whose impact on its community is both large and disproportionately large relative to its abundance (Power et al., 1996).

It is apparent that, although historically considered a keystone species, excessive densities of *C. volutator* preclude the consideration of this species as keystone. A more appropriate designation would be key species, defined as a species which drives ecosystem processes or energy flows (Piraino and Fanelli, 1999). Although key and keystone species are occasionally considered interchangeable in the literature, it is important to note that the two terms are not equivalent (Higdon, 2002). The major difference between a keystone and key species is the species’ importance relative to its biomass. *C. volutator* is extraordinarily abundant and as such does not meet the criteria

for a keystone species. In order to avoid the confusion of two very similar terms, the term “ecological dominant” has also been suggested as an alternative for keystone (Davic, 2000). An ecological dominant species is defined as “a species or species group that largely controls the energy flow and strongly affects the environment of other species” (Odum, 1971). Therefore, *C. volutator* can be considered either a key species or an ecological dominant species. Determining the factors which affect or regulate populations of this mudshrimp is none the less seen as a very important undertaking by researchers worldwide.

This thesis deals specifically with the investigation of the effects of an unknown microsporidian on host sex ratios and direct effects on host **fecundity** and brood sizes; two factors which should relate to population growth or recruitment and/or stability. For example, in a recent paper, Forbes et al. (2005) showed that male *C. volutator* become limiting as the season advances on mudflats in the Bay of Fundy. Their limitation, relative to abundant females, is associated indirectly with reduced rates of female ovigery and reduced brood sizes. The factors underpinning male limitation have not been studied but may well relate to parasitic sex ratio distorters.

Specific Objectives

In Chapter 1, I begin by examining variation in prevalence of the novel microsporidian in male and female gonads. Female-biased prevalence would indicate a potential for sex distortion by the parasite (Galbreath et al., 2004; Terry et al., 2004). Following the investigation of prevalence of infection in adult sexes, I then determine the sex ratio of broods from infected and uninfected mothers. The difference in sex ratio

between infected and uninfected broods suggests whether the parasite (or a correlated factor) has an effect on host sex ratio (Cook, 2002). Generally, Koch's postulates must be fulfilled in order to establish a causative link between parasite and disease. First, the parasite must be present in all diseased hosts, but not in healthy ones. Secondly, the parasite must be isolated from a diseased host and grown in pure culture. The cultured parasite should cause disease when introduced into a healthy host, and finally, the parasite must be isolated from the experimentally infected host (Evans, 1976). However, microsporidia are obligate intracellular parasites and cannot be directly cultured. Additionally, experimental infection is improbable, as the route of infection of this parasite is unknown and therefore impossible to mimic at this time. As it is not possible to fulfill Koch's postulates at this point, I restrict myself to the investigation of a correlation between parasitic infection and sex ratio distortion. Finally, I consider a measure of fitness (namely, brood size) of infected and uninfected mothers, using female length as a covariate. In amphipods specifically, female length is strongly correlated with the number of embryos (Fish and Mills, 1979; Dunn and McCabe, 1995; Llodra, 2002). The study comprising this chapter is hypothesis-driven. I predict that infection with the novel microsporidian will be correlated with host sex ratio distortion.

In Chapter 2, I further investigate the fitness of infected and uninfected ovigerous females by quantifying two measures of fecundity; brood size (the total number of embryos) and brood volume (the total volume of the embryos). I examine the variation in brood volume between infected and uninfected mothers, again using body length as a covariate. I test the null hypothesis that there are no differences in fecundity of infected

and uninfected females, and that there is no difference in the length of females, however this study is mainly a collection of observations.

Finally, I also have two ancillary objectives. In one of my appendices, I provide the first ultrastructural and molecular characterization of the novel microsporidian infecting the intertidal amphipod, *C. volutator*. This work suggests that we may well be dealing with a species new to science. In another appendix, I more fully investigate the incidence and functional significance of intersexuality in the amphipod, *C. volutator*.

These two ancillary objectives support my main objectives. By providing ultrastructural and genetic information, researchers can later examine whether this microsporidian is related to others which also have been suggested to be sex ratio distorters. Further, one possibility for this host species is that intersexes are incompletely feminized males and my work may have some bearing on this possibility.

CHAPTER 1

Offspring Sex Ratio and Infection of Females of the Intertidal Amphipod, *Corophium volutator* by an Unknown Microsporidian

Introduction

Sex determination is the process by which an individual is assigned a gender. The process can involve genetic and environmental factors, or even a combination of the two. Many vertebrates have genetic sex determination, with X and Y chromosomes acting as the repositories for genes by which sex is determined. Invertebrate sex determining mechanisms may be conserved within taxa, or differ between closely related species, making sex determination much more difficult to elucidate (Bull, 1983; Rigaud, 1997). The major categories of invertebrate sex-determining mechanisms include both genetic and environmental sex determination. Chromosomal sex determination can be a result of male or female heterogamety. Haplodiploidy is also a mechanism by which sex is genetically determined in invertebrates, and includes arrhenotoky and paternal genome loss. In both types of haplodiploidy, fertilized eggs become diploid females and unfertilized eggs, or eggs which have lost the paternal contribution, develop as haploid males (Bull, 1983; Cook, 2002). Environmental sex determination mechanisms include temperature, photoperiod, nutrition level and mate availability as cues. This method of sex determination is widespread in Crustacea, where females are the favored sex at long photoperiods, high temperatures and high nutrition levels. Another less common cue for

environmental sex determination is mate availability. Females are the default sex and males occur only when females are present (Bull, 1983; Hamilton, 1967; Cook, 2002).

Although the sex determining mechanisms of most invertebrate species are unknown, it is likely that one of the mechanisms outlined above is an important mechanism for sex determination for species where it is currently unknown. While sex determination may be primarily genetic or environmental, there are also polyfactorial systems, in which many factors, possibly both genetic and environmental, influence sex determination.

To further complicate the mechanisms of sex determination, consideration must be given to cytoplasmic sex factors (Bull, 1983). Also known as sex ratio distorters (SRDs), these factors are inherited elements that are capable of host sex ratio modification, promoting their own transmission presumably at some cost to host fitness. Host offspring are female-biased as the SRD feminizes male progeny. Due to **anisogamy**, the SRD is transmitted from mother to daughter and males are thought to be incapable of SRD transmission. Specific types of SRDs include heritable bacteria (such as Wolbachia) and eukaryotes (such as microsporidia) (Hamilton, 1967; Eberhard, 1980; Cosmides and Tooby, 1981; Hurst, 1993; Bandi et al., 2001; Hurst and Werren, 2001; Cook, 2002).

Heritable transmission from mother to daughter is termed vertical transmission and is widespread in the eukaryotic phylum Microspora (Terry et al., 2004). This route of transmission ensures parasite survival and transmission during periods when hosts are present at low densities within the habitat, such as over-wintering (Dunn and Smith, 2001). Several species of vertically transmitted microsporidia kill male host offspring, possibly increasing the probability of survival of female offspring by reducing sib-sib

competition, or by providing extra nutrients to female young who eat the dead males. Vertically transmitted microsporidia are also known to override normal sex determination mechanisms in infected amphipods, converting male offspring into functional females (Stouthamer et al., 2002). The mechanism by which genetic males are feminized, becoming phenotypically and functionally female, involves parasitic inactivation of the host androgen gland (Rodgers-Gray et al., 2004). Previous research determined that the removal of the androgen gland from male crustaceans led to the development of the female phenotype. It appears that individuals possess all the genes necessary for male or female sex differentiation, and the action of the androgen gland is the key to sexual development (Legrand et al., 1987). However, the specific mechanisms by which the androgen gland is activated are largely unknown, and may be a result of genetic or environmental cues, or a combination of the two.

The mechanisms of feminization are deserving of much study, however, in this study I focused on whether there is any evidence for host sex ratio distortion by this microsporidia. I also considered measures of parasite **virulence**. Further, I investigated the route of transmission of the microsporidian. In the evolution of host-parasite interactions and parasite virulence, the route by which microsporidia are transmitted is a driving force (Dunn and Smith, 2001). By employing vertical transmission, microsporidia are tying their reproductive success to that of their hosts, and any negative effect on host fecundity would directly impact the success of the parasite. As such, theory predicts that the virulence of vertically transmitted parasites would logically evolve to a symbiotic relationship with the host (Rigaud, 1997). However, sex ratio distortion employed by some vertically transmitted microsporidia may lead to small-scale extinction events as a

result of the scarcity of males in a sexually-reproductive population (Hatcher et al., 1999). Alternatively, if the parasite does not reach fixation within a population, the excess of females may provide for exploding population growth rates (Hamilton, 1967; Werren, 1987; James and Jaenike, 1990; Werren and Beukuboom, 1993).

The population dynamics of the intertidal amphipod, *C. volutator*, have been well documented. Reproduction is synchronous at the local level in at least some populations, and may be a result of dependence on spring tides for offspring dispersal (McCurdy et al., 2000). Extreme female-bias in both adult and juvenile populations has been documented (e.g., Schneider et al., 1994). Differential predation of male amphipods exhibiting mate-searching behaviour does not account for the extreme sex ratio bias, and does not provide an explanation for the similar bias observed in juveniles (Forbes et al., 1996). There are two generations of *C. volutator* per year in most Bay of Fundy populations. The overwintering generation consists primarily of juveniles, and is subject to high mortality as a result of slow growth rates, ice scour and periodic freezing. The mortality of these populations during winter has been measured as 70%. Those individuals that survive form the spring breeding population, which gives rise to the second generation. This late summer generation displays a slower growth rate, and overwinters as juveniles, continuing the cycle (Gratto et al., 1983). Localized extinction of mudshrimp populations has been observed and investigation of the cause of such mortality has been undertaken. Commercial bait worm harvest which involves disturbing the sediment, and therefore *C. volutator* burrows, has been found to have a devastating and immediate effect on mudshrimp populations (Shepherd and Boates, 1999). Additionally, bacterial and viral

infections have been implicated in rapid population declines, as well as parasitism-induced mortality (McCurdy, 2000).

As mentioned, this first study will investigate the offspring sex ratios of *C. volutator* and the potential role of a microsporidian in contributing to the female-bias observed in mudshrimp populations. I will address specifically the following questions: do males and females differ in prevalence of infection by microsporidia where only reproductive tissues are extracted? Secondly, does offspring sex ratio depend on the infection status of the mother?

Methods

Collection Methods

C. volutator were collected from the mudflat at Blomidon (Figure 1.1, 45°13'N; 63°22'W) (McCurdy, 2000). This site was chosen due to high densities and because of a lack of predation by migratory shorebirds and the absence of parasitic helminths, both known to influence reproduction of *C. volutator* (McCurdy et al., 2000). Amphipods were collected from a single 10 x 10 m patch, approximately 40 m from the shoreline. The first 5-10 cm of substrate was removed and placed in coolers. *C. volutator* reside in U-shaped burrows (Meadows, 1964), so substrate was removed until no part of the burrows was observed in the remaining substrate. 1 L of seawater obtained from the site was added to each cooler, which contained between 10 and 15 kg of substrate. Once returned to the lab in Ottawa, Ontario 30 hours later, the substrate was gently sieved (0.85 mm mesh) and adult *C. volutator* were obtained using live-specimen forceps. The mesh

Figure 1.1: Map of the Minas Basin. Blomidon site where *Corophium volutator* were sampled on May 21st 2005 is indicated (1). Redrawn from Hicklin, 1987.

size used ensured that juvenile individuals (<4.5 mm) would pass through. Individuals >4.5 mm are retained by 0.85 mm mesh (Crewe et al., 2001). The adults obtained from sieving were further sorted, with ovigerous females being isolated. Ovigerous females were readily distinguishable based on the mass of visible embryos contained within an external brood pouch (Figure 1.2). These embryos ranged in color from yellow to white, depending on the stage of development. The remaining adults were housed communally, forming breeding colonies for future research.

Brood Release

Three hundred ovigerous females were housed individually in 250 mL plastic cups containing 200 mL of artificial salt water (18% w/v Instant Ocean and 100 µg/ml each of Streptomycin sulfate and Penicillin-G (Sigma)). *C. volutator* tolerates salinities ranging from 2-50‰, but highest population densities are found at 15-20‰ salinity (McLusky, 1967). The addition of antibiotics reduces the mortality of amphipods due to bacteria, and also reduces variability in amphipod growth that can also be caused by bacterial populations (Pelletier and Chapman, 1996). Additionally, 15 mL of original substrate which had been sieved (0.425 mm mesh) to remove larger particles and macrofauna, and autoclaved to kill microorganisms, lined the bottom of the cup. *C. volutator* is a deposit feeder and selects particles between 4 and 63 µm in size when feeding. Therefore, the presence of silt and clay sediment is important for *C. volutator* feeding (Fenchel et al., 1975). Using a 0.425 mm sieve allowed the larger particles to be removed and the substrate supplied to *C. volutator* to be of appropriate quality for

Figure 1.2: Brood pouch of an ovigerous *C. volutator* female, viewed at 16x magnification using a dissecting microscope.

burrowing. Without substrate, individuals become stressed and survival is reduced (Meadows and Reid, 1966).

The plastic cups were placed on racks in environmental chambers on a 15°C temperature regime and a light regime of 16 h light and 8 h dark. The temperature of 15°C is associated with greatest *C. volutator* growth rate and the light regiment mimics summer conditions, in which *C. volutator* reproduces (Peters and Ahlf, 2005). The ovigerous females were fed twice weekly with commercial fish food (SmallFry, Wardley) in solution with salt water. Each cup received 20 mL of a solution of 250 µl of fish food in 1 L of salt water (Instant Ocean, 18% w/v), which contained antibiotics (100 µg/ml each of Streptomycin sulfate and Penicillin-G, Sigma) on alternate weeks. When a female released her brood, she was killed with 100% ethanol. Brood release was determined by the lack of visible embryos within the female's brood pouch, the presence of many small burrow holes in the substrate, and/or the observation of young swimming. Females released broods between 5 and 20 days after sorting. From fertilization to hatching, the development of embryos takes 14 days. The hatched juveniles remain in the female brood pouch for up to 5 days, during which the juveniles undergo a first molt (Fish and Mills, 1979).

Dissection

The blotted wet weight of each female was determined (Dunn and McCabe, 1995) and length was measured with digital calipers (Mitutoyo, accurate to the nearest 0.01mm) from the tip of the rostrum to the telson (Schneider et al., 1994). Dissection was performed using a dissecting microscope (Wilde 73060). The female was placed with the

dorsal side facing up, and pinned through the head with a number 0 insect pin. Approximately 250 μ l of saline (0.13 M NaCl, 0.005 M KCl and 0.003 M CaCl in dH₂O) was added to the specimen. Forceps were used to grasp the posterior section of the animal, just above the first pleopod segment (Figure 1.3). A number 1 insect pin placed above the first pleopod segment was used to initiate a tear in the exoskeleton and the posterior section of the animal was carefully removed with forceps, using a continuous pulling motion. Ideally, various internal organs and guts of the animal also were removed with the posterior section, being attached to the body segments below the first pleopod segment. If the internal organs and guts separated from the posterior section, they were removed individually. The internal organs and guts were readily distinguishable from the ovaries as the gut membrane is folded. As a result of the dissection, the ovaries were exposed and visible (Figure 1.4). Forceps were then used to remove the ovaries from the anterior body section. The ovaries were placed in a sterile 1.5mL eppendorf tube in 10 μ l of saline. All dissecting instruments were sterilized in 100% ethanol between dissections, to avoid contamination of samples.

In order to determine if the parasite was localized only in female gonads and not male testes, 24 male *C. volutator* were also dissected. Males were obtained from the communally housed individuals resulting from the original sorting, described above. Adult males are readily distinguishable from females based on the prominent second antennae, a secondary sexual characteristic present on male individuals (Barbeau and Grecian, 2003; Figure 1.5). The males were killed in 100% ethanol and, with the ventral side facing up, pinned through the head with a through the head with a number 0 insect pin. Approximately 250 μ l of saline (0.13 M NaCl, 0.005 M KCl and 0.003 M CaCl in

Figure 1.3: External anatomy of *Corophium volutator*, with head (a), thorax (b), abdomen (c), antenna (d), walking legs (e), pleopods (f) and uropods (g) indicated. This individual is approximately 7 cm in length, from (a) to (g).

Figure 1.4: Partial dissection of the ovaries (indicated by arrow) of a *C. volutator* female, viewed at 16x magnification using a dissecting microscope.

Figure 1.5: Male and female (bottom) *C. volutator*, side view, at 8x magnification.

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dH₂O) was added to the specimen. Forceps were used to grasp the posterior section of the animal, at the top of the segment just below the penial papillae: another characteristic found on males (Figure 1.10). A number 1 insect pin placed on the opposite side of the segment was used to initiate a tear in the exoskeleton and the posterior section of the animal was carefully removed with forceps, using a continuous pulling motion. Ideally, the assorted internal organs and tissue of the animal also were removed with the posterior section, being attached to the body segments below the first pleopod segment (Figure 1.4). If the internal organs and tissue separated from the posterior section, they were removed individually. Using the forceps to grasp and the pin to initiate a tear, the segment containing the penial papillae was removed from the remaining anterior body section. The testes remained attached to the penial papillae and were composed of two sausage-shaped segments (Figure 1.6). Forceps were then used to remove the testes from the isolated body segment. The testes were placed in a sterile 1.5 mL Eppendorf tube in 10 µl of saline. All dissecting instruments were sterilized in 100% ethanol between dissections, to avoid contamination of samples. To prevent cross contamination from infected females, male dissection, DNA extraction and PCR screening was performed completely separate from female dissection, DNA extraction and PCR screening.

Molecular Screening

Molecular screening involved three discrete steps. The preparation of genomic DNA comprised the first step, followed by a quality assessment of the genomic DNA using amphipod-specific primers for PCR amplification. Finally, microsporidia infection was assessed using microsporidia-specific primers for PCR amplification.

Figure 1.6: Partial dissection of the testes (indicated by arrows) of a *C. volutator* male, viewed at 16x magnification using a dissecting microscope.

Genomic DNA was extracted from dissected ovaries by standard Phenol-Chloroform purification (Sambrook et al., 1989) following a 2 hour digestion step at 65°C in CTAB buffer (2% Cetyl Trimethyl Ammonium Bromide, Sigma, 0.1 M Tris HCl, 0.02 M EDTA and 1.4 M NaCl) and proteinase K (0.74 µg/ml, Invitrogen). All samples were amplified using invertebrate Cytochrome C oxidase subunit I primers to ensure successful DNA extraction (Folmer et al., 1994, forward primer 5'-ggtaacaaatcataaagatattgg-3', reverse primer 5'-taaacttcagggtgacaaaaaatca-3') and because the Forbes' lab wanted to have potential haplotype markers for *C. volutator*. The PCR reaction had a total volume of 25 µl and contained 20 ng of DNA extraction, 10X PCR buffer, 160 µM of each dNTP, 1.5 mM MgCl₂, 0.8 pmol of each primer and 1.5 units of Taq polymerase (Invitrogen). Amplification began with a denaturation at 95°C for 5 minutes. 40 cycles of denaturation at 95°C for 50s, annealing at 50°C for 70s and elongation at 72°C for 90s ensued, followed by a final elongation step at 72°C for 10 minutes. Microsporidian primers 18sf (5'-gttgattctgcctgacgt-3') and 964r (5'-cgcggtgagtgcaaattaagccgcaca-3') (Terry et al., 1999) were used to amplify part of the parasitic 16S rDNA in the following PCR reaction: a total volume of 25 µl containing 20 ng of DNA extraction, 10x PCR buffer, 160 µM of each dNTP, 0.75 mM MgCl₂, 0.8 pmol of each primer and 1.5 units Taq polymerase (Invitrogen). After an initial denaturation at 95°C for 5 min, samples were subjected to 40 cycles of denaturation at 95°C for 50 sec, annealing for 60 sec at 50°C and extension at 72°C for 90 sec. Finally, there was one extension cycle at 72°C for 10 min.

Following PCR, samples were screened by agarose electrophoresis. A 1.5% w/v agarose gel was prepared with 1X TAE buffer (50X stock: 2 M Tris base, 1 M Acetic

Acid and 0.05 M EDTA in 1 L ddH₂O, pH 8.5; diluted to working concentration in H₂O). 5 µl of each PCR sample was combined with 3 µl of loading buffer (0.25% w/v xylene cyanol, 40% w/v sucrose in ddH₂O) diluted to 1.5X in TAE. Samples were loaded onto the agarose gel in an Owl EasyCast gel electrophoresis chamber (Diamed). 3 µl of a 1:5 dilution of 100bp DNA size standard (Invitrogen) was loaded in the first, last and middle lanes. The gel was run at 80 V for 45 minutes and stained in an ethidium bromide bath (4 µg/mL EtBr in H₂O) for 30 minutes. PCR products were visualized using GelDoc (AphaInnotec). Samples were scored as successful DNA extractions based on the presence of an appropriately sized PCR product resulting from the Cytochrome C oxidase primer amplification (Figure 1.7). Once the presence of PCR-amenable DNA was confirmed, samples were subjected to PCR with microsporidia-specific primers. Infection with microsporidia was indicated by the presence of an appropriately sized PCR product (Figure 1.8).

Rearing of Young

Once the infection status of the mothers was known, broods corresponding to infected mothers were reared to sexual maturity (n=40). An equal number of broods from uninfected mothers were also reared to sexual maturity (n=40). After approximately 2 weeks in the plastic cups with mothers, young were counted and placed in fresh cups as described above. No more than 10 young were placed in a single cup, to prevent overcrowding. The young were fed twice weekly with commercial fish food (SmallFry, Wardley) in solution with artificial salt water. Each cup received 20 mL of a solution of 250 µl of fish food in 1 L of artificial salt water (Instant Ocean, 18% w/v), which

Figure 1.7: Electrophoresis of 700bp PCR products resulting from amplification with amphipod-specific cytochrome oxidase I primers on a 1.5% agarose gel. Lanes 1, 10 and 19 contain 100bp ladder (Invitrogen). Lane 17 contains the positive control and lane 18 the negative control. Lanes 6, 13 and 14 did not contain product and were not used further.

Figure 1.8: Electrophoresis of 850bp PCR products resulting from amplification with microsporidia-specific 16S rDNA primers on a 1.5% agarose gel. Lanes 1, 11 and 20 contain 100bp ladder (Invitrogen). Lane 18 contains the positive control and lane 19 the negative control.

contained antibiotics (100 µg/ml each of Streptomycin sulfate and Penicillin-G, Sigma) on alternate weeks. After one month, the young were transferred to a fresh cup, in response to algae growth in the original environment. After approximately 3 months, the young reached sexual maturity (approximately 3 mm in size) and were killed in 100% ethanol. Using a dissecting microscope (Wilde 73060), individuals were sexed based on the presence or absence of penial papillae and/or oostegites (Figure 1.9, 1.10).

Statistical Analyses

Most statistical analyses were performed with SigmaStat v.3.11 (Systat), however, generalized linear models (GLMs) were performed with R (Ihaka and Gentleman, 1996). A GLM was determined to be the most appropriate test for the proportional data of survival and sex ratio, as it is semi-parametric and encompasses models that have non-normally distributed deviations (Wilson and Hardy, 2002). While arcsine-squareroot transformation may normalize proportional data, heavily biased ratios are not transformable (Wilson and Hardy, 2002). In this study, the sex ratios are strongly female-biased and arcsine-squareroot transformation fails to normalize the data.

The GLMs performed consisted of a quasibinomial error function, a linear predictor and a logit-link function. Quasibinomial errors were specified as the residual mean deviance was larger than 1.5 for both the survival and sex ratio GLMs. For the survival GLM, the linear predictor was specified as the proportion of surviving individuals with respect to the length and infection status of the mother. As indicated, the proportion of surviving offspring was the number of offspring surviving to sexual maturity divided by the number of offspring initially recovered at time of hatching. The

Figure 1.9: Female secondary sexual characteristics used to differentiate sexes of *C. volutator* included the presence of feathered appendages (known as öostegites, indicated by arrows) which form the brood pouch.

Figure 1.10: Male secondary sexual characteristics used to differentiate sexes of *C. volutator* included the presence of penial papillae (indicated by arrows), small flaps located on the ventral side of the abdomen, on the segment directly anterior to the pleopods.

length of the mother was included in the model to allow for the age of the female. The logit-link function of the GLM makes the model linear and has asymptotes at 0 and 100%, preventing the prediction of values unrealistic to nature (Wilson and Hardy, 2002). The linear predictor of the sex ratio GLM was defined as the number of female offspring divided by the total number of surviving offspring. Using the number of surviving offspring rather than the initial number of offspring allows for consideration of sex-specific mortality. Further, broods with very high mortality (>50%) were removed from statistical consideration *a priori* for the same reason. Basically, if there is sex specific mortality, then this becomes a problem for determining whether infection status of mothers relates to sex of offspring and mostly a problem when sample size is low (and error high). The other parameters of the sex ratio GLM were the same as the survival GLM, except that the sex ratio GLM was weighted to number of surviving young. The purpose of the weighting was to give more consideration to an all-female brood of 60 offspring compared to an all-female brood of 6 offspring. The GLM is a one-tailed test, with the null hypothesis that there is no difference in brood sex ratio associated with microsporidian infection. The alternative hypothesis is that there is a female bias in brood sex ratio resulting from infection by the microsporidia.

Results

Prevalence

The overall microsporidian prevalence was 27.1% in female gonads (Clopper-Pearson 95% confidence interval 22.2-32.4%, n=306) and 1.5% in male gonads (0.04-8.2%, n=65). The parasite was present in significantly more ovaries than testes ($p < 0.001$, X^2 goodness of fit, Figure 1.11).

Offspring Survival

As described in the methods, a generalized linear model (GLM) was used to analyze the proportional data of offspring survival. The equation used was: $\text{glm}(\text{final}/\text{initial} \sim \text{length} * \text{infection}, \text{family} = \text{quasibinomial}, \text{weights} = \text{final})$. The proportion of surviving offspring did not differ between infected (n=37) and uninfected (n=49) females ($P=0.75$, GLM with quasibinomial errors, Figure 1.12). The average initial brood size was 21 with a standard deviation of 11. It is important to note that broods with fewer than 10 surviving offspring were removed from all further statistical consideration *a priori* to account for possible sex specific mortality, as mentioned previously.

Figure 1.11: Microsporidian prevalence in female and male gonads, graphed as mean prevalence with Clopper-Pearson 95% confidence intervals. 22.2-32.4%, n=306) and 1.5% in male gonads (0.04-8.2%, n=65). The parasite was present in significantly more ovaries than testes ($p < 0.001$, X^2 goodness of fit).

Figure 1.12: Boxplot of the proportion of surviving offspring from infected and uninfected broods, plotted as median, 25th and 75th percentiles. Error bars indicate 10th percentiles and closed circles indicate outliers. ($P=0.75$, GLM with quasibinomial errors; uninfected $n=37$, infected $n=49$). As the average brood size was 20, broods with fewer than 10 surviving offspring were removed from further statistical consideration *a priori*.

Brood Sex Ratio

Infected females (n=27) had a higher proportion of female offspring than uninfected females (n=34) ($P < 0.001$, GLM with quasibinomial errors, weighted with surviving number of offspring). The equation used for this GLM was:
`glm(females/final~length*infection, weights=final, family=quasibinomial)` (Figure 1.13).

Length of Mothers

Infected and uninfected females did not differ in body length ($P = 0.073$, t-test; Power of performed test with $\alpha = 0.050$: 0.297).

Discussion

The purpose of this study was to determine if microsporidian infection of female *C. volutator* was associated with sex ratio distortion. The results of this study suggest that the microsporidian is typically localized in female gonads and infection is correlated with extreme female-bias in brood sex ratios.

Broods from infected mothers were $87 \pm 16\%$ female and broods from uninfected mothers were $70 \pm 34\%$ female. Mothers infected with the microsporidian had significantly more female offspring ($P < 0.001$). This result clearly demonstrates an association between infection and host sex ratio bias. Further evidence of the parasite's role in host sex ratio distortion is the female bias in parasite distribution, which is a preliminary indication of host sex ratio distortion (Galbreath et al., 2004; Terry et al., 2004). The microsporidian parasite of interest was determined to be more prevalent in ovaries (27.1%) than testes (1.5%). The overall prevalence of the microsporidian in the

Figure 1.13: Boxplot of the proportion of female offspring from infected and uninfected broods, plotted as median, 25th and 75th percentiles. Error bars indicate 10th percentiles and closed circles indicate outliers. ($P < 0.001$, GLM with quasibinomial errors; uninfected $n=34$, infected $n=27$).

female gonads of *C. volutator* was 27%, similar to a previously determined prevalence of 26% (Mautner et al., submitted). Similarly, prevalence of a vertically transmitted microsporidian in a related amphipod, *Gammarus duebeni*, was determined to be 31% (Smith and Dunn, 1991).

Population theory suggests that cytoplasmically inherited sex ratio distorters will spread quickly throughout a population, reaching fixation, or 100% prevalence (Bull 1983; Skinner, 1987; Werren, 1987). However, models have more recently been developed that consider incomplete transmission from mother to offspring, as well as incomplete feminization (Hatcher and Dunn, 1995). Specifically, if transmission and feminization are less than 100%, a vertically inherited feminizer is predicted to reach an intermediate equilibrium frequency within a population. Fixation is limited by host variability (Dunn et al., 1993), seasonal processes (Kelly et al., 2002) and host resistance (Taylor, 1990). Studies that investigate transmission efficiency of this parasite from mother to offspring would help to address the relatively low prevalence of the parasite in *C. volutator* populations. This study implies that there is indeed variation in transmission efficiency between individuals. The sex ratios of individual broods in this study demonstrate that infected mothers give rise to between 40% and 100% female offspring (Appendix 3).

The single infected male found in this study was determined to be infected with the same species of microsporidia as that infecting the female gonads. This determination resulted from sequencing of the parasite 16s rDNA, which returned identical sequences from male and female-isolated microsporidia. It is possible that the male individual was actually an intersex. As outlined in the methods, males were sorted from the collected

samples by the prominent second antenna. Intersex individuals have antenna that are of intermediate size, and it is possible that an intersex would be sorted as a male. In *G. duebeni*, 26% of intersex individuals are infected with a vertically transmitted microsporidian, but no infected males have been found (MacNeil et al., 2003). Alternatively, if parasite sex ratio distortion is due to feminization of genetic males to phenotypic females, an infected male would be evidence of an incomplete feminizing effect (Haine et al., 2004). *Gammarus roeseli* males were found to be infected with three vertically transmitted microsporidia, but the prevalence was significantly lower than in females (Haine et al., 2004).

This study found no difference in offspring mortality from infected ($81 \pm 16\%$) and uninfected ($83 \pm 14\%$) females. Other studies of vertically transmitted microsporidia in *G. duebeni* also found no reduction in survival of offspring from infected mothers (Kelly et al., 2002, 2003). The similar mortality rate of offspring from infected and uninfected mothers is evidence against horizontal transmission, which usually is associated with greater virulence of the parasite, resulting in host death to transmit the parasite. Additionally, the similar mortality rate implies that this parasite feminizes offspring, rather than killing males (Dunn et al., 2003).

Manipulations of reproductive parasites include the conversion of a female host to **thelytokous parthenogenesis** (Bandi et al., 2001). However, *C. volutator* females housed individually never became ovigerous unless a male was present (personal observation). While this observation does not completely exclude parthenogenesis, it makes the possibility highly unlikely. The combination of the observations of mortality

and ovigery make host feminization the most likely route by which the microsporidian distorts host sex ratios.

In this study, broods consisted of an average of 21 individuals, and ranged from 4 to 73 individuals. After rearing to sexual maturity, broods ranged from 1 to 66 individuals, with an average of 15 individuals. Literature values for *C. volutator* brood sizes focus on developing embryos rather than free-living young, as hatching is asynchronous. However, brood sizes of embryos at the last stage of development consist of an average of 20 individuals (Fish and Mills, 1979). Mortality of *C. volutator* young from hatching to sexual maturity has never been measured before. In *Corophium spinicorne*, mortality of juveniles under similar experimental conditions was expressed as 0.003 deaths in the population per day (Pelletier and Chapman, 1996). In this *C. volutator* rearing study, 1777 individuals were initially sorted. After approximately 90 days, 1257 individuals were recovered. This mortality is equivalent to 0.0033 deaths in the population per day. Future studies can expect a similar mortality of approximately 30% in laboratory reared broods.

In mosquitoes infected with microsporidia, infected hosts have reduced intraspecific competitiveness, taking longer to develop and, when in competition with uninfected individuals, they slow development even more (Bedhomme et al., 2005). The study undertaken in Chapter 1 controlled intraspecific competition by reducing the densities of developing young and providing adequate food. However, the size of offspring was not determined at time of sexual maturity, and individuals were killed and sexed over a period of several weeks. Further studies should evaluate the effects of microsporidia on intraspecific competition in *C. volutator*.

In addition to the lack of difference in offspring mortality, the parasite was not associated with differences in female size. Infected and uninfected mothers did not differ in body length. However, the power of the performed t-test (0.297) is below the desired power of 0.800. Less than desired power indicates you are more likely to not detect a difference when one actually exists (Zar, 1996). As there is a strong linear relationship between brood size and female body length, the potential lack of a size difference between infected and uninfected mothers implies that the microsporidian has little or no effect on host **fecundity** (Haines et al., 2004). Predictions of characteristics for a cytoplasmically inherited microorganism include no effect of the organism on host fecundity and no impairment of host offspring survival (Smith and Dunn, 1991).

Overall, the results of this study indicate that the microsporidian infecting *C. volutator* is of low virulence, vertically transmitted, and associated with sex ratio bias. Further studies need to investigate the transmission efficiency of this parasite and the possibility of mate discrimination. Additionally, further characterization of the microsporidian should be undertaken to assist with classification of this novel parasite. Finally, there is the unresolved issue of why clutches are not 50:50 for uninfected mothers, and investigation of sex determination mechanisms of *C. volutator* is warranted.

Chapter 2

Fecundity and Brood Volume of *Corophium volutator* females in Relation to Parasitism by a Novel Microsporidian

Introduction

By definition, parasites are in some way detrimental to their hosts (Price, 1980). Parasites divert host resources, which results in fewer resources being available for host growth, survival and reproduction. The selective pressures of parasitism have been well documented, and, within a host population, may be mediated or compounded by ecological pressures (Toft and Karter, 1990). For example, observations of parasitized individuals in times of environmental stress help to determine the effect of ecological pressures, which may lead to a further reduction in fecundity, or even an enhancement at the cost of some other life-history trait, such as survival (Kelly et al., 2002). Studies conducted in the lab, under optimal conditions, can help determine the direct effects of parasitism on a life history trait, if such effects exist. Often, parasitized individuals demonstrate reduced fecundity in laboratory conditions (Bull et al., 1991; Herre, 1993; Ewald, 1994; Dunn and Smith, 2001). However, we may even expect increased short term fecundity if fecundity is traded off against some other life history trait, and parasitism influences the shape of the trade-off curve (Forbes, 1993).

As previously discussed, vertically transmitted parasites, such as some species of microsporidia, are predicted to evolve towards **commensulism**, or even **mutualism** (Toft and Karter, 1990). By employing vertical transmission, microsporidia are tying their

reproductive success to that of their hosts, and any negative effect on host fecundity may directly impact the success of the parasite. It has been postulated that vertically transmitted parasites would evolve to a symbiotic relationship with the host (Ewald, 1987; Yamamura, 1993; Smith and Dunn, 1991; Hurst and Majerus, 1993; Dunn et al., 1995; Bandi et al., 2001), possibly by increasing host fitness, or increasing the production of infected females (Rigaud, 1997). Cytoplasmically inherited micro-organisms, which include vertically transmitted microsporidia, are predicted to have reduced pathology, preferentially infect host gonads, have no negative effect on host fecundity, and have no negative effect on survival or growth of offspring (Smith and Dunn, 1991). In addition to the reduction of parasite costs on hosts, it is also predicted that the parasite may manipulate host behaviour to increase the mating success of parasitized females (Kelly et al., 2001). However, male discrimination of infected females could provide a mechanism by which the spread of a vertically transmitted parasite is limited in a population (Hatcher, 2000). In fact, a fitness gain from male avoidance of parasitism is predicted to drive males to selectively mate with uninfected females (Kelly et al., 2001).

C. volutator adult females greatly outnumber males, and display synchronous breeding at some sites in the Bay of Fundy (Forbes et al., 1996; McCurdy et al., 2000). Females are only fertile for a few days following a parturial molt, and are unable to hasten the onset of molting in the presence of a male (McCurdy et al. 2000). Males choose mates based on time to receptivity, and not based on size of the female (Forbes et al., 1996). Females release waterborne chemicals which indicate receptivity (Krang and Baden, 2004). This species has an external brood pouch, formed by oostegites. The number of embryos contained within the brood pouch is constrained by the size of the

female. As such, number of embryos is strongly correlated with female size (Fish and Mills, 1979; Dunn and McCabe, 1995; Llodra, 2002).

C. volutator has a high colonization rate, due to high egg production and juvenile dispersal (Jensen and Kristensen, 1990). The semi-lunar rhythm in breeding is thought to be linked to spring tides, which would allow for greater juvenile dispersal (Fish and Mills, 1979).

Between amphipod species, reproductive parameters may vary greatly as a result of diverse life-history strategies. Specifically, embryo size and number, duration of embryo development, size of hatched offspring and size of adults at sexual maturity are all reproductive parameters which display variation across species (Sainte-Marie, 1991). These parameters may also vary within species, as a result of season or geographic location (Shedder, 1995; Forbes et al., 2005). Parasitism may also influence reproductive parameters in a reproductively flexible species. In this study, I consider embryo number, brood volume and size of ovigerous females in relation to infection with a vertically transmitted microsporidia.

Methods

Collection Methods

C. volutator sample collection and sorting was carried out as described in Chapter 1. Three hundred ovigerous females were obtained by sorting and housed communally in 1L containers, containing 400mL of artificial salt water (Instant Ocean, 18% w/v) and 60ml of sieved and autoclaved substrate, for up to 48 hours while processing was carried out. Over 48 hours, individual females were anaesthetized by brief immersion in 20 mL

of carbonated mineral water (Pellegrino, USA). Embryos were flushed from the brood pouch with saline (0.13 M NaCl, 0.005 M KCl and 0.003 M CaCl in 1 L dH₂O). The embryos from each female were counted and classed as “early” or “late” stage. Early stage embryos (Figure 2.1) consisted of stage A embryos, as described by Ford et al., 2003. Late stage embryos were comprised of all other stages described by Ford et al., 2003 (Figure 2.2). All females were allowed to recover in aerated artificial salt water until pleopod activity had resumed.

Females were then housed individually in 250 mL environments, containing artificial salt water (Instant Ocean 18 % w/v, and 100 µg/ml each of Streptomycin sulfate and Penicillin-G, Sigma) and 15 mL of sieved and autoclaved substrate. These adult females were maintained for up to three weeks to allow the immature eggs in the ovaries to ripen, resulting in large ovaries which could be more easily dissected. Food and additional salt water were supplied bi-weekly. Feeding consisted of adding 3mL of a solution of 250 µl of commercial fish food (Small Fry, Wardley) in 1 L of salt water to each 250 mL environment. Salt water was supplemented as needed to maintain the 250 mL volume.

Embryo Size

In determining embryo volume for a given clutch, only females with stage A embryos were used, in order to minimize the effect of brood loss. During embryonic development, *C. volutator* loses up to 26% of a brood (Fish and Mills, 1979). The length and width of five embryos randomly selected from each clutch was determined using the

Figure 2.1: Early stage *C. volutator* embryos, staged according to Ford et al., 2003, viewed at 16x magnification using a dissecting microscope.

Figure 2.2: Late stage *C. volutator* embryos, staged according to Ford et al., 2003, viewed at 16x magnification using a dissecting microscope.

350 μm

scaling function of a digital microscope (Zeiss Axioplan 2ie), accurate to ± 0.01 mm. The volume of each embryo was approximated by the equation for the volume of an ellipsoid: $V = 4/3\pi lw^2$ (Dunn and McCabe, 1995). A brood volume was calculated for each female by multiplying the average of the individual volumes of the five representative eggs from each female by the total number of embryos.

Molecular Screening

DNA was extracted from the readily available embryonic tissue and screening for the parasite was performed as outlined in Chapter 1. Females determined to be infected were killed in ethanol and their ovaries dissected out for TEM (Appendix 2). The remaining females were killed in ethanol. The blotted wet weight of each female was determined (Dunn and McCabe, 1995) and length was measured with digital calipers (Mitutoyo) from the tip of the rostrum to the telson (Schneider et al., 1994). Ovaries from each female were obtained by dissection, as described in Chapter 1. DNA was extracted from ovarian tissue, as outlined in Chapter 1. Additionally, screening for the microsporidian parasite using PCR was undertaken as outlined in Chapter 1. Additional microsporidian infections were observed by screening DNA extracted from ovarian tissue. Samples that scored positive with either ovarian tissue or embryonic tissue in at least two PCRs were considered to be infected. This ensured that effects of limitations in PCR detection of parasite copy number within very early stage embryos were mitigated.

Statistical Analyses

Most statistical analyses were performed with SigmaStat v.3.11 (Systat Inc.). Analysis of covariance (ANCOVA) was performed with Systat (Systat Inc.). ANCOVA was used to determine if the number of embryos (the dependent variable) was influenced by infection status (a categorical factor), with the length of the mother as a covariate. The length of the mother was included in the statistical tests as brood size and female length are strongly correlated (e.g., Peer, 1986). Early and late stage embryo data were analyzed separately as volume measurements were only obtained for early stage embryos. In addition, combining early and late stage embryos would introduce variation from brood loss which might obscure any effect of infection on brood size.

Results

Early Stage Embryos

Uninfected (n=82) and infected (n=32) ovigerous females with early stage embryos were not different in length ($P=0.804$, t-test). The number of early stage embryos did not significantly differ between infected and uninfected ovigerous females, controlling for female length ($P=0.073$, power=0.901, ANCOVA, Figure 2.3). However, uninfected and infected ovigerous females did differ significantly in brood volume (i.e. the volume of all embryos in a single brood) ($P=0.054$, power=0.868, ANCOVA, Figure 2.4). There is a trend of infected females having fewer early stage embryos and significantly smaller brood volumes, indicating potential costs of parasitism to host fecundity.

Figure 2.3: Boxplot of the number of early stage embryos from uninfected (n=82) and infected (n=32) ovigerous females ($P=0.073$, ANCOVA). Plotted as median, 25th and 75th percentiles. Error bars indicate 10th percentiles and closed circles indicate outliers.

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Figure 2.4: Boxplot of the brood volume of early stage embryos from uninfected (n=82) and infected (n=32) ovigerous females ($P=0.054$, ANCOVA). Plotted as median, 25th and 75th percentiles. Error bars indicate 10th percentiles and closed circles indicate outliers.

Late Stage Embryos

In comparison to the results above, uninfected (n=60) and infected (n=21) ovigerous females with late stage embryos differed in length ($P=0.002$, t-test), with uninfected females being larger. After controlling for length statistically using ANCOVA, I found that uninfected and infected did not differ in the number of late stage embryos ($P=0.194$, Figure 2.5). Thus, commitment of females to late reproduction does not appear to vary depending on infection status.

Discussion

The purpose of this study was to investigate if the commitment of female *C. volutator* to reproduction varies with infection by the novel microsporidian. I determined that a reduction in host fecundity is associated with parasitism by the microsporidian. Specifically, infection is associated with a significant reduction in overall brood volume of early stage clutches, and a trend towards fewer early stage embryos. However, there is no apparent effect of infection on late stage embryos.

To begin with, the prevalence of the microsporidia in the sample of ovigerous females for this study was 39% (Clopper-Pearson confidence interval 32.5-46.1%, n=212). In point of fact, this is barely outside the 95% confidence limits of prevalence of infection for females used in the first study. However, this significant difference is not unexpected. Although females were collected at the same site (Blomidon, NS), they were collected in different years and yearly variation in parasitemia is not unexpected for many organisms. The more important point was that as in the previous years of study, prevalence was not near to fixation in the population, suggesting that there should be

Figure 2.5: Scatterplot of the number of late stage embryos from uninfected (n=60) and infected (n=21) ovigerous females (P=0.194, ANCOVA).

observations of costs to parasitism and/or low efficiency of transmission. Also, there is the logistical point that a prevalence of ca. 40% also allows for good samples of both infected and uninfected females after collecting a few hundred amphipods.

In addition to the female-bias in parasite infection found in Chapter 1, further evidence for vertical transmission was revealed in this study. DNA was obtained, for several samples, from both the gonads of mothers and their embryos. Microsporidia were never detected in embryos from mothers that were determined to be uninfected (n=142), and females infected with the microsporidian had infected embryos (n=9). There were a total of 83 females determined to be infected with the microsporidia, and only 50 of those females had embryos screened. The observed transmission efficiency is therefore 9/50 or 18% (Clopper –Pearson 95% confidence intervals range from 8.6-31.4%), however this should be interpreted with caution as the mothers were housed in laboratory conditions for several days prior to the harvest of embryos, followed by up to several weeks of laboratory housing to allow oogenesis to occur, making the dissection of gonads more successful. The transmission efficiency of this parasite within populations, and generations, of *C. volutator* should be further investigated, both in the lab and in the field. It is likely to be quite higher than 18%, but it none the less indicates that transmission efficiency may be low enough to preclude microsporidia from going to fixation in populations even if they enjoyed an advantage from producing more daughters: the limiting sex. It is also relevant from Chapter 1 that males can be produced from infected mothers, which either suggests failed transmission or failed feminization.

In addition to environmental conditions which may hamper transmission efficiency, there are detection limits to the PCR screening for the parasite. To ensure that

DNA obtained from the extractions of samples is amenable to PCR, a control amplification is undertaken with amphipod-specific primers. However, extremely low copy number of microsporidian DNA, relative to that of amphipod DNA, may result in a failed microsporidian-specific PCR regardless of the amenability of the amphipod DNA. Additionally, microsporidia spores have thick spore walls which may not be efficiently degraded in the standard extraction protocol, preventing the extraction of parasite DNA. An alternative method to PCR screening for determining transmission of this parasite would be DAPI staining (see Appendix 2). This is especially important whenever it is expected that the parasite's DNA is less likely to be extracted (during resting spore stages) rather than when infecting new tissues or initiating feminization of older embryos. The mechanism and timing of feminization is unknown for this species association, and therefore it is not possible to optimally extract microsporidia DNA at a certain stage of the parasitic life cycle.

The microsporidian was associated with a significant reduction in host fecundity. The number of early stage embryos did not significantly differ between individuals infected with the parasite and those uninfected, but there was a visible trend towards the reduction of embryo number with infection ($P=0.0703$). Infected females had 47 ± 20 early stage embryos, and uninfected females had 53 ± 21 early stage embryos. Although a vertically transmitted microsporidian is predicted to have no effect on host fecundity, this study has provided further evidence of direct fitness costs of a feminizing microsporidian on its host (Kelly et al., 2001). Further, there was a significant reduction in total brood volume for infected females, controlling for female length ($P=0.054$), with infected females having a mean brood volume of $0.17 \pm 0.02 \text{ mm}^3$ and uninfected females having

a mean brood volume of $0.18 \pm 0.03 \text{ mm}^3$. This result suggests that infected females suffer a decrease in fecundity as a result of parasitism. Theoretical models for the spread of sex ratio distorters within a host population allow for this reduction in host fitness if sex ratio distortion is significant (Hatcher and Dunn, 1995; Werren and O'Neill, 1997). However, further investigation of additional fitness parameters would help to determine if this measured reduction of fecundity results in an overall decrease in net fitness. Future studies should investigate the effects of microsporidian infection on different size classes of females, as parasitism may have a greater effect on the fecundity of smaller females (Glazier, 2000). The length of infected and uninfected females also did not differ. This does not mean that there is no fitness effect of infection with the microsporidian, but that an effect was not apparent with the single parameter of female condition measured in this study. Considering females with late stage embryos, the mean brood size of infected females was 20 ± 5 , and the mean brood size for uninfected females was 28 ± 10 . There was no difference in the number of embryos, controlling for female length ($P=0.194$). Females did differ significantly in length, with infected females being smaller. Further studies must be undertaken to determine if parasitism is associated with differences in female size at various stages of host reproduction, as well as possible implications of mate choice.

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Appendix 1: Glossary of Operational Definitions

Anisogamy – Unequally sized gametes, with the egg being larger than the sperm, resulting in differential contribution to embryo cytoplasm (Agnew et al., 2003).

Commensulism – Parasite virulence reduced to a net effect of zero (Toft and Karter, 1990).

Dioceous – Male and female sexes are separate individuals (Rout et al., 1996).

Fecundity – The number of embryos in a single reproductive event, or the volume of such embryos.

Horizontal transmission – The transmission of a parasite from host to host within the same generation (Dunn et al., 2001).

Mutualism – The state of parasitic virulence becoming positive (Toft and Karter, 1990).

Phenotypic plasticity – A change in the expressed phenotype of a genotype as a function of the environment (Scheiner, 1993).

Polar filament – A tube-shaped organelle anchored at the anterior pole of the spore and coiled inside the spore (Vavra and Sprague, 1976)

Setose – Covered in bristles.

Sporoblast – A cell formed by the division of the sporont, which develops directly into a spore (Levine, 1971).

Sporont – A cell which divides into sporoblasts (Kudo, 1924).

Sporoplasm – The infective portion of the spore (Debaisieux, 1928).

Thelytokous parthenogenesis – Production of female offspring from unfertilized eggs (Schilder et al., 1999).

Vertical transmission – The direct transmission of infection from the parental host to the progeny (Yamamura, 1993).

Virulence – Negative effects on host fitness caused by parasite infection, measured in terms of reproduction (Kelly et al., 2003).

Appendix 2: Ultrastructural and molecular characterization of a novel microsporidian infecting an intertidal amphipod, *Corophium volutator*

Introduction

Prior to the advent of molecular phylogenetic tools, microsporidia were primarily characterized by spore morphology and life cycle. Microsporidia have diverse life cycles, with varying complexity and transmission routes (Canning, 1993). Horizontal transmission can occur between related or unrelated hosts of the same or different generations as well as between hosts from the same or different species (Vizoso and Ebert, 2005). The usual route of transmission is via ingestion, which results in the infection of gut epithelium. Additionally, microsporidian infection can also be transmitted venereally and directly through the host epithelium. Vertical transmission (VT) occurs within the host lineage and is passed from generation to generation of hosts (Yamamura, 1993). Many species of microsporidia use VT at some point during their respective life cycles (Terry et al., 2004). Within invertebrates, VT occurs transovarially. In addition to the variation in life cycles, there is also significant morphological variation in Microspora, even within species. This variation includes overall size and shape of the spore, the thickness of the exospore and endospore, the number of nuclei, the structure of the polaroplast lamellae, the structure of the polar filament and the number of polar filament coils (Canning, 1993). Spores which participate in within-host cell to cell transmission, including VT, tend to have thinner spore walls and shorter polar filaments than spores responsible for horizontal transmission (Agnew et al., 2003). Exclusive use of

life cycle characteristics and the morphology of spores as taxonomic indicators must be done cautiously.

The genus *Microsporidium* is designated as a temporary home for species with incomplete life cycle descriptions (Dunn and Smith, 2001). More recently, the characteristics used to determine not only species level identification, but also higher levels of classification of microsporidia have been declared unsuitable as these characteristics change states quickly at the genus, species and population levels. Comparing classification by morphological characteristics to molecular phylogenies using *ssrDNA* sequences reveals that the use of ultrastructural features for taxonomic divisions of microsporidia is unreliable (Vossbrinck and Debrunner-Vossbrinck, 2005).

The unknown microsporidia infecting populations of *C. volutator* was characterized using traditional morphological features, as well as *ssrDNA* sequencing. I have provided a rationale for why DAPI staining may be important to examining transmission success. The sequence data is needed to determine whether we are likely dealing with one or more species (the rationale for using 16 *ssrDNA* for classification of microsporidia is presented elsewhere). Basically, it is a reasonably conserved area that quickly allows identification of microsporidia with enough variability to allow resolution of different genera (Baker et al., 1994; Refardt et al., 2002).

Materials and Methods

DAPI Staining

C. volutator sampling and sorting was carried out as described of Chapter 1. The ovigerous females processed as described in Chapter 2 were also used for DAPI staining. Five to ten embryos from each early stage clutch were rinsed with 5 M HCl and frozen at -20°C in 100% acetone until needed for staining (Terry et al., 1997). Embryos were obtained from every early stage clutch to ensure that samples would include clutches from females later determined to be infected with the microsporidian. Prior to staining, embryos were rinsed in saline (0.13 M NaCl, 0.005 M KCl and 0.003 M CaCl in dH₂O) for 15 minutes at room temperature (22-25°C). Embryos were then transferred to a slide, air dried for 5 minutes, and stained with 100µl of 0.2µg/ml 4',6-Diamidino-2-phenylindole (DAPI, Invitrogen) for 5 minutes. A coverslip was added and excess stain removed by blotting with a kimwipe. The slides were examined using a digital microscope (Zeiss Axioplan 2ie) with UV light (excitation: 365nm, emittance: 420nm).

TEM

In Chapter 2, following molecular screening, 35 females that were determined to be infected with the microsporidian were killed by brief immersion in 100% ethanol. The blotted wet weight of each female was determined (Dunn and McCabe, 1995) and length was measured with digital calipers (Mitutoyo) from the tip of the rostrum to the telson (Schneider et al., 1994). Ovaries from each female were obtained by dissection, as outlined in Chapter 1. Ovaries were fixed in 1.6% (v/v) gluteraldehyde in 0.1 M

cacodylate buffer, pH 7.4. Further processing was performed by Peter Rippstein, ART, MLT, at the Core Pathology Laboratory of University of Ottawa Heart Institute (Ottawa, Ontario). Tissue was post-fixated in 1% (w/v) osmium tetroxide in the same gluteraldehyde buffer, dehydrated through a graded ethanol series, and embedded in Spurr's resin (Spurr, 1969). Ultra-thin sections were stained with uranyl acetate and lead citrate and examined on a JEOL 1230 Transmission electron microscope.

Sequencing

Microsporidian DNA amplified with specific 16S primers was obtained as outlined in the Materials and Methods of Chapter 1. Cloning was performed using the TOPO TA Cloning kit (Invitrogen) according to kit directions. Agar slants were inoculated and sent to DNA Landmarks, Montreal for sequencing. DNA sequences were aligned using CLUSTALW and analyzed using NCBI's BLAST service (Altschul et al., 1997).

Results

DAPI

Using DAPI stain, microsporidia nuclei were visible surrounding host nuclei in late stage *C. volutator* embryos. Embryos from the same brood were determined to be infected by PCR with microsporidian-specific primers, prior to DAPI staining. One or two parasite nuclei were situated near a given host nucleus.

TEM

Images resulting from TEM were used to determine that the microsporidia spores were oval in shape, and occurred in groups surrounded by a membrane of undetermined origin (Figure A2.1). The average spore size was $2.7 \pm 0.3\mu\text{m}$ in length and $1.9 \pm 0.2\mu\text{m}$ in width. Polar filaments were isofilar and in two rows, with 18 coils per row (Figure A2.2). The average exospore was 78 ± 32 nm thick, and the average endospore was 94 ± 20 nm thick.

Molecular Sequencing

PCR products from amplification with microsporidian-specific primers were subject to sequencing. In total, 13 partial parasite 16s rDNA sequences were obtained, isolated from 13 separate hosts from both the rearing study and the brood size study. The sequences were between 99 and 100% similar, over 900bp. A representative DNA sequence of the parasite's 16S rDNA was submitted to GenBank (Accession number: DQ521753).

Discussion

DAPI staining of microsporidia nuclei is useful in determining the location of parasites within host cells. In addition, this type of staining indicates the intensity of infection, evidenced as one or more parasite nuclei surrounding a host nucleus. Most importantly, DAPI staining has been used to investigate the transmission efficiency of microsporidia from ovaries to eggs. It is in this final application that more studies need to be undertaken with respect to *C. volutator* and *Microsporidium* sp. In the few infected *C.*

Figure A2.1: TEM of microsporidia spores, within a section of *C. volutator* ovary at 2000x magnification. Developing microsporidia spores are indicated (a,b) and the spores are surrounded by a membrane, which may be host or parasite-derived (c).

Kathy Cook.005.tif

C-81

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10 microns

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Univ. of Ottawa Heart Institute

Figure A2.2: TEM of a single microsporidia spore within a section of *C. volutator* ovary, at 8000x magnification. The exospore (a), endospore (b), nucleus (c), polar lamellae (d) and polar filaments (e) are indicated.

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500 nm

HV=60kV

Direct Mag: 8000x

Univ. of Ottawa Heart Institute

volutator embryos observed in this study, intensity of parasitic infection was quite low, with a very small percentage of host cells being associated with a single parasite nucleus. This low parasite burden implies that the parasite has low pathogenicity (Terry et al., 1998). The determination of transmission efficiency was not a key objective in this thesis, and further investigation using DAPI staining is warranted.

Using TEM, it is evident that the parasite is localized within the ovary, but the host tissue is very degraded and the exact cellular location of the spores is indeterminable. This degradation may be a result of processing, or may be an indication of pathology. The spore morphology is more consistent with a horizontally transmitted microsporidia, as evidenced by the high number of polar filament coils and the thick spore walls. Generally, spores responsible for intrahost and vertical transmission have short polar filaments and thin spore walls (Dunn and Smith, 2001). The ridged exospore is consistent with microsporidia infecting marine invertebrates (Canning, 1993). Additional investigation of life cycle characteristics, including the number of nuclei in each life stage, will help to determine the taxonomic classification of this parasite. Based on the currently described characteristics, it is not possible to determine a classification, and the parasite will remain *Microsporidium* sp. until further characterization can be performed.

Sequence similarity of 99% or more indicates the same species (Terry et al., 2004). The 16S rDNA sequence obtained from *Microsporidium* sp. did not return any matches with this level of similarity, when subjected to BLASTn. The most similar sequence was the microsporidia *Dictyocoela* sp. infecting the freshwater amphipod *Gammarus roeseli*. Based on sequence data, it is not possible to determine the probable

route of transmission as vertical transmission has been found throughout the phylum Microspora, and may evolve frequently (Terry et al., 2004). Further sequencing should be done for *Microsporidium* sp., spanning not only the small subunit ribosomal RNA gene sequenced in this study, but also the internal transcribed spacer and large subunit ribosomal RNA gene. In short, vertical transmission is not a pleisomorphic character and as such we may not be able to infer likely transmission mode from sequences alone. This species is a candidate for testing whether sequences in the embryos are the same as those seen in the mothers regardless if the ultra structural evidence indicates horizontal transmission.

Based on the ultrastructural and molecular characterization of the microsporidia infecting *C. volutator*, it is not currently possible to suggest a classification. The novel microsporidian infecting *Corophium volutator* remains *Microsporidium* sp. until further investigation can be carried out.

Appendix 3: Rearing Study (Chapter 1) Raw Data

Table A3.1: Rearing Data from Infected Females

Sample Number	Initial Number of Offspring	Final Number of Offspring	Males	Females	Intersex	Proportion of Surviving Offspring	Proportion of Female Offspring
R100	10	2	2	0	0	20.00	0.00
R183	10	5	1	4	0	50.00	80.00
R181	10	6	1	5	0	60.00	83.33
R161	12	8	2	6	0	66.67	75.00
R146	12	12	0	10	1	100.00	83.33
R167	13	6	0	6	0	46.15	100.00
R160	14	2	0	2	0	14.29	100.00
R122	14	10	1	9	0	71.43	90.00
R300	14	12	0	12	0	85.71	100.00
R168	15	14	0	14	0	93.33	100.00
R81	15	15	0	15	0	100.00	100.00
C265	15	15	5	10	0	100.00	66.67
R275	16	13	0	13	0	81.25	100.00
R297	16	13	0	13	0	81.25	100.00
R56	17	10	0	10	0	58.82	100.00
R200	17	13	5	8	0	76.47	61.54
C287	17	14	4	10	0	82.35	71.43
R18	17	17	0	17	0	100.00	100.00
R290	18	14	0	12	2	77.78	85.71
R292	19	18	1	17	0	94.74	94.44
C296	20	18	2	11	5	90.00	61.11
R140	20	20	0	20	0	100.00	100.00
R76	21	9	6	3	0	42.86	33.33
C267	21	21	12	9	0	100.00	42.86
R192	23	7	0	7	0	30.43	100.00
R220	23	23	7	14	0	91.30	66.67
R278	24	19	1	15	2	79.17	78.95
R92	25	8	0	8	0	32.00	100.00
R171	25	8	0	8	0	32.00	100.00
R293	25	9	2	7	0	36.00	77.78
R242	29	15	6	9	0	51.72	60.00
R295	30	28	0	28	0	93.33	100.00
R210	32	25	10	15	0	78.13	60.00
C221	32	30	0	30	0	93.75	100.00
R90	34	19	1	18	0	55.88	94.74
R134	42	38	0	38	0	90.48	100.00
R190	69	56	0	56	0	81.16	100.00
R55	73	66	0	66	0	90.41	100.00

Table A3.2: Rearing Data from Uninfected Females

Sample Number	Initial Number of Offspring	Final Number of Offspring	Males	Females	Intersex	Proportion of Surviving Offspring	Proportion of Female Offspring
C223	4	1	1	0	0	25.00	0.00
R98	10	7	1	5	0	70.00	85.71
C237	11	10	0	10	0	90.91	100.00
C260	12	12	1	11	0	100.00	91.67
R120	13	4	0	4	0	30.77	100.00
R226	13	7	0	7	0	53.85	100.00
R216	13	9	0	9	0	69.23	100.00
R19	13	11	6	2	0	84.62	45.45
C277	14	7	2	4	0	50.00	71.43
C234	14	9	7	1	1	64.29	11.11
R41	14	10	1	9	0	71.43	90.00
R194	14	11	0	10	1	78.57	90.91
R215	14	12	0	12	0	85.71	100.00
C248	14	13	0	13	0	92.86	100.00
R123	15	4	0	4	0	26.67	100.00
R20	15	8	0	8	0	53.33	100.00
C280	15	11	0	9	1	73.33	90.91
R227	16	6	0	6	0	37.50	100.00
R94	16	8	0	8	0	50.00	100.00
C263	16	13	9	4	0	81.25	30.77
R32	16	14	8	4	0	87.50	42.86
R57	16	15	0	15	0	93.75	100.00
R96	17	11	0	11	0	64.71	100.00
R188	17	15	14	1	0	88.24	6.67
R274	17	16	0	16	0	94.12	100.00
R64	18	13	13	0	0	72.22	0.00
C255	18	17	0	17	0	94.44	100.00
C274	18	18	8	10	0	100.00	55.56
C242	19	19	17	2	0	100.00	10.53
R196	20	8	3	9	1	50.00	0.00
R195	20	19	0	19	0	95.00	100.00
C244	20	20	19	1	0	100.00	5.00
R155	23	21	12	9	0	91.30	42.86
R65	24	19	6	12	0	79.17	68.42
C290	24	22	6	8	7	91.67	40.91
R9	25	21	0	21	0	84.00	100.00
C264	25	21	4	17	0	84.00	80.95
R10	25	22	0	17	5	88.00	77.27
C251	27	26	1	25	0	96.30	96.15

Table A3.2 continued: Rearing Data from Uninfected Females

Sample Number	Initial Number of Offspring	Final Number of Offspring	Males	Females	Intersex	Proportion of Surviving Offspring	Proportion of Female Offspring
R199	28	22	0	22	0	78.57	100.00
R44	30	27	0	20	7	90.00	74.07
R252	34	30	16	14	0	88.24	46.67
R284	37	28	28	0	0	75.68	0.00
R93	40	1	0	1	0	2.50	100.00
R75	44	38	15	23	0	86.36	60.53
R60	47	20	2	16	2	42.55	80.00

Figure A3.1: Brood sex ratios of individual uninfected and infected mothers. Proportion of female offspring ranged from 0 to 100% for infected females. The mean was 86% female. Brood sex ratios of individual uninfected mothers ranged from 0 to 95% female. The mean was 68% female.

Appendix 4: Investigation of Intersexuality in *Corophium volutator*

Intersexuality in dioecious species refers to an individual that possesses both male and female characteristics and functions only as male or a female (Kelly et al. 2004).

Intersexuality can be widespread among species, yet uncommon with specific populations, in gonochoristic or dioecious crustaceans (Ford et al., 2004). Prevalence of intersexuality can vary between populations as well as with time (Barbeau and Grecian, 2003). Intersex individuals can be identified by their behaviour, their external or endocrine morphology, and/or their gonadal differentiation (Dunn et al., 1990).

Specifically, individuals with both genital papillae and öostegites are considered to be intersex (Rodgers-Gray et al., 2004).

There are many purported causes of intersexuality. Genetically, the transmission of intersexuality does not follow Mendelian laws and may occur maternally through the eggs or paternally through the sperm (Juchault et al., 1991). Environmental sex determination in crustaceans may result in intersex development during periods of intermediate environmental conditions (Dunn et al., 1990, 1993). Chemical contamination also results in intersex individuals. The androgenic gland is responsible for male differentiation in crustaceans, and both regulates spermatogenic activity and inhibits ovarian maturation (Brown et al., 1999; Taketomi et al., 1996; Taketomi and Nishikawa, 1996). Removal or inhibition of the androgenic gland results in female differentiation and formation of an intersex individual (Brown et al., 1999; Rodgers-Gray et al., 2004; Nagamine et al., 1980; Sagi et al., 1990; Zou and Fingerman, 2000). Endocrine disrupting chemicals, such as estrogen mimics, have been linked with intersexuality (Depledge and

Billingshurst, 1999; Ford et al., 2004). Additionally, incomplete feminization as a result of parasitism is also a leading cause of intersexuality (Juchault et al., 1991; Rigaud and Juchault, 1998). Host feminization is widespread in the phylum Microspora, and these parasites have been linked to intersexuality as a result of incomplete feminization (Kelly et al., 2003; Rodgers et al., 2004).

Corophium volutator is a **dioceous** species. Intersexuality has been observed in populations of *C. volutator* at an incidence of 0 to 5% (Barbeau and Grecian, 2003). These intersex individuals possess both male and female characteristics. Barbeau and Grecian observed intersex *C. volutator* possessing non-setose öostegites and penial papillae. No intersex individuals were observed with setose öostegites, and none were ovigerous (Barbeau and Grecian, 2003). However, more recently, two types of intersexes have been identified in *C. volutator*, both of which function as males. The first type was symmetrical, possessing non-setose öostegites and two penial papillae, and was found at a prevalence of 2%. The second type of intersex possesses öostegites with setose and one or two penial papillae and occurred in 1.3% of the population examined. Upon dissection, both ovaries and testes were found in Type II intersex individuals (McCurdy et al., 2004). As stated above, both types of intersex individuals were determined to function as males. In personal observations, two intersexes were found to carry embryos in the brood pouch, but these two ovigerous intersex are the only ovigerous intersex observed to date. Both individuals possessed setose öostegites and two penial papillae, classifying them as Type II intersex (Figure A4.1).

It is possible that Type II intersexes molt less frequently than true females. Although McCurdy et al. did not observe the molting of Type II intersex, it is unknown

Figure A4.1: Ovigerous *C. volutator* intersex, characterized by both embryos within the brood pouch (a) and penial papillae (b).

over what time period individuals were observed. As amphipods are only fertile after a molt, Type II intersexes that molt less frequently than true females would also become ovigerous less frequently than true females. Further, it may be possible that these intersexes function both as males and females, and may even be capable of self-fertilization. In the course of the rearing study undertaken in Chapter 1, intersex individuals were encountered among the offspring of both uninfected females and those females infected with a potentially feminizing microsporidian parasite. Both Type I (2.4%) and Type II (0.5%) intersex were encountered. The observation of intersex individuals in broods from uninfected mothers is evidence against the incomplete feminization route of intersexuality. The proportion of intersex individuals in the offspring observed was higher in uninfected broods than infected broods (4% compared to 1.7%), but this difference was not statistically significant ($p=0.912$, GLM). These results suggest that a factor other than microsporidian infection is influencing the occurrence of intersexuality in *C. volutator*.

Table A4.1: *C. volutator* intersex frequencies from the literature

Study	Microsporidia	Type	Intersex	Population	Proportion
Cook, 2006	Infected	1	8	600	1.3
		2	2	600	0.3
	Uninfected	1	21	610	3.4
		2	4	610	0.7
McCurdy et al., 2004	N/A	1	20	1000	2.0
		2	13	1000	1.3
Barbeau and Grecian, 2003	N/A	N/A	217	11148	1.9
Barbeau unpublished	N/A	N/A	168	3298	5.1
Lui and Forbes unpublished	N/A	N/A	355	7715	4.6