THE REPRODUCTIVE ECOLOGY OF A NORTHEASTERN PACIFIC NUDIBRANCH, *JANOLUS FUSCUS*, WITH AN EXAMINATION OF ITS ENDOPARASITIC COPEPOD, *ISMAILA BELCIKI*

by

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A DISSERTATION

Presented to the Department of Biology
and the Graduate School of the University of Oregon
in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy

December 2010

DISSERTATION APPROVAL PAGE

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Title: The Reproductive Ecology of a Northeastern Pacific Nudibranch, *Janolus fuscus,* with an Examination of Its Endoparasitic Copepod, *Ismaila belciki*

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Degree awarded December 2010

DISSERTATION ABSTRACT

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The arminacean nudibranch *Janolus fuscus* (family Zephyrinidae) is found in rocky intertidal habitats along the northeast Pacific coast. Adult *J. fuscus* are conspicuous from April to October but absent in the early winter at two sites, North Cove and Fossil Pt., in the Coos Bay region of Oregon. Over four years of intertidal surveys at these sites, the density of *J. fuscus* peaked with the abundance of their bryozoan prey, *Bugula pacifica* and *Tricellaria circumternata,* in spring and summer, while adult absence in winter was correlated with strong winter storms. To describe the timing of development and determine the life cycle of *J. fuscus*, embryos and larvae were reared in the laboratory and examined with light, scanning electron, and confocal microscopy. Larvae reared in the lab and juveniles collected from the field were monitored to quantify growth. *Janolus fuscus* exhibited typical spiral cleavage and hatched as planktotrophic veligers that grew for over a month before they reached competency, settled, and metamorphosed on their prey, *B. pacifica*. Juvenile growth was rapid, and adults reached maximum sizes of over 50 mm before dying. These demographic and developmental studies suggest that *J. fuscus* is a subannual species with a life span of approximately five

months. *Janolus fuscus* is often infected with an endoparasitic copepod, *Ismaila belciki*. In the field, prevalence of *I. belciki* increased with host density and size. The distribution of *I. belciki* was weakly aggregated in the host population. The large female parasite was generally found in the anterior portion of the host hemocoel, and one or more dwarf males were typically associated with each female. Infected *J. fuscus* produced significantly smaller egg masses with fewer larvae than did uninfected individuals. Infection did not influence growth rate but did cause decreased survival in older nudibranchs. To examine the life cycle of *I. belciki*, naupliar larvae were reared in the lab and incubated with potential hosts. Additionally, copepodid stages were described from dissected *J. fuscus* collected from the field. *Ismaila belciki* has a least three planktotrophic naupliar stages and four copepodid stages in its life cycle.

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Wolf, M. 2008. Seastar flip experiment. Northwest Aquatic Marine Educators. Scuttlebutt. Pp 4-5.

ACKNOWLEDGMENTS

I wish to express sincere appreciation to my advisor, Craig Young, for his substantial support and advice throughout the work embodied by this dissertation and for encouraging me to participate in a variety of activities, including courses at other institutions, that greatly enriched my graduate experience. Craig Young and Sandra Brooke provided welcome opportunities to participate in deep-sea research cruises and projects. I wish to thank Richard Emlet for graciously allowing me use of his laboratory facilities, for statistical advice and editing, and for instruction and enthusiasm in all things larval. I extend my gratitude also to Bitty Roy, Frances White, and Alan Shanks for their statistical expertise, inspiring ideas in experimental design, and expressions of enthusiasm for this project. The work would not have been possible without Sandra Brooke, Tracey Smart, Shawn Arellano, Ahna van Gaest, Paul Dunn, and Laurel Hiebert for their help in the field, for discussing experimental design and statistics and especially for editing parts of this dissertation. Armand Kuris and Ryan Hechinger, who kindly hosted me for a term in the Kuris lab and invited me to participate in the parasitology course at UCSB. They provided invaluable insights into the world of parasites. Drs. Svetlana Maslakova and George von Dassow welcomed me into their laboratory, trained me on the confocal microscope, and assisted with other related techniques. I thank Dr. Cynthia Trowbridge for editing a chapter of this dissertation, for enlightening discussions about sea slugs, and for providing the opportunity to participate in research abroad. Dr. Terue C. Kihara from the Universidade de Sao Paulo shared her experience on the staining copepod exoskeletons and Dr. Geoffrey Boxshall of the Natural History Museum assisted in the identification of contaminants in cultures. Dr. Dave Bilderback assisted in

identifying bryozoan species. I thank Dr. Jeff Goddard for encouragement and for sharing unpublished data on *Janolus fuscus* in North Cove. Scott Groth served as my diving partner and shared his amazing *in situ* photographs of sea slugs. Heidi Harris provided GIS expertise and help in finding intertidal maps. I wish to thank Barb Butler and the University of Oregon librarians for helping me acquire a constant stream of literature for this dissertation. This work would not have been possible without the help of many graduate and undergraduate students at the Oregon Institute of Marine Biology, especially Heather Austin, Katie Bennett, Sam Bund, Zair Burris, Ezzy Cooper, Paul Cziko, Christina Geierman, Ben Grupe, Mike Holmes, AnnMarie Jones, Holly Keammerer, Alix Laferriere, Ryan Lenz, Josh Lord, Jose Marin Jarrin, Sara Matthews, Myndee McNeil, Wyatt Miller, Annie Pollard, Kristina Sawyer, Stephanie Schroeder, and Kira Treiberg. Salary support during this investigation came in part from a National Science Foundation GK-12 grant to Drs. Jan Hodder and Alan Shanks and National Science Foundation grants to Dr. Craig Young. Last, I must thank my incredibly supportive family, including the Wolf, Punnett, Estridge, Rodenbush, and Watts clans and my awesome husband, Sean Watts for his endless patience and love.

To Jo and Ed Wolf and Sean Watts for their love, support and patience.

And

To Betsy who read “Girls Can Be Anything” to me when I was young.

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CHAPTER I

GENERAL INTRODUCTION

Opisthobranch gastropods are an ecologically diverse group and conspicuous in a diversity of marine habitats. Opisthobranchs are hermaphroditic and short-lived, with cohorts appearing and growing rapidly, reproducing, and completing their life cycle within a year. Developmental timing, embryonic periods, developmental mode, larval duration, metamorphic inducers, and growth rate are diverse in these gastropods. Though a number of studies have addressed the demography, reproduction, and development of superfamilies Doridoidea and Aeolidoidea within the Nudibranchia (Opisthobranchia), few studies have targeted members of the superfamily Arminoidea. Additionally, some opisthobranchs are hosts to endoparasitic copepods of the family Splanchnotrophidae, These large, highly modified copepods are typically found within the host's cerata or hemocoel, with the female copepod protruding her posterior through the host integument to produce paired egg sacs. While the morphology and systematics of splanchnotrophids has been well studied, there is little information on the basic ecology and life cycle of these copepods and their effect on host condition.

Opisthobranch Demography

Opisthobranchs are typically classified into three ecological groupings based on the duration of their life cycle. 'Subannual' species produce several generations in one year, while 'annual' species produce a single generation a year before dying. 'Biennial' species have a post-larval life of almost two years, during which they spawn once, then

die (Miller, 1961; Potts, 1970; Clark, 1975; Todd, 1981). Most opisthobranchs have annual life cycles with a single discrete spawning period and total post-reproductive mortality (Comfort, 1957; Thompson, 1961, 1964; Miller, 1962; Potts, 1970; Todd, 1981).

Various biotic and abiotic factors may influence the abundance and distribution of a population over time. Changes in the demography of an opisthobranch population are often tightly correlated with changes in prey abundance (Miller, 1961; Swennen, 1961; Thompson, 1964; Potts, 1970; Todd and Doyle, 1981; Lambert, 1991; Knowlton and Highsmith, 2000). Prey not only serve as food for juveniles and adults, but may impact larval recruitment as a substrate and cue for settlement and metamorphosis of opisthobranch larvae (Thompson, 1958; Harris, 1975; Hadfield, 1977; Harrigan and Alkon, 1978; Hadfield and Scheuer, 1985; Avila, 1998). Extremes in abiotic factors such as changes in temperature (Clark, 1975; Lambert, 1991), wind speed (Berry, 1994), and wave action (Crozier, 1917; Costello, 1938; Nybakken, 1978) may also affect opisthobranch populations.

Opisthobranch populations often exhibit large fluctuations in abundance with periodic disappearance and reappearance of entire populations (Thompson, 1958, 1961, 1966; Potts, 1970). Some studies suggest complete post-spawning mortality and large-scale synchronous migration of opisthobranchs to explain such boom and bust intertidal populations (Garstang, 1890; Hecht, 1895; Eliot, 1910). However, most studies suggest intertidal populations of opisthobranchs establish by larval recruitment and subsequent rapid growth into adult populations (Alder and Hancock, 1845-1855; Chambers, 1934; Miller, 1962; Thompson, 1964; Potts, 1970; Clark, 1975).

Development and Growth of Opisthobranchs

Embryogenesis in opisthobranchs occurs via spiral, typically holoblastic, cleavage (Casteel, 1904; Thompson, 1958). Gastrulation progresses by invagination, epiboly, or some combination of the two (Casteel, 1904; Thompson, 1958; Gohar and Soliman, 1967a,b; Soliman, 1978). Embryos pass through a preveliger (trochophore-like stage) into a veliger stage within the egg capsule. Larvae hatch as lecithotrophic (non-feeding) larvae with a short obligate planktonic period, planktotrophic (feeding) larvae with a longer planktonic period, or as a crawl-away juveniles (direct development) that progresses through all stages within the egg mass (Thompson, 1967; Clark and Jensen, 1981; Todd, 1981, 1983; Todd and Doyle, 1981; Hadfield and Miller, 1987).

Larval period varies widely among opisthobranch species with planktotrophic larvae, ranging from a few days to months with indiscernible to substantial growth of the larval shell (Hadfield and Switzer-Dunlap, 1984). When veligers become competent to metamorphose, larval growth typically slows (Perron and Turner, 1977; Harrigan and Alkon, 1978; Chia and Koss, 1978; Todd, 1981; Trowbridge, 2000). Also, the larva develops eyespots and a propodium on the enlarged metapodium (Perron and Turner, 1977; Harrigan and Alkon, 1978; Chia and Koss, 1978; Todd, 1981). Chemical cues from conspecifics and adult prey items (among other cues) induce settlement and metamorphosis in competent opisthobranch veligers (e.g. Hadfield, 1977; Harrigan and Alkon, 1978; Hadfield and Pennington, 1990; Lambert et al., 1994; Avila et al., 1997; Trowbridge and Todd, 2001). Juvenile growth to reproductive maturity is often rapid in opisthobranchs (Eyster, 1981; Todd, 1981; Hadfield and Switzer-Dunlap, 1984). Once

mature, slugs mate and deposit egg masses repeatedly over one reproductive season, typically, before dying (Todd, 1981; Hadfield and Switzer-Dunlap, 1984).

Splanchnotrophid Copepods

A few marine parasites are known to have major effects on the physiology, behavior, and population dynamics of their hosts (Reinhard, 1956; Kuris, 1974; Baudoin, 1975; Moore, 1980, 1983; Sousa, 1983; Sloan, 1984; Blower and Roughgarden, 1987, 1989; Lafferty & Kuris 2002). The majority of marine parasites, however, are known only taxonomically; fundamental aspects of their biology, including complete life cycles, modes of nutrition and reproduction, and impacts on hosts, remain virtually unknown. The copepod endoparasites (family Splanchnotrophidae) of opisthobranchs are among these poorly understood parasites.

Copepods from the family Splanchnotrophidae are endoparasites of marine opisthobranch gastropods that inhabit the hemocoel of opisthobranchs worldwide (Huys, 2001). While extensive studies have addressed the morphology and systematics of copepods from the family Splanchnotrophidae, (Bergh, 1868; Belcik, 1965, 1981; Ho, 1981, 1987a; Jensen, 1987; Huys 2001; Haumayr and Schrödl, 2003), reports on their ecology and impact on host condition are rare, vary significantly, and are typically based on their position in the host and organ and gonad condition in dissected specimens (Bergh, 1868; Jensen, 1987, 1990; Haumayr and Schrödl, 2003; Marshall and Hayward, 2006; Salmen et al., 2008a,b) with one notable experimental exception (Schrödl, 1997). Most studies have found low prevalence (percent of the host population that is infected) of splanchnotrophid copepods within the host population (Jensen, 1987; Schrödl , 2002; Marshall and Hayward, 2006). Great variation has been observed in the intensity

(number of parasites per host) and sex ratios of splanchnotrophids in their opisthobranch hosts (Hancock and Norman, 1863; Ho, 1981; Schrödl, 2002; Haumayr and Schrödl, 2003; Marshall and Hayward, 2006; Salmen et al. 2008a, 2008b), but no studies have examined the distribution of intensity of these copepods in a host population. For example, Marshall and Hayward (2006) found only one female and one male in every *Splanchnotrophus willemi* dissected, while Ho (1981) found 425 copepods (34 female and 77 male) of *Ismaila occulta* in one nudibranch host (*Dendronotus iris*) (Ho, 1981; Marshall and Hayward, 2006).

Reports suggest that some splanchnotrophid species inflict no obvious internal damage to their hosts (Jensen, 1990; Haumayr and Schrödl, 2003; Salmen et al., 2008a,b), while others imply these copepods damage the host's gonad or decrease the host's ability to copulate (Bergh, 1968; Jensen, 1987, 1990; Schrödl, 1997; Haumayr and Schrödl, 2003; Marshall and Hayward, 2006). In the only experimental study to address the effect of a splanchnotrophid copepod on host survivorship and fecundity, Schrödl (1997) found that a Chilean opisthobranch, *Flabellina* sp., infected with *Ismaila damnosa* experienced greater mortality and total castration. Jensen (1987) suggested that somatic growth was not impaired in a sacoglossan opisthobranch *Ercolania funerea* infected with *Ismaila monstrosa*.

Copepod Life Cycles

Parasitic copepod adults are often highly modified compared to their free-living counterparts (Boxshall, 2005). However, the life cycle and larval stages can be similar, including six naupliar stages followed by five copepodid stages before the final molt to a juvenile (Izawa, 1987; Ruppert et al., 2004; Boxshall, 2005). In contrast, those orders of

the Copepoda that have parasitic representatives, the number of larval stages can vary considerably from zero to six naupliar stages and one to five copepodid stages (Costanzo, 1959; Dudley, 1964; Gotto, 1979; Do et al., 1984; Izawa, 1987). Those species with abbreviated larval stages often have lecithotrophic development, do not feed or grow in the plankton and rapidly infect their hosts (Do et al., 1984; Izawa, 1987).

Only two studies have described larval stages from species belonging to the family Splanchnotrophidae (Cyclopoida) (Belcik, 1981; Ho, 1987b). Belcik (1981) described the first nauplius of *Ismaila belciki* from Oregon and inferred that there are at least two nauplius stages in the life cycle. Additionally, three copepodid stages (copepodid II-IV) of *Ismaila occulta* were described from a single nudibranch host, *Dendronotus iris*, collected in Long Beach, California (Ho, 1987b).

Scope and Objectives

My primary objectives in this dissertation are to examine the demography, reproduction and development of a northeast Pacific nudibranch *Janolus fuscus*, and the impacts of parasitic infection by the endoparasitic copepod *Ismaila belciki* on reproduction in this host. Additionally, I aim to describe the ecology (i.e., changes in their prevalence and overall distribution in the host population and site specificity in the host body) and larval stages of the parasite.

Janolus fuscus is an arminacean nudibranch found from Klu Bay, Alaska to the Gulf of California. This nudibranch is conspicuous in rocky intertidal habitats of Oregon from April to October. They feed on arborescent bryozoans such as *Bugula pacifica*, *Tricellaria circumternata*, and *Scrupocellaria diegensis* (Sphon, 1972; Belcik, 1975; McDonald and Nybakken, 1978; Goddard, 1984, 1992, 1998). In chapter II, I describe

intertidal surveys conducted over four years to determine whether *J. fuscus* is a subannual, annual, or biennial species. The density of *J. fuscus* is compared between two populations, one exposed and one protected site, and among seasons. Last I address potential correlations between the density of *J. fuscus* and prey abundance and storm effects. In chapter III, I examine embryonic timing, larval growth and duration, larval settlement and metamorphosis as well as juvenile growth through adulthood in *J. fuscus*. These data provide additional evidence to characterize the life history of *J. fuscus* as a subannual, annual, or biennial species.

In and near Coos Bay, Oregon, up to 62% of the population of the arminacean nudibranch *Janolus fuscus* can be infected with *Ismaila belciki*, an obligate endoparasite (Belcik, 1965). In chapter IV, I examine changes in the prevalence and intensity of infection of *I. belciki* within two populations of *J. fuscus* during the aforementioned intertidal surveys. Surveys and dissections were conducted to document the distribution of *I. belciki* in the host population. Additionally, I address site specificity and the potential for intraspecific competition in *I. belciki* within the host. In chapter V, I examine the impact of infection with *I. belciki* on the reproduction, growth and survival of *J. fuscus*. This was achieved by collecting egg masses from infected and uninfected *J. fuscus* in the lab to compare reproductive output. Additionally, growth and survivorship were monitored in the lab. These ecological features were then compared to those of typical parasites and parasitic castrators, to put the association of *I. belciki* with its host in a broader context.

In chapter VI, I examine the life cycle of *Ismaila belciki* by rearing nauplius larvae in the lab, documenting changes in morphology with each larval stage, and

attempting to experimentally infect *Janolus fuscus*. Additionally, dissections were conducted to search for internal copepodid stages of *I. belciki*. Light, scanning electron, and confocal microscopy were used to visualize and describe larval stages.

CHAPTER II

THE DEMOGRAPHY OF A NORTHEAST PACIFIC NUDIBRANCH, *JANOLUS FUSCUS*, IN RELATION TO PREY ABUNDANCE AND STORM EFFECTS

Problem

One perplexing pattern found in many demographic studies of nudibranchs is the reported rapid disappearance and reappearance of entire nudibranch populations, a pattern that makes most nudibranch populations temporally unstable and difficult to study (Thompson 1958, 1961, 1966; Potts 1970). Post-spawning mortality is one potential explanation for sudden absences of adults (Garstang 1890; Hecht 1895; Comfort 1957; Miller 1962; Thompson 1964; 1981), but death does not always follow spawning (Chambers 1934). Some early studies proposed large-scale synchronous migration of juvenile nudibranchs, with adults perishing after spawning while their offspring migrate from intertidal to subtidal habitats, returning to the intertidal as mature adults to breed (Garstang 1890; Hecht 1895; Eliot 1910). However, most authors consider such migrations unlikely, instead suggesting that intertidal populations of nudibranchs are established by larval recruitment and subsequent rapid growth to adult populations (Alder and Hancock 1845-1855; Chambers 1934; Miller 1962; Thompson 1964; Potts 1970; Clark 1975). In support of this idea, some studies have found that new recruits and small juveniles occupy inconspicuous habitats (e.g., under boulders or in crevices) near adults,

then move to more conspicuous positions as adults, leading to the 'sudden appearance' of an adult population (Potts 1970; Nybakken 1978).

Numerous studies over the past two centuries have described the abundance and distribution of assemblages of opisthobranch species from particular geographic areas (Garstang 1890; Swennen 1961; Schmekel 1968; Miller 1961; Nybakken 1974; 1978; Clark 1975; Eyster 1980) but relatively few address the numerous biotic and abiotic factors that could drive population changes over time (Thompson 1958, 1961, 1966; Potts 1970). Abundance of nudibranchs is often tightly correlated with abundance of their prey (Miller 1961; Swennen 1961; Thompson 1964; Potts 1970; Todd and Doyle 1981; Lambert 1991; Knowlton and Highsmith 2000). Prey abundance may influence recruitment patterns not only because of the direct effect of food supply, but also because prey often serve as preferred substrata for settlement and metamorphosis (Todd 1981). Veliger larvae often respond to chemical cues from their prey in the laboratory (Thompson 1958; Harris 1975; Hadfield 1977; Harrigan and Alkon 1978; Hadfield & Scheuer 1985; Avila 1998) though settlement distribution in the field has seldom been documented, since new recruits are often inconspicuous in the adult habitat (Potts 1970; Lambert 1991). Abundance of opisthobranchs may also be related to abiotic factors such as temperature (Clark 1975; Lambert 1991), wind speed (Berry 1994), and wave action (Crozier 1917; Costello 1938; Nybakken 1978). High wave action associated with more exposed intertidal sites or periods of severe storms may be negatively correlated with the abundance of opisthobranchs and their new recruits (Crozier 1917; Potts 1970; Nybakken 1978; DeFreese 1987; Trowbridge 1992).

Nudibranchs are typically classified into three ecological groupings based on the duration of the life cycle: a) ‘subannual’ - species that produce several generations in one year, b) ‘annual’ - species that produce a single generation in one year before dying, or c) ‘biennial’ - species that have a post-larval life of almost two years, during which they spawn once, then die (Miller 1961; Potts 1970; Clark 1975; Todd 1981). Most nudibranchs are semelparous and have annual life cycles with a single discrete spawning period and total post-reproductive mortality (Comfort 1957; Thompson 1961, 1964; Miller 1962; Potts 1970; Todd 1981). Annual species are often large and generally feed on temporally stable prey (e.g., sponge), allowing for more stable populations (Thompson 1964; Clark 1975). Subannual nudibranchs are typically small aeolids that prey upon seasonally ephemeral resources (e.g., hydroids) and fluctuate in abundance over short time periods (Swennen 1961; Miller 1962; Thompson 1964; Nybakken 1974, 1978; Clark 1975; Todd 1981). Most studies of nudibranch life cycles have focused on species belonging to the superfamilies Doridoidea and Aeolidoidea (Miller 1962; Thompson 1961, 1966; Potts 1970; Todd 1981). Little is known about the life cycles of species belonging to the superfamily Arminoidea, although subannual and annual life cycles have been suggested for species in this group (Clark 1975; Eyster 1981; Todd 1983; Battle & Nybakken 1998).

Janolus fuscus O’Donoghue was first documented along the coast of Oregon in the 1940s and has been noted in a number of subsequent studies (Belcik 1965; Gosliner, 1982; Goddard 1984, 1998). This arminacean nudibranch is conspicuous from April to October in large tidepools at North Cove, Cape Arago, OR with peak abundances around August (Goddard 1984; pers. obs.). Preliminary observations and work by Goddard

(unpubl. data) of the abundance of *J. fuscus* suggest that slugs are most abundant in summer but are absent or rare as small recent recruits during winter months. I hypothesized that winter storms may have a negative effect on the density of *J. fuscus*. North Cove is located on the open coast and exposed to seasonal high wave action during winter storms. At this site, *J. fuscus* prey primarily on the epiphytic arborescent bryozoan *Tricellaria circumternata* (Soule, Soule and Chaney 1995, formerly *T. ternata*, Osburn 1950), attached to red algae on rock surfaces (Goddard 1998; Soule et al. 2007; pers. obs.). Another prey species, *Bugula pacifica* (Robertson 1905), is found more rarely at North Cove, where it is typically attached to the leeward or undersides of undercut rocks. At Fossil Pt., *J. fuscus* are found in a large cobble/boulder field, with adults crawling in tidepools and small slugs attached to *B. pacifica* hanging from undercut rocks (pers. obs.). Fossil Pt. is located in Coos Bay, OR and is thus more protected from wave action and winter storms than North Cove.

In this study I examine the demography of *Janolus fuscus*, to determine whether it has a subannual, annual or biennial life cycle. I examine seasonal patterns in the density of this nudibranch species at two sites, one protected and the other exposed. Last, I address correlations of prey abundance and seasonal storm effects with changes in slug density at both sites.

Methods

Sites

Populations of *Janolus fuscus* at two sites, North Cove at Cape Arago (43°18’30.6N, 124°23’58.92W) and an unnamed point (43°21’32.4N, 124°18’45.36W) midway between Fossil Point and Pigeon Point, Charleston, OR, USA were surveyed in

this study (Fig. 1.1). North Cove is a large intertidal area containing sandstone outcrops, and boulders of varying sizes. Surveys were conducted in the inner boulder field, a protected area of the cove that contained the majority of *J. fuscus*. The inner boulder field is composed of fissured and pocketed bedrock and boulders ranging in size from 0.25 to 0.5 m in diameter (Goddard 1984). *Janolus fuscus* were found in large tidepools and under and among boulders with two of their arborscent bryozoan prey, the dominant epiphytic *Tricellaria circumternata* and, more rarely *Bugula pacifica* (Soule et al. 2007). While more protected than the other coves on the outer coast, North Cove is more exposed than Fossil Pt.; therefore, I use North Cove and 'exposed site' synonymously in this study. The second site is located inside and north of the entrance of Coos Bay and will be referred to as Fossil Pt. for convenience in this paper, though it is not Fossil Pt. per se. This site has large sandstone shelves and boulder and cobble fields ranging in size from (0.25-2.5 m) arising from a surrounding mudflat. Surveys were conducted in a narrow (12 m) boulder and cobble field located just to the south of the large sandstone shelf that comprises this unnamed point. *Janolus fuscus* were found in small tidepools and under undercut boulders that support primarily *Bugula pacifica* and occasionally another arborescent bryozoan, *Scrupocellaria diegensis* (Soule et al. 2007). As this site lies within Coos Bay, it is not exposed to the open ocean and is therefore also called 'protected site' in this study.

Field surveys

Populations of *Janolus fuscus* at each of the two sites were surveyed 23 times between August 2005 and December 2009: seven times between August 2005 and April

2007, and every other month from July 2007 to December 2009. To determine density of *J. fuscus*, every slug was collected along five haphazard, 30 m x 2 m and 30 m x 1 m belt transects through the low intertidal boulder fields of North Cove and Fossil Pt., respectively (Fig. 1.1). A narrower transect was used at Fossil Pt. because the population and habitat were concentrated in a smaller area than at North Cove. All five transects were pooled into a single value for abundance and density at each site and date, as slug abundances were extremely variable among transects. Each slug was transported to the Oregon Institute of Marine Biology (43°34.51N, 124°32.90W) in an individual 50-ml Falcon tube to prevent mating. For each field survey starting in February 2007 (with the exception of June 2008 at Fossil Pt.), approximately 500 cm^3 of arborescent bryozoans, *Bugula pacifica*, *Tricellaria circumternata*, and/or *Scrupocellaria diegensis* (Soule et al. 2007) were collected at each site, returned to the lab in seawater, and examined under a

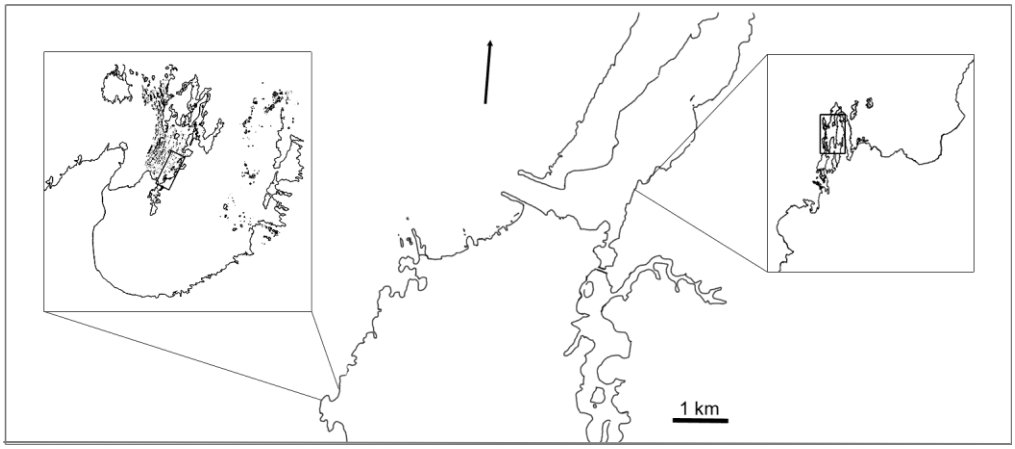


Fig. 1.1: Map of outer coast and Coos Bay, Oregon. North Cove (43°18'30.6N, 124°23'58.92W) is displayed in detail on the left and a site midway between Fossil Point and Pigeon Point (43°21'32.4N, 124°18'45.36W), referred to as Fossil Pt. for convenience, is on the right. Boxes within each inlay outline the survey area. The height of the small boxes represents 60 meters. Arrow indicates North.

dissecting microscope for new recruits (<10 mm long) of *J. fuscus*. While bryozoans were collected during 2005-2006 transects, the volumes of bryozoans were smaller and less consistent. Thus fewer recruits were found during these first two years.

Variation in the density of *Janolus fuscus* was examined as a function of site (North Cove = exposed and Fossil Pt. = protected) and season, and year of collection as well as the percent cover of their bryozoan prey, *Bugula pacifica* and *Scrupocellaria diegensis* from Fossil Pt. and *Tricellaria circumternata* and *B. pacifica* from North Cove. Season, year, and percent cover of bryozoans could not be included in the same models as every season was not sampled in every year and bryozoan cover was only documented from April 2008 to December 2009. Seasons were designated as winter (January - March), spring (April - June), summer (July - September), and fall (October - December) with respect to patterns seen in the demography of *J. fuscus* after the first year of collection. Variation in density of *J. fuscus* according to site and season and site and year, were analyzed with two, two-factor, mixed-model ANOVAs with site as a random factor and year and season as fixed factors in SPSS 14.0 statistical software. As sites were significantly different, a single-factor ANOVA examining variation in slug density by season, was run for each site separately. Slug density was square root transformed to meet assumptions of normality and homogeneity of variance in all General Linear Model analyses.

To determine if variation in total slug density ((adults + new recruits)/m^2), adult slug density ((slugs >10 mm in length)/m^2) and new recruit abundance (number of slugs ≤10 mm) of *J. fuscus* were related to fluctuations in the amount of food (i.e., arborescent bryozoans) available throughout the year, I surveyed each site during each collection

period from April 2008 through December 2009. Five transect lines were laid (as described above) and the percent cover of each arborescent bryozoan (*B. pacifica*, *S. diegensis* and/or *T. circumternata*) was estimated and recorded in ten random quadrats on each transect line. Because virtually no bryozoans were ever found on one of the five Fossil Point transects, this transect was not used in the analysis. The variation in adult density, new recruit abundance, and total density of *J. fuscus* were examined using three ANCOVAs, with site as a random factor and average percent cover of bryozoan as a covariate. New recruit abundance was used (as opposed to density) as these small slugs were typically found on approximately the same amount of bryozoan collected from both sites, and thus require no standardization. Total density of *J. fuscus*, adult density, and new recruit abundance were all square root transformed and percent cover of bryozoan was arcsine transformed to meet GLM assumptions of normality and homogeneity of variance.

Nudibranch maintenance and processing

After collection, *Janolus fuscus* were kept in the lab in seawater tables in separate plastic (10.5x10.5x12.5 cm) flow-through containers and fed *Bugula pacifica, Tricellaria circumternata,* and/or *Scrupocellaria diegensis* every few days to satiation. Slug length was measured with calipers as the slug crawled upside down on the water surface tension. Slugs over 8 mm were gently blotted on a paper towel before recording a wet-weight measurement. Length was used as the primary measure of slug size, as blotting and measuring wet weights of very small slugs (<8 mm) is damaging to the slugs and causes them to lose cerata. These data were used to create size frequency histograms for each

site and date of collection. Also, these data were used to create a best-fit non-linear regression curve of slug length and weight (in Sigmaplot 11.0), that was used to convert slug length to wet weight in concurrent studies comparing growth rates in larger slugs. Slugs not used in concurrent experiments were returned to their respective sites.

Correlations with abiotic factors

Abiotic data (i.e., sea surface temperature, salinity, wind speed, and wave height) were downloaded from the NOAA National Data Buoy website for years 2007-2009 to compare to bimonthly measures of the density of *J. fuscus* from February 2007 to December 2009. Temperature and salinity data (at 15 minute intervals) were collected from the South Slough National Estuarine Reserve station, CHNO3 (43.345N, 124.329W), approximately 7.0 and 2.04 km from North Cove and Fossil Pt., respectively. Maximum and average wave height (averaged by hour) were collected off Port Orford, Station 46015 (42.747N, 124.823W), approximately 71.31 and 79.69 km south of North Cove and Fossil Pt., respectively. Maximum and average wind speed (averaged by hour) were from the Cape Arago weather station, CAR03 (43.342N, 124.375W), approximately 4.20 and 5.41 km from North Cove and Fossil Pt., respectively. Data were first processed to remove any errors or false readings, typically displayed as 99s within the files. The average and minimum temperature and salinity and average and maximum wave height and wind speed were calculated for each day of every year between 2007 and 2009.

As all abiotic measures collected were strongly correlated ($p<0.01$), I used maximum wave height as a proxy for storm effect. Two regression analyses were used to examine the effect of average maximum wave height on the density of *J. fuscus* at

North Cove (exposed site) and Fossil Pt (protected site). The average daily maximum wave height of the prior month was used, as the population of *J. fuscus* was expected to be influenced by prior rather than current conditions. Two subsequent regression analyses were used to determine whether prey abundance (arcsine transformed cover of bryozoan) explained any of the residual variation in slug density from the previous analysis of slug density by wave height. As new recruits of *J. fuscus* may be found in winter at both sites when adults are absent, I ran virtually identical analyses to examine the effect of average maximum wave height on the abundance of new recruits of *J. fuscus* at both sites. However, daily maximum wave height was averaged two weeks prior to collection (instead of one month) as new recruits are likely to be 1-3 wks old (chapter III). Density of slugs and abundance of new recruits were square root transformed and percent bryozoan cover arcsine transformed in these analyses to meet GLM assumptions of normality and homogeneous variance.

Overwintering and informal surveys

Janolus fuscus were collected from Fossil Pt. on December 11, 2008 and kept in the laboratory to see if they survived over the winter when adults are absent in the intertidal populations. *Janolus fuscus* were fed bryozoan every 3-7 days (except from December 19-31, when slugs were fed an excess of food but not replenished or checked for 12 days). While I did not conduct formal surveys in January 2009, I searched for adult *J. fuscus* for one hour and collected (~500 ml) bryozoans, *T. circumternata* (*B. pacifica* could not be found) from North Cove and *B. pacifica* from Fossil Pt., and the Charleston boat docks in search of new recruits.

Results

Field surveys

Density of *Janolus fuscus* changed dramatically throughout the year at both sites (Fig. 1.2), but was significantly higher at Fossil Point than North Cove in all analyses (Tables 1.1, 1.2, 1.3). *Janolus fuscus* were absent at both sites in January 2006 and 2008 and from North Cove only in November 2007 and December 2008 and 2009. They were typically absent at North Cove during the late fall and winter. A few recruits appeared in February and March at North Cove, and overall densities of *J. fuscus* slowly increased through the spring and reached peak densities during the summer (Fig. 1.2 and 1.3A). At Fossil Pt., *J. fuscus* were most abundant in spring, persisted through summer and fall but disappeared during the early winter. Recruits returned in February (Fig. 1.2, 1.3B). The abundance of recently recruited *J. fuscus* roughly mirrors adult density at both sites, except in February and March when new recruits represented 100% of the population in the absence of adults (Fig. 1.3A, B). At North Cove recruitment did not occur or was very low in late fall and early winter (November – February), increased in March and trickled in throughout the year (Fig. 1.3A, B). Recruitment was fairly continuous throughout the year at Fossil Pt., but did not occur in a few collection periods such as November 2007 and January 2008. One extreme recruitment event occurred at Fossil Pt. in February 2009 (Fig. 1.3B).

Density of *Janolus fuscus* did not differ significantly by season if site was included in the model (Table 1.1). However, the near significant interaction of site and season, suggested the density of *J. fuscus* may differ by season between the two sites (Table 1.1). If sites were analyzed separately, slug densities were significantly lower in

winter than spring at Fossil Pt. (Bonferroni pairwise comparison p=0.005) and lower in winter than summer at North Cove (p=0.004) (Table 1.2, Fig. 1.4). Density of *J. fuscus* did not differ by year of collection at North Cove ($F_{18,4}$=0.447, p=0.773) or Fossil Pt. ($F_{18,4}$=0.030, p=0.998) (Fig. 1.2). However, average slug densities were lower than previous years in the spring and summer of 2009 at both sites (Fig. 1.2).

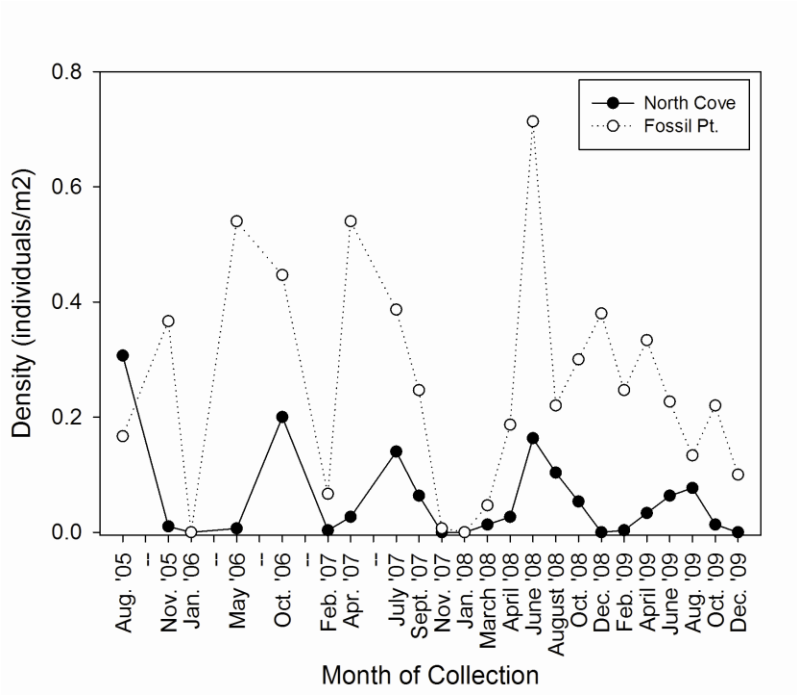


Fig. 1.2: Density of *Janolus fuscus* by each collection date from August 2005 to December 2009. Collection dates before July 2007 were not evenly distributed in time. Intervals greater than two months are represented by dashed lines on the x-axis.

The non-linear relationship of length to weight of *Janolus fuscus* was best fit by a cubic polynomial equation ($y=0.224-0.038x+0.003x^2-(8.5^{-6})\ x^3$) ($r^2$=0.794, p<0.0001, n=1117) (Fig. 1.5). Length of *J. fuscus* varied throughout the year at North Cove and Fossil Point. Slug lengths in winter collection dates (when slugs are present) were smaller than other seasons for both sites corresponding to the absence of adult *J. fuscus* and predominance of new recruits (Fig. 1.6, 1.7, 1.8). Average length of *J. fuscus* rapidly

increased at both sites, as slugs grew rapidly from early recruits in February and March into large adults by April and May (Fig. 1.6, 1.7, 1.8). At both sites recruitment was fairly continuous at a slow rate throughout the year with slugs present in 0-5 or 6-10 mm size classes during most collection periods except early winter (and late fall at North Cove) (Fig. 1.7, 1.8). At North Cove, the average length of *J. fuscus* increased through spring (av.=21.99 ± 2.76 mm) and typically reached peak lengths in the summer (av.=23.86 ± 3.45 mm) before they declined slightly in the fall (av.=17.67±1.8 mm) and disappeared by November or December (Fig. 1.6A). In 2008 however, lengths decreased steadily from April to October (Fig. 1.6A). At Fossil Point, *J. fuscus* reached peak

Table 1.1: Results of ANOVA analysis examining variation in density of *Janolus fuscus* according to site (North Cove = exposed site and Fossil Pt. = protected site) and season (winter = January-March, spring = April-June, summer = July-September, fall = October-December) of collection. Density of *J. fuscus* was square root transformed to meet GLM assumptions of normality and homogeneous variance.

Factor	SS	df	MSE	F	p
Site	0.806	1	0.806	14.454	0.031
Season	0.592	3	0.197	3.510	0.165
Site x Season	0.169	3	0.056	2.479	0.076
Error	0.861	38	0.023		

Table 1.2: Results of ANOVA analyses examining variation in density of *Janolus fuscus* by season (winter = January-March, spring = April-June, summer = July-September, fall = October-December) at North Cove and Fossil Pt. Density of *J. fuscus* was square root transformed to meet GLM assumptions of normality and homogeneous variance.

	North Cove (Exposed)					Fossil Pt. (Protected)				
Factor	SS	df	MSE	F	P	SS	df	MSE	F	P
Season	0.284	3	0.095	6.151	0.004	0.472	3	0.159	5.303	0.008
Error	0.292	19	0.015			0.569	19	0.030		

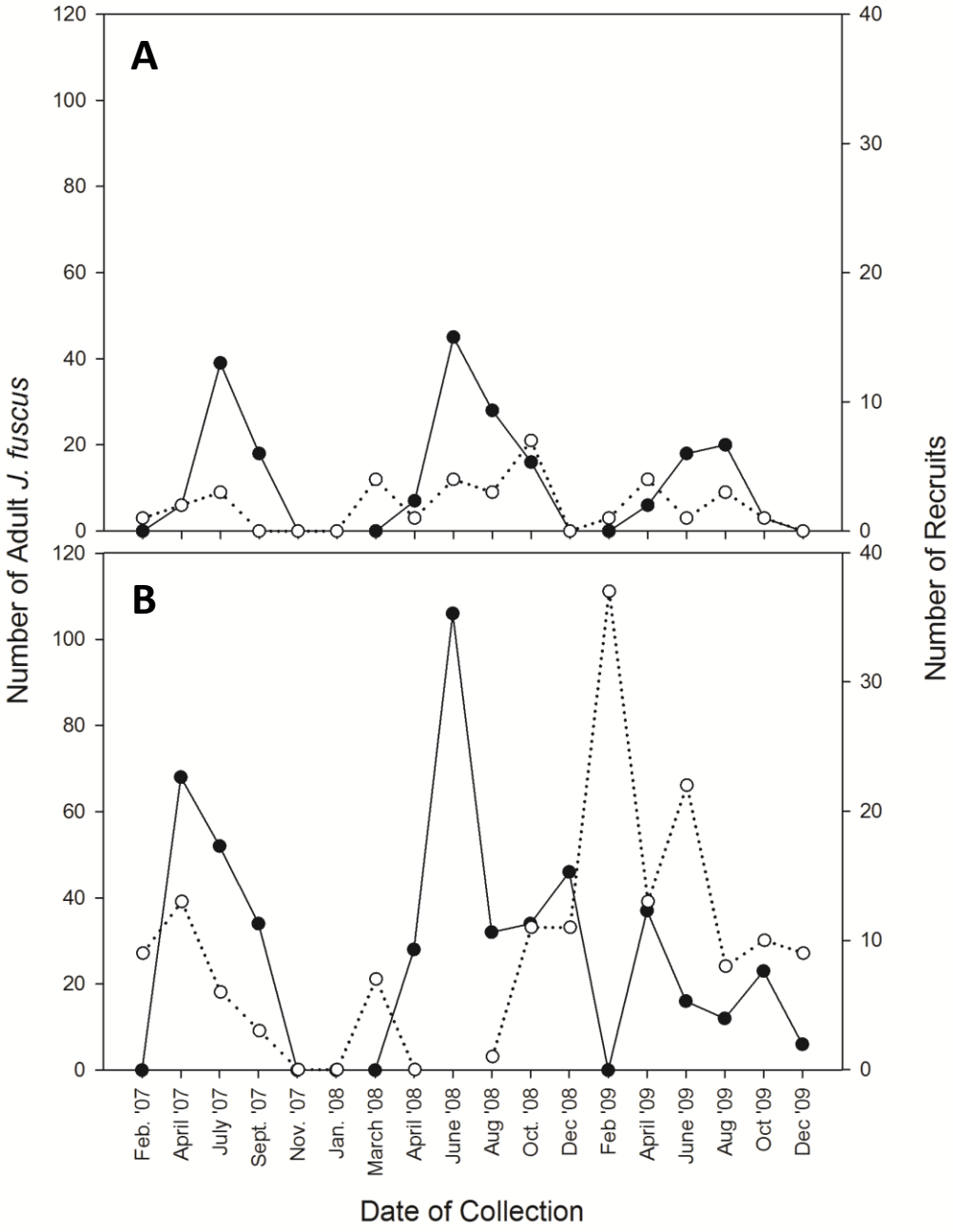


Fig. 1.3: Number of adults (>10 mm) (solid line/closed circles) and new recruits (≤10 mm) (dotted line/open circles) of *J. fuscus* at (A) North Cove; and (B) Fossil Pt. collected every other month from February 2007 to December 2009. Data for collection dates before 2007 were not included as the volume of bryozoans collected to search for newly recruited *J. fuscus* was not consistent.

Table 1.3: Results of three ANCOVA analyses examining the effect of collection site (North Cove and Fossil Pt.) (random factor), and average percent cover of bryozoan (covariate) on density of adult *J. fuscus* (i.e. number of slugs >10 mm/m^2), abundance of new recruits (number of slugs ≤10 mm), and total density of *J. fuscus* (i.e. (adults + recruits)/m^2) (all square root transformed to meet GLM assumptions of normality and homogeneous variance). Percent bryozoan cover is arcsine transformed to meet GLM assumptions of normality and homogeneous variance.

Analysis	Factor	SS	df	MSE	F	p
Adult Density	Site	0.125	1	0.125	4.147	0.056
	Bryozoan Cover	0.053	1	0.053	1.770	0.199
	Error	0.574	19	0.030		
New Recruit Abundance	Site	5.992	1	5.992	6.322	0.022
	Bryozoan Cover	9.164	1	9.164	9.668	0.006
	Error	16.113	17	0.948		
Total Slug Density	Site	0.252	1	0.252	16.023	0.001
	Bryozoan Cover	0.070	1	0.070	4.478	0.048
	Error	0.299	19	0.299	0.016	

density and length in the spring (av.=20.4 ± 1.52 mm), though adults were present through summer (av.=20.2 ± 1.52 mm) and fall (av.=17.05 ± 3.45 mm) (Fig. 1.6B, 1.8B). There was substantial fluctuation in slug length throughout the year, with some years showing secondary increases or stabilization of slug length after an initial peak (Fig. 1.8B). This fluctuation in size and obvious overlap of generations made distinguishing cohorts impossible past the initial late winter/early spring cohort (Fig. 1.7 and 1.8).

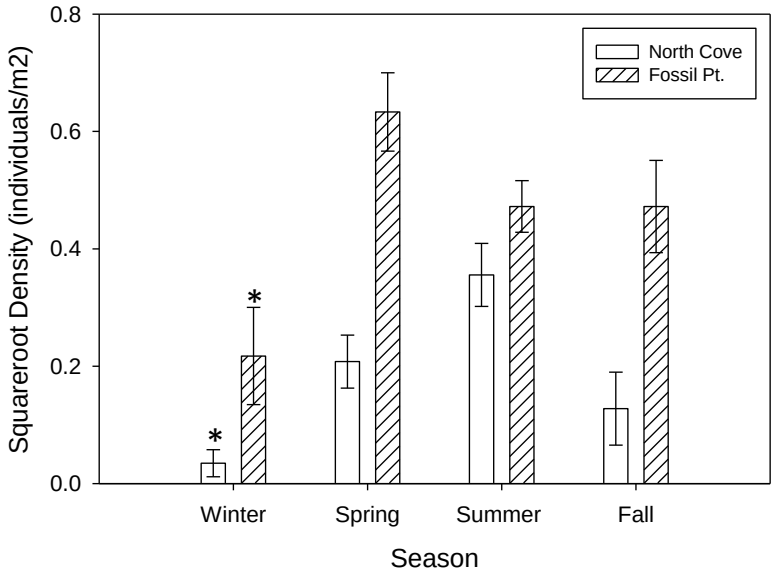


Fig. 1.4: Density of *Janolus fuscus* by season, (winter = January-March, spring = April – June, summer = July-September, fall = October-December). Asterisks denote that densities of *J. fuscus* were significantly lower in winter than spring at Fossil Pt. (Bonferroni pairwise comparison p=0.004) and summer at North Cove (p=0.005). Error bars represent standard error.

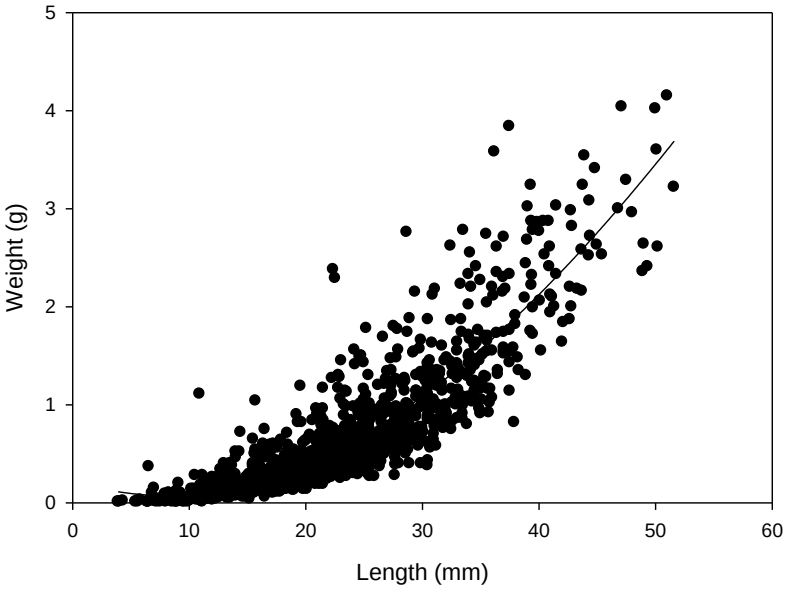


Fig. 1.5: Curvilinear relationship of length and weight of *Janolus fuscus* with best fit cubic polynomial line, $y=0.224-0.038x+0.003x^2-((8.5^{-6})\ x^3)$ ($r^2=0.794$, $p<0.0001$, $n=1117$).

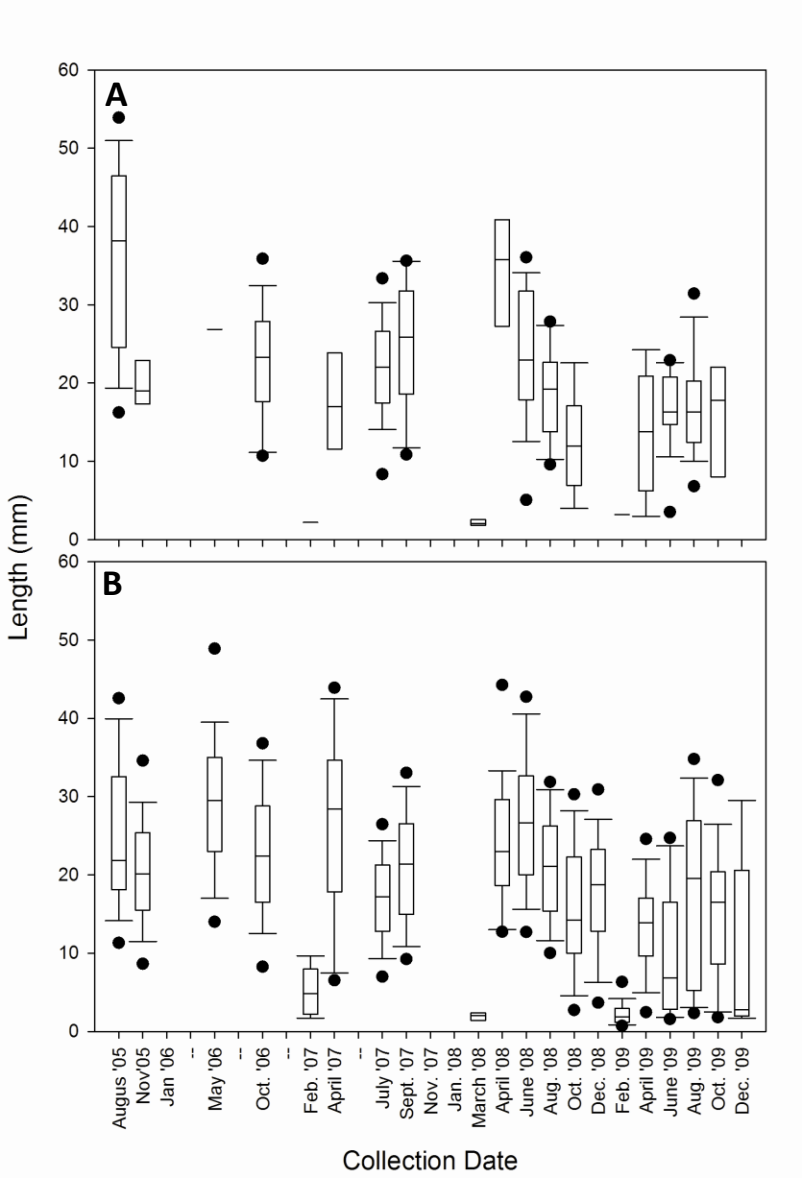


Fig. 1.6: Whisker box plots of the length of *Janolus fuscus* over 23 collection dates (between August 2005 and December 2009) at (A) North Cove; and (B) Fossil Pt. Boxes represent 25% and 75% quartiles with the median value in the middle. Whiskers represent 5th and 95th percentiles. Black circles represent points outside the 5th and 95th percentiles. Boxes without whiskers had fewer than nine samples and single dashes occur during collection dates in which fewer than three slugs were found. Collection dates before July 2007 were not evenly distributed in time, ranging in intervals between two and five months. These gaps are represented by dashed lines on the x-axis.

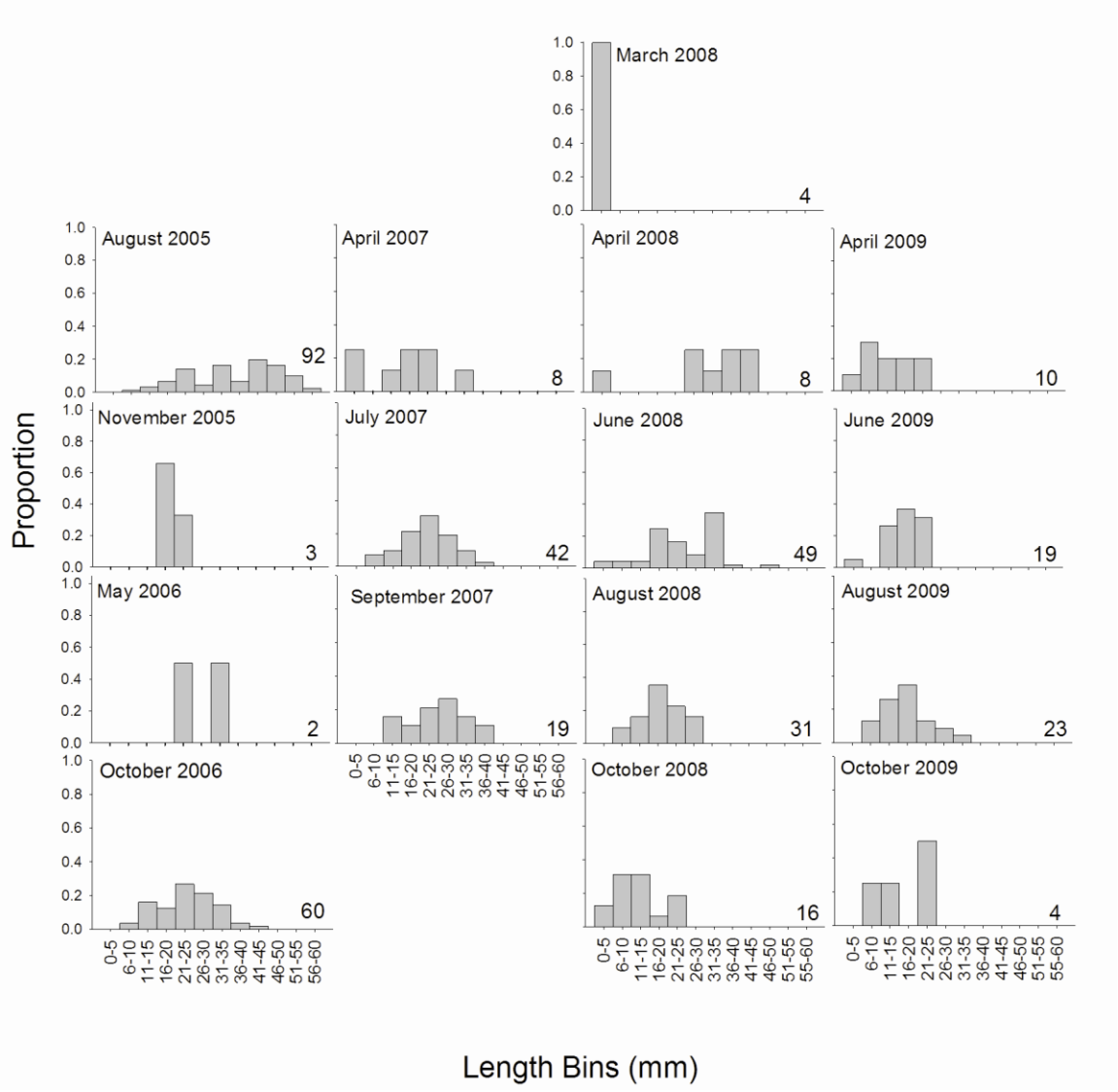


Fig. 1.7: Size frequency histograms of *Janolus fuscus* from North Cove over 16 collection dates (i.e., dates when more than one slug was present) of the 23 total collection dates from August 2005 to December 2009. Month and year of collection and abundance of *J. fuscus* are displayed on each graph. Surveys were conducted every other month from July 2007 to December 2009. Collection dates before July 2007 were not evenly distributed in time, with a range of temporal intervals between two and five months. Graphs are not shown for collection dates in which sea slugs were absent or only a single individual was found (i.e. January 2006, February 2007 (n=1), November 2007, January 2008, December 2008, February 2009 (n=1), December 2009).

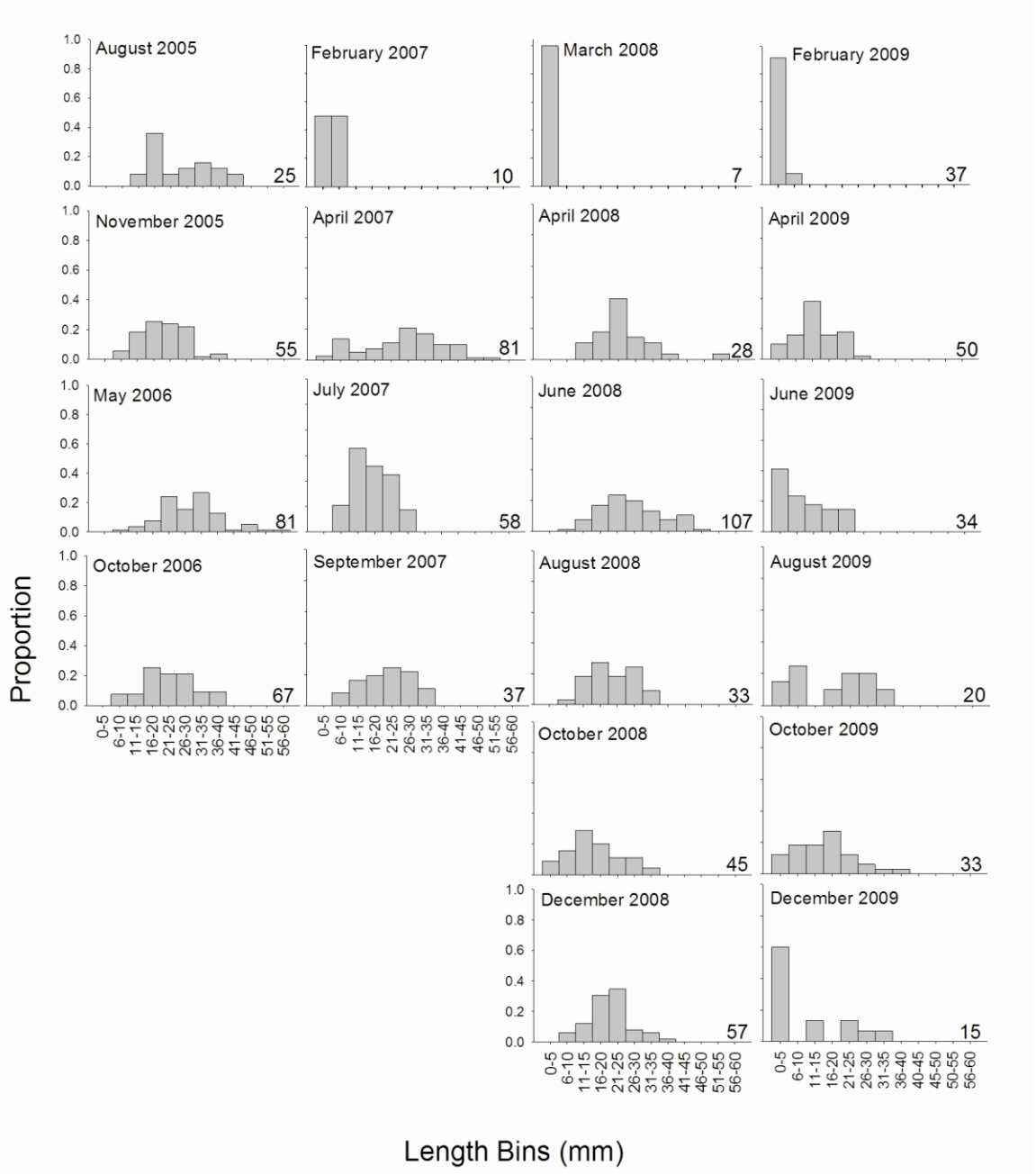


Fig. 1.8: Size frequency histograms of *Janolus fuscus* from Fossil Point over 20 collection dates (i.e., dates in which slugs were present) of the 23 total from August 2005 to December 2009. Month and year of collection and abundance of *J. fuscus* are displayed on each graph. Surveys were conducted every other month from July 2007 to December 2009. Collection dates before July 2007 were not evenly distributed in time, with a range of temporal intervals between two and five months. Graphs are not shown for collection dates in which sea slugs were absent (i.e. January 2006, January 2008, November 2007).

Bryozoan cover

Bugula pacifica was the primary bryozoan at Fossil Pt., while the epiphytic bryozoan *Tricellaria circumternata* dominated the bryozoan composition in most months at North Cove (Fig 1.9). Overall, young *T. circumternata* appeared at North Cove in late winter but did not reach peak abundance until late summer/early fall and declined again in early winter (Fig. 1.9A). At North Cove, *B. pacifica* dominated the bryozoan community in April 2008 and were common in April 2009 but sparse August through February (Fig. 1.9A). Colonies of *B. pacifica* were present as rare, small colonies in February at this site. At Fossil Pt., *B. pacifica* was most abundant in winter and spring, decreased in late summer and early fall and increased again in late fall (Fig. 1.9B). Colonies of *B. pacifica* in summer often appeared "well-grazed" and had fewer active lophophores than in winter and spring colonies. In October-December, patches of *Scrupocellaria diegensis* (Robertson 1905) were also present at Fossil Point (Fig. 1.9B). Both sites showed higher peaks in percent cover of bryozoans in 2008 than in 2009 (Fig. 1.9A, B).

Overall, North Cove had significantly lower percent cover of bryozoans (0 - 1.9%) than Fossil Pt. (0 – 7%) ($F_{21,2}$=8.634, p=0.008) (Fig.1.10A, B, C). Total slug density and new recruit abundance increased significantly with percent cover of bryozoans, but there was not a significant positive relationship between adult slug density and bryozoan cover (Table 1.3, Fig. 1.10A, B, C). This difference may be explained by the fact that adults were absent at North Cove in December 2008 and 2009 when moderate cover of *Tricellaria circumternata* still occurred and at Fossil Pt. in February 2009 when *Bugula pacifica* were abundant (Fig. 1.9A, B). Recruits of *Janolus fuscus*

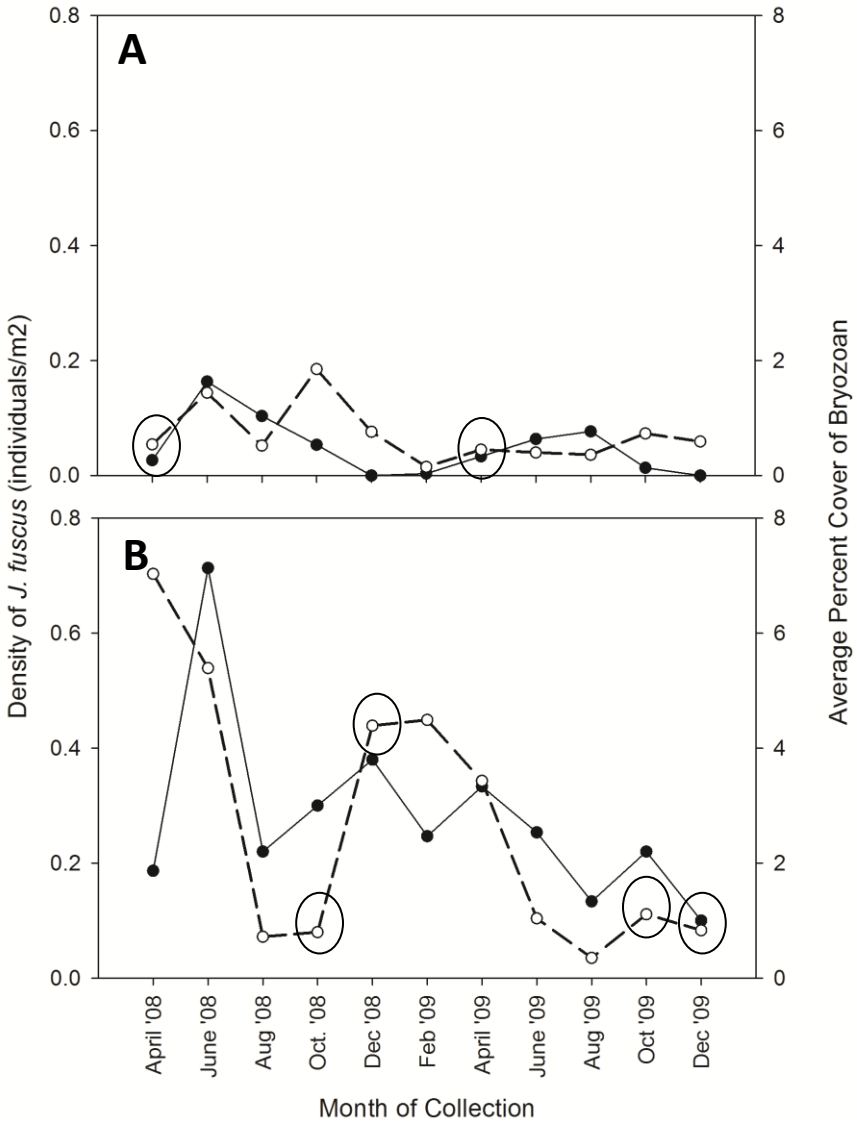


Fig. 1.9: Density of *Janolus fuscus* (solid line/closed circles) and average percent cover of bryozoan (dotted line/open circles) at (A) North Cove (where bryozoan cover is dominated by *Tricellaria circumternata*); and (B) Fossil Point (where bryozoan cover is dominated by *Bugula pacifica*) during bimonthly transects from April 2008 to December 2009. Circles designate dates when non-dominant bryozoan species were conspicuous at each site. At North Cove *B. pacifica* was the dominant bryozoan species on April 2008 and was fairly abundant in April 2009. At Fossil Pt. *Scrupcellaria diegensis* was present in October and December 2008 and 2009.

were only ever found crawling on *B. pacifica* from both sites when bryozoans were examined in the lab. Even when the bryozoan population at North Cove was dominated by *T. circumternata*, recruits were only found on the few small colonies of *B. pacifica* collected.

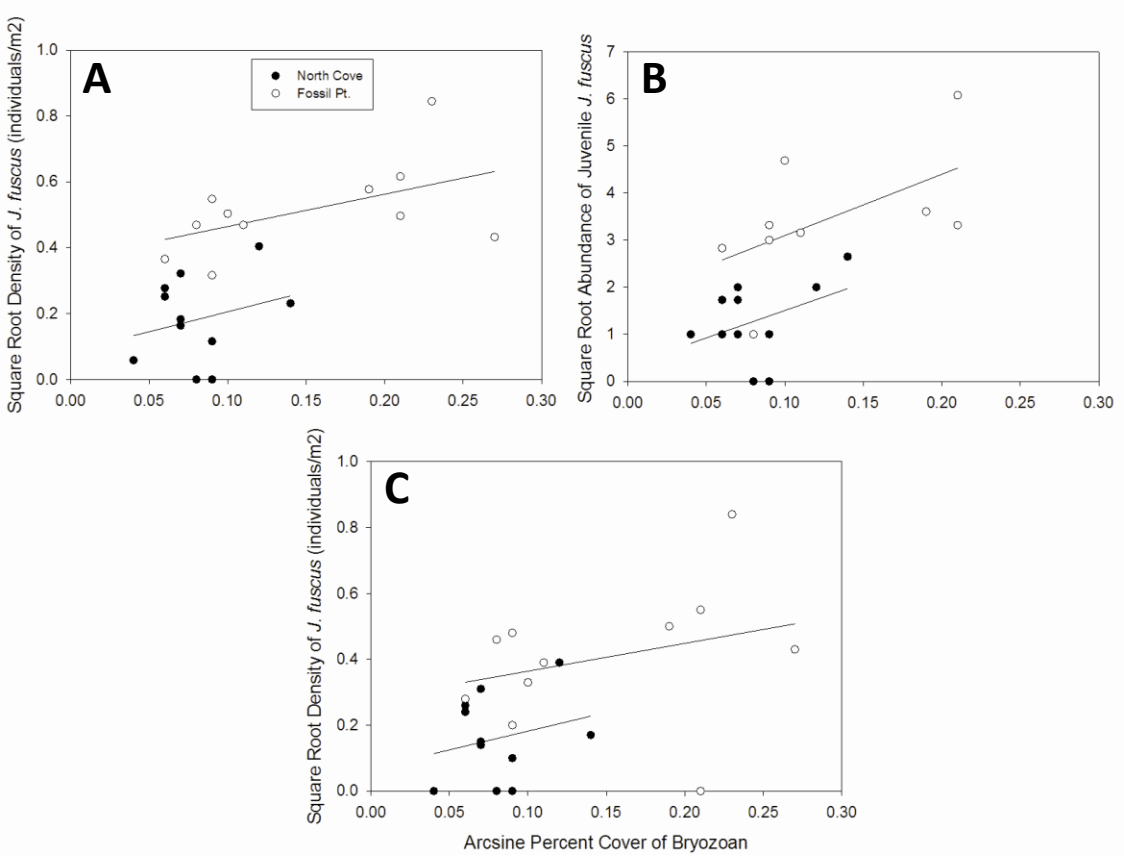


Fig. 1.10: Regression of the percent cover of bryozoan by (A) total density of *J. fuscus* (new recruits + adults); (B) abundance of new recruits (≤10 mm); and (C) density of adult *J. fuscus* (i.e. >10 mm) during bimonthly surveys between April 2008 and December 2009. The datum point for new recruits at Fossil Pt. April 2008 was removed as an outlier (see text for more details).

Abiotic factors

Density of *Janolus fuscus* decreased significantly with increasing average maximum wave height one month prior to collection at both the exposed (North Cove)

and protected site (Fossil Pt.) (Table 1.4, Fig. 1.11A, B). Wave height in the month prior to collection explained ~65% and 37% of the variation in the density of *J. fuscus* at North Cove and Fossil Pt., respectively (Table 1.4). No *J. fuscus* were found at North Cove (exposed) and Fossil Pt. (protected) when average max wave heights (at the offshore buoy) were higher than 4.3 meters and 4.6 meters, respectively (Fig. 1.11A, B). Residuals of the density of *J. fuscus* (from the previous analyses) increased with bryozoans cover, significantly at Fossil Pt. but not at North Cove (Table 1.5, Fig. 1.11C, D). Bryozoan cover explained ~27% and 45% of the remaining variation in the density of *J. fuscus* at North Cove and Fossil Pt., respectively (Table 1.5). The abundance of recent recruits of *Janolus fuscus* decreased significantly with average maximum wave height at the exposed site (North Cove) but not at the protected site (Fossil Pt.) (Table 1.6, Fig. 1.12A, B). Wave height two weeks prior to collection explained ~28% and <1% of the variation in abundance of new recruits at North Cove and Fossil Pt., respectively (Table 1.6). Residuals of the density of *J. fuscus* (from the previous analyses) did not vary significantly with bryozoan cover at North Cove (r^2=0.169, F=1.218, p=0.312) or Fossil Pt. (r^2=0.026, F=0.158, p=0.705). One outlier (April 2008 at Fossil Pt.) was removed from the analysis of variation in new recruit abundance by site and percent cover of bryozoan as no recruits were found during this month. This is surprising as numerous recruits were found both in April 2007 and 2009 as well as three weeks earlier in March 2008 at Fossil Pt. Additionally, in a regression analyses of juvenile abundance and percent cover of bryozoans at Fossil Pt., the leverage coefficient of the April 2008 datum point (0.458) compared to those of all other collection data points (0.022-0.093), suggest this datum point strongly skews the regression line.

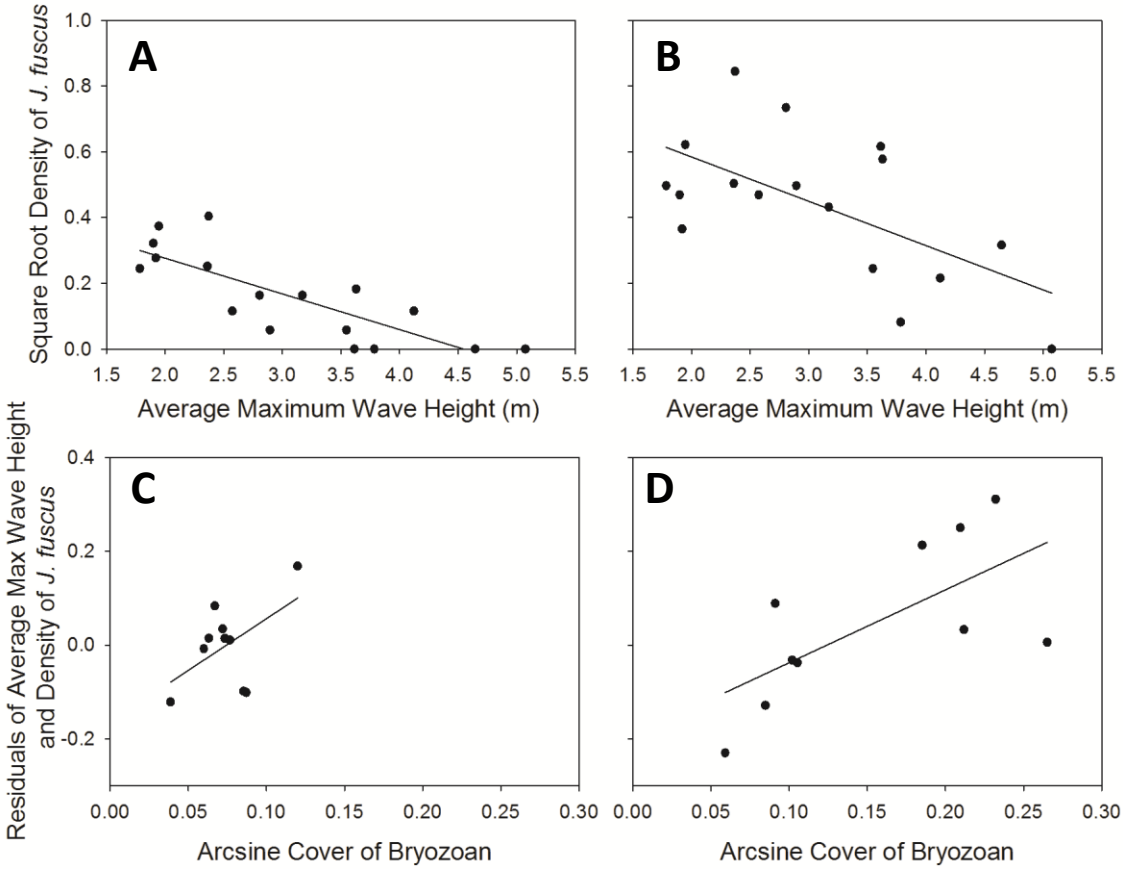


Fig. 1.11: Regression of the total density of *Janolus fuscus* by the average maximum daily wave height one month prior to the month of collection of *J. fuscus* at (A) North Cove (exposed site); and (B) Fossil Pt. (protected site) from February 2007-December 2009 (n=17). Wave height data were not collected by the Port Orford Station 46015 during October 2008. The residual variation in total slug density (after the removal of variation explained by the average maximum daily wave height) by bryozoan cover at (C) North Cove; and (D) Fossil Pt. from April 2008-December 2009 (n=11). Total slug density was square root transformed and percent cover of bryozoan arcsine transformed to meet GLM assumptions of normality and homogeneous variance.

Table 1.4: Results of regression analyses examining the effect of maximum daily wave height one month prior to collection on density of *Janolus fuscus* at North Cove (exposed site) and Fossil Pt. (protected site) from February 2007-December 2009. Total slug density was square root transformed to meet GLM assumptions of normality and homogeneous variance.

	North Cove (Exposed)						Fossil Pt. (Protected)					
Factor	SS	df	MSE	F	p	r^2	SS	df	MSE	F	p	r^2
Av. Max Wave Height	0.187	1	0.187	27.754	<0.0001	0.649	0.289	1	0.289	8.883	0.009	0.372
Error	0.101	15	0.009				0.488	15	0.033			

Table 1.5: Results of regression analyses examining the relationship between bryozoan cover and the residuals of the regression line describing total slug density by average maximum daily wave height one month prior to the month of collection of *J. fuscus* at North Cove (exposed site) and Fossil Pt. (protected site) (see above analysis) from April 2008-December 2009. Bryozoan cover was arcsine transformed to meet GLM assumptions of normality and homogeneous variance.

	North Cove (Exposed)						Fossil Pt. (Protected)					
Factor	SS	df	MSE	F	P	r^2	SS	df	MSE	F	p	r^2
Bryozoan Cover	0.020	1	0.020	3.023	0.120	0.274	0.118	1	0.118	6.484	0.034	0.448
Error	0.052	8	0.006				0.145	8	0.018			

Table 1.6: Results of regression analyses examining the effect of average maximum daily wave height two weeks prior to the month of collection on abundance of *Janolus fuscus* recruits (<10 mm) at North Cove (exposed site) and Fossil Pt. (protected site) from February 2007-December 2009. Average maximum wave height was used as a proxy for storm effect. Abundance of recruits was square root transformed to meet GLM assumptions of normality and homogeneous variance.

	North Cove (Exposed)						Fossil Pt. (Protected)					
Factor	SS	df	MSE	F	P	r^2	SS	df	MSE	F	p	r^2
Av. Max Wave Height	2.265	1	2.265	5.153	0.041	0.284	3.559	1	3.559	1.246	0.285	0.087
Error	5.715	13	0.440				37.139	13	2.857			

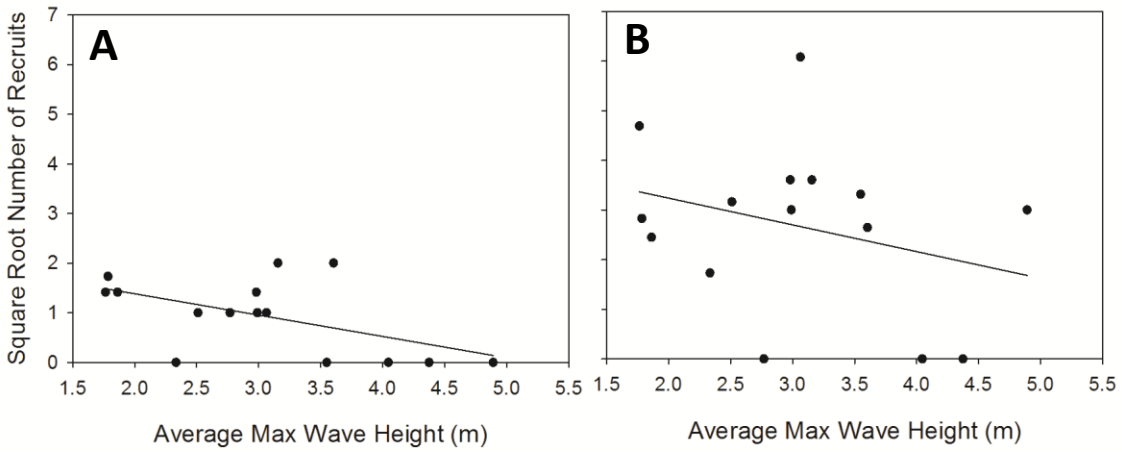


Fig. 1.12: Variation in abundance of new recruits of *Janolus fuscus* due to the average maximum daily wave height two weeks prior to the date of collection at (A) North Cove (exposed site); and (B) Fossil Pt. (protected site) from February 2007-December 2009 (n=15). Wave height data were not collected by the Port Orford Station 46015 in the two week interval prior to collections in June, August, and October 2008.

Informal surveys

Over half of the slugs (n=20) from the December 11, 2008 (average length 17.75 mm) survey collection at Fossil Pt. survived in the lab past December 31, 2008 and many survived into March 2009. No adult *Janolus fuscus* were found at Fossil Pt., North Cove, or the Charleston boat docks during January surveys (January 6-12, 2009). No recruits were found on *Bugula pacifica* collected from Charleston Docks on December 31st, but six (1.37-3.172 mm) were found a week later (January 6, 2009) on another batch of bryozoans. Six, large *J. fuscus* (36.3-50.0 mm) were found at the Charleston Docks ~50 days later on February 25, 2009. At Fossil Pt., on January 12, 2009 three recruits (1.5 - 2.35 mm) were found on *B. pacifica*. However, no adults (but >30 recruits from 0.556 – 6.15 mm) were found a month later during the regular survey on February 11, 2009. In contrast, a similar survey at North Cove on January 9, 2009 found no newly recruited *J.*

fuscus on *T. circumternata.* One recruit (but no adults) was found on a small colony of *B. pacifica* during the regular February 2009 survey.

Discussion

Intertidal surveys were conducted to determine whether *Janolus fuscus* has a subannual, annual, or biennial life cycle. *Janolus fuscus* has a subannual life cycle. They exhibit continuous recruitment throughout the year, rapid growth from a juvenile population in the winter to adult populations by early spring, and have multiple overlapping generations, all characteristics of subannual nudibranchs (Miller 1961, 1962; Potts 1970; Clark 1975; Todd 1981). Furthermore, *J. fuscus* reared in the lab grow rapidly and have an approximate lifespan of 5 months with an egg to egg generation time of ~3.5 months suggesting a subannual life cycle (chapter III). However, most subannual species are small aeolids that feed on ephemeral prey such as hydroids and occur for only a few months each year (Swennen 1961; Miller 1962; Thompson 1964; Nybakken 1974, 1978; Clark 1975, Todd 1981). In contrast, *J. fuscus* grow to a large size (>50 mm) and feed on prey that are more stable (Grave 1930; Clark 1975). While both *Bugula pacifica* and *Tricellaria circumternata* show seasonal peaks, they do not disappear entirely from either site. Moreover, during winter and spring when *T. circumternata* is less abundant at North Cove, *B. pacifica* occur. At Fossil Pt., when *B. pacifica* are less abundant in fall, *Scruopocellaria diegensis* can serve as an alternate food source. This more stable community of arborescent bryozoans may allow *J. fuscus* to persist for a greater portion of the year and grow larger than most subannual species.

Studies examining the demography of opisthobranch populations often find a positive correlation with prey abundance (e.g., Miller 1961; Potts 1970; Lambert 1991; Knowlton and Highsmith 2000; Ito *et al*. 2006). Prey abundance may influence

recruitment patterns as a direct effect of food supply and as preferred substrata for settlement an metamorphosis of veliger larvae (Thompson 1958; Harris 1975; Hadfield 1977; Harrigan and Alkon 1978; Todd 1981; Avila 1998). For example, Potts (1970) found the abundance of the dorid nudibranch, *Onchidoris fusca*, to closely correspond to the abundance of its barnacle prey, *Balanus balanoides.* Likewise, the composition and percent cover of bryozoan prey has a significant effect on the density of *Janolus fuscus*. Overall slug density and recent recruit abundance increases significantly with bryozoan cover at both sites. North Cove has consistently lower bryozoan cover than Fossil Pt, perhaps explaining lower slug densities at the former site. Also, bryozoan composition differs between the two sites, with *Bugula pacifica* dominating the bryozoan community at Fossil Pt. and *Tricellaria circumternata* dominating at North Cove. Peak densities of *J. fuscus* in spring at Fossil Pt. and summer at North Cove correspond to peaks in abundance of *B. pacifica* and *T. circumternata*, respectively.

Additionally, recruits of *Janolus fuscus* are only found on *Bugula pacifica*, suggesting this prey species may be an important settlement substratum/cue for the larvae of *J. fuscus.* Most studies examining nudibranch settlement suggest that veliger larvae preferentially settle in response to chemical cues of their prey (e.g., Thompson 1958; Harris 1975; Hadfield 1977; Harrigan and Alkon 1978; Avila 1998). *Janolus fuscus* reared in the lab settle and metamorphose on *B. pacifica*, though I had too few larvae to test *Tricellaria circumternata* and *Scrupocellaria diegensis* as potential sources of settlement cues (chapter III). *Bugula pacifica* is most abundant in winter and spring at Fossil Pt. However *B. pacifica* is much less abundant and present only as small young colonies in winter at North Cove, before returning in spring. If *B. pacifica* acts as an

important settlement cue, differences in their abundance and timing of return could explain the lower number of slug recruits and the later appearance and lower density of adults in North Cove compared to Fossil Pt.

Adult *Janolus fuscus* are absent from both North Cove and Fossil Pt. in the winter. There are at least five possible explanations for this absence: 1) prey abundance is too low in winter to sustain the populations 2) *J. fuscus* experience complete post-spawning mortality in late fall/early winter 3) *J. fuscus* do not recruit in late fall and winter 4) *J. fuscus* populations complete a subtidal migration in winter and return migration to the intertidal in early spring 5) winter storms may remove the adult populations and/or prevent new recruits from establishing and growing into adult populations. The absence of adult *J. fuscus* in winter at North Cove and Fossil Pt. is probably not due to low abundance of bryozoan prey. At North Cove bryozoan cover is low in early winter with only small colonies of *Bugula pacifica* and *Tricellaria circumternata*. However, adults are absent in the population by November and December when *T. circumternata* is still abundant at this site. At Fossil Pt., *B. pacifica* is most abundant in winter and spring. Thus, a lack of food is unlikely to be the reason for the winter absence of adult *J. fuscus* at Fossil Pt. or the reason for their initial disappearance in late fall at North Cove.

Numerous studies suggest post-spawning mortality as the explanation for the sudden disappearance of most adult nudibranchs at a particular time of year (Comfort 1957; Miller 1962; Clark 1975; Nybakken 1978; Todd 1981). However, these studies are typically of annual nudibranch species that develop and spawn synchronously and die as a collective adult population (Comfort 1957; Miller 1962; Clark 1975; Nybakken 1978;

Todd 1981). In the lab, adult *J. fuscus* mate and lay egg masses during all seasons of the year. They do die following extensive egg mass laying, which could lead to adult absence in the populations at the end of the year (pers. obs). However, this species has overlapping generations and does not develop synchronously as a population. New recruits, juveniles, and adults can be found as late as December and numerous slugs survive over winter in the lab. As new recruits of *J. fuscus* typically survive for over two months before dying in the lab (chapter III), I would expect new recruits and juveniles to survive and develop into adults by January and February at intertidal sites.

Our formal and informal surveys suggest that recruitment is continuous throughout the year. New recruits are found as late as December and as early as January in some years (at Fossil Pt. and the Charleston docks), suggesting that recruitment is not limiting the population in winter at these two sites. At North Cove, the scarcity of *B. pacifica* as a suitable substratum may limit the number of recruits in early winter. Yet at Fossil Pt., where *B. pacifica* is abundant in early winter, adults do not appear in February after recruits are found in January. In contrast, large adult *J. fuscus* can be found in February on the Charleston docks, suggesting new recruits from January are surviving to adulthood in this artificial subtidal site or that the docks provide a refuge for overwintering *J. fuscus*. Therefore some other factor may be causing adult absence in the intertidal populations.

I cannot confirm or deny that *Janolus fuscus* migrates synchronously to the subtidal zone in winter. Some studies do suggest that storms and increased currents and wave action may facilitate offshore movement of nudibranchs (Crozier 1917; Chambers 1934; Costello 1938). If *J. fuscus* are swept via winter storms into suitable local subtidal

habitats with appropriate bryozoan prey, it may be possible for a subset of the population to survive the winter. However, I do not see evidence of a synchronous rapid return migration of an overwintering subtidal population to our intertidal sites. The appearance of large adult *J. fuscus* in April at North Cove and Fossil Pt. is not sudden. Recruits of *J. fuscus* are found in February and March at both sites. Development and growth studies of *J. fuscus* reared in the lab and collected as recruits from the field show this nudibranch can grow rapidly, from a 2 mm juvenile to a sexually mature (~19 mm) individual in 25 days or to a large adult (~50 mm) in 63 days (chapter III). Such rapid growth probably accounts for the 'appearance' of adults one to two months after recruits appear in the intertidal. Rapid growth has been reported in other subannual nudibranchs as well, though most species reach smaller maximum sizes (Thompson 1964; Clark 1975; Eyster 1981). This study suggests that *J. fuscus* establish in intertidal populations by means of larval recruitment and rapid juvenile growth, as suggested for other nudibranch species (Miller 1962, Thompson 1964; Potts 1970; Clark 1975; Nybakken 1978).

At both the exposed (North Cove) and the protected (Fossil Pt.) site, winter storms may have a negative effect on the density of the intertidal slug populations, potentially explaining their winter absence. Density of *Janolus fuscus* decrease significantly with average maximum weight height at both sites. Sixty-five percent of the variation in slug density at the exposed site (North Cove) is explained by wave height compared to only 37% at the protected site (Fossil Pt.). As North Cove is more exposed on the coastline and Fossil Pt. found within Coos Bay one may expect the former to experience more direct wave action. Additionally, the abundance of new recruits decrease significantly with wave height at this exposed site, but not at the protected site.

This suggests that wave action (or storms in general) may have a more substantial negative affect and greater disturbance potential on the density of the overall population of *J. fuscus* at this exposed site (North Cove) compared to the protected site (Fossil Pt.). Winter storms are the most likely explanation (of the list above) for the absence of *J. fuscus* in winter at both sites.

While wave dislodgement of opisthobranchs has rarely been observed (Potts 1970) numerous studies imply that high wave action may decrease the abundance and diversity of intertidal slugs (Crozier 1917, Nybakken 1978, DeFreese 1987). Nybakken (1978) found that the abundance and diversity of intertidal nudibranch assemblages from Pacific Grove, CA were negatively correlated with wave action. Crozier (1917) found that following severe storms, the dorid nudibranch, *Chromodoris zebra* could only be found in deeper waters. Additionally, Trowbridge (1992) found significantly fewer recruits of the sacoglossan *Placida dendritica* from a wave-exposed site compared to a wave-protected site along the Oregon coast.

Abiotic factors such as winter storms may also indirectly affect *Janolus fuscus* populations by influencing the species composition or abundance of their bryozoan prey at different sites. *Bugula pacifica* is more abundant in winter and spring at Fossil Pt. (protected) than North Cove (exposed). This bryozoan is only present as small colonies at the exposed site in the winter when mature colonies are most abundant at the protected site. Grave (1930) found that a congener, *B. flabellata*, from Woods Hole, Massachusetts does not survive if transplanted into sites with strong currents. Large mature colonies of *B. flabellata* do not survive the winter, especially when they are in exposed sites where waves "may tear them to pieces" (Grave 1930). Instead, *B. flabellata* overwinter as more

resistant young colonies that have less surface area exposed to wave action, before resuming growth in the spring (Grave 1930). If *B. pacifica* is more sensitive to wave action associated with winter storms, this may explain its protected position on the undercut surfaces and leeward sides of rocks, its lower overall abundance, and its presence as small colonies in winter months at my exposed site. In turn, these fluctuations in prey may explain the later arrival of recruits and the lower abundance of both new recruits and adults at North Cove in spring. While nothing is known of the response of *Tricellaria* species to wave action, I find colonies attached to algae on more exposed rock surfaces, suggesting they may have greater tolerance for currents or wave action.

The larval period of *Janolus fuscus* is long enough that they may overwinter in the plankton, regardless of the reason for adult absence in the intertidal populations during this time. Planktotrophic veliger larvae of *Janolus fuscus* hatch from egg masses after 13 days and spend ~50 days in the plankton (chapter III). Sixty-three days (the combined embryonic and larval period) is sufficient for egg masses laid by adults in late November or early December to hatch and overwinter as veliger larvae that return to intertidal sites as new recruits by February, bridging the gap of adult absence. Sites that support adult populations into November and December (e.g., Fossil Pt., Charleston docks) may serve as larval source populations for new recruits arriving in winter at all sites. In contrast, adult *J. fuscus* are typically absent from November to March at North Cove, a period exceeding their larval duration. Thus new recruits settling in February probably originate outside of the intertidal North Cove population. However, many nudibranch larvae have the ability to delay metamorphosis for extended periods of time under unfavorable

conditions, such as periods of low food availability (reviewed in Hadfield and Switzer-Dunlap 1984). I reared larvae of *J. fuscus* in the lab at spring and summer seawater temperatures (11-13°C), whereas average sea surface temperatures from December through February range from 8.2-9.8°C depending on the month and year. Because developmental rates are inversely related to temperature (reviewed in Strathmann 1987), larval duration may be greater during winter months.

Density of *Janolus fuscus* did not differ by year, but the within year variation due to seasonal fluctuations makes it difficult to see differences between years. Density of slugs was much lower at both sites during 2009 than in previous years. Peaks in bryozoan cover were lower in 2009, perhaps explaining part of this decline in the nudibranch population. Keough and Chernoff (1987) observed large variation in the abundance of a congener, *B. neritina*, within and among years. This species experienced local extinction after severe storm disturbance when occurring as an epiphyte. *Bugula neritina* may occur at low abundance and experience local extinction because of their short-lived lecithotrophic larvae and low recolonization potential (Keough and Chernoff 1987). While either low bryozoan cover or storm effects could explain low densities during 2009, my repeated sampling of these populations from 2005-2008, may be a contributing factor. While large *J. fuscus* die naturally after several spawning events and rarely reach sizes above 50 mm in the lab (chapter III) losses due to collection could have caused an artificial decrease in density and average length in these populations. In an attempt to mitigate such impacts on the population, slugs were returned to their respective populations if not needed for experiments, and a limited volume of bryozoans were only collected from survey sites to look for new recruits. However, the influence of my study

cannot be eliminated as a possible factor in altering the demography of North Cove and Fossil Pt. populations of *J. fuscus*.

Differences in the density of *Janolus fuscus* between sites and among seasons are likely due to a combination of biotic and abiotic influences acting on these slug populations. Prey abundance and composition may explain site differences and seasonal peaks in density of *J. fuscus* at both sites, but it cannot explain adult slug absence in the winter. Additionally, recruitment does not appear to be limiting during the winter. While synchronous post-spawning mortality and mass migrations have been suggested as explanations for the sudden disappearance and reappearance of nudibranch populations in some species, these appear unlikely for *Janolus fuscus*. Winter storms seem the most likely explanation for the absence of *J. fuscus* from intertidal sites in winter.

Conclusion

Janolus fuscus is a subannual nudibranch with continuous recruitment, exhibiting rapid growth and overlapping generations. Density of *J. fuscus* differed between sites, with greater densities at the protected site that harbors greater cover of the bryozoan than an exposed site with lower bryozoan density. Seasonal peaks in nudibranch density were correlated with peaks in bryozoan abundance. Seasonal abundance of *B. pacifica* at both sites may impact the abundance and timing of larval recruitment as this bryozoans may be a preferred settlement substratum. The absence of adults at both sites over winter may be attributed to winter storms. Fewer *B. pacifica*, fewer recruits, and greater wave exposure might explain the longer period of adult absence every year at North Cove. This study suggests that *J. fuscus* establish in intertidal populations via larval recruitment

and rapid juvenile growth, not as the result of a return migration of an overwintering subtidal population.

Bridge I

Field surveys of populations of *Janolus fuscus* suggest this nudibranch has a subannual life cycle characterized by continunous recruitment, rapid growth and overlapping generations. Additionally, new recruits of *J. fuscus* are only found on the arborescent bryozoan *Bugula pacifica* in field collections. This suggests this prey species may induce settlement and metamorphosis in competent veligers of *J. fuscus*. In chapter III, I examine the complete development and life cycle of *J. fuscus*. By estimating the life span, I provide further evidence for determining whether *J. fuscus* is a subannual, annual, or biennial species. Competent veligers of *J. fuscus* are exposed to *B. pacifica* in the laboratory to see if this prey item induces settlement and metamorphosis of *J. fuscus* as suggested in field observations in chapter II. Also, the growth rate of juvenile and adult *J. fuscus* is measured to expand on the hypothesis that this slug grows rapidly in the field after recruiting, explaining the sudden appearance of the populations in spring and their overlapping generations throughout the year.

CHAPTER III

THE COMPLETE DEVELOPMENT OF A NORTHEAST PACIFIC ARMINACEAN NUDIBRANCH, *JANOLUS FUSCUS*

Introduction

Reproduction and development of the Opisthobranchia have been studied for more than a century (e.g., Casteel, 1904; Thompson, 1958; Todd, 1981; reviewed in Hadfield and Switzer-Dunlap, 1984), but only a few studies have addressed the entire development and growth of a single species, including details of embryonic development, larval growth, metamorphosis and post-metamorphic development (e.g., Thompson, 1958, 1966; McFarland, 1966; Perron and Turner, 1977; Chia and Koss, 1978). Such studies are important because opisthobranchs are highly variable in developmental timing, embryonic periods, developmental mode, larval duration, metamorphic inducers, and growth rate (reviewed in Todd, 1981, 1983; Hadfield and Switzer-Dunlap, 1984). Only two studies, to my knowledge, have thoroughly addressed multiple aspects of reproductive and developmental biology in species of the nudibranch superfamily Arminoidea (Eyster, 1981; Page, 2007).

Over a century of study on opisthobranch reproduction and development has revealed some common trends. Opisthobranch egg masses are typically laid on rocks or prey in the field, and have been classified into four characteristic types (Hurst, 1967).

Opisthobranch embryology involves spiral, typically holoblastic, cleavage which is highly conserved within annelids, molluscs, and nemerteans (Wilson, 1892; Casteel, 1904; Thompson, 1958; Maslakova et al., 2004a, b; Nielson, 2004). Gastrulation occurs by emboly, epiboly, or some combination of the two (Casteel, 1904; Thompson, 1958; Gohar and Soliman, 1967a, b; Soliman, 1978). Embryos pass through a preveliger (trochophore-like stage) into a veliger stage within the egg capsule before hatching as lecithotrophic (non-feeding) larvae with a short obligate planktonic period, planktotrophic (feeding) larvae with a longer planktonic period, or as a crawl-away juvenile (direct development) that progresses through all stages within the egg mass (Thompson, 1967; Clark and Jensen, 1981; Todd, 1981, 1983; Todd and Doyle, 1981; Hadfield and Miller, 1987).

The size of embryos and number per egg mass and size of egg capsules, as well as shell-type have been used as diagnostic features that characterize the three major modes of development in most opisthobranchs (described above). Thus, opisthobranchs with planktotrophic larvae typically have smaller embryos or capsules and more embryos per capsule and egg mass than those species with lecithotrophic or direct development (Thompson, 1967; Clark and Jensen, 1981; Todd, 1981, 1983; Todd and Doyle, 1981; Hadfield and Miller, 1987; Soliman, 1991; Goddard, 1992, 2004; Jensen, 2001). Species with planktotrophic veligers often have a Type 1 protoconch (i.e. larval shell) with ¾ to 1 whorl, as described by Thompson (1961a), as this shell type permits shell and somatic growth during the pelagic phase (Todd, 1981).

Larval period varies widely among opisthobranch species with planktotrophic larvae, ranging from a few days to months with indiscernible to substantial (i.e., >200μm)

protoconch growth (Hadfield and Switzer-Dunlap, 1984). Larval growth cesses as veligers become competent to metamorphose (Perron and Turner, 1977; Chia and Koss, 1978; Harrigan and Alkon, 1978; Todd, 1981; Trowbridge, 2000). Shell growth ceases with retraction of the mantle fold from the shell aperture at the onset of metamorphic competence. Other morphological signs of competence include the appearance of eyespots and the development of a propodium on the enlarged metapodium (Perron and Turner, 1977; Chia and Koss, 1978; Harrigan and Alkon, 1978; Todd, 1981). Numerous studies have found that chemical cues from conspecifics and adult prey items induce settlement and metamorphosis in competent opisthobranch veligers (e.g., Hadfield, 1977; Harrigan and Alkon, 1978; Hadfield and Pennington, 1990; Lambert et al., 1994; Avila et al., 1997; Trowbridge and Todd, 2001).

Juvenile opisthobranchs can grow rapidly (e.g., growth rates >7% in length per day) to reproductive maturity, whereupon they mate, deposit egg masses repeatedly, then die, typically after one reproductive season (Eyster, 1981; Todd, 1981; Hadfield and Switzer-Dunlap, 1984). While most nudibranchs are annual species, species with subannual and biennial life cycles are also known (reviewed Todd, 1981). To characterize a nudibranch species as subannual, annual or biennial requires information on development and growth as well as demography of natural populations of nudibranch species.

Reproduction and development has been understudied in arminacean nudibranchs and mostly restricted to notes on egg mass characteristics, from which developmental modes have been surmised. The only study of embryonic development in an arminacean nudibranch (*Madrella sanguine*) reports a novel form of embryogenesis never seen in

other nudibranchs (Page, 2007). In her study, Page found that fertilized eggs of *M. sanguinea* contain a large mass of red-pigmented cytoplasm that is extruded as a type of polar lobe and later detached from the gastrula stage, remaining as a "red body" within the egg capsule. Despite this odd beginning, *M. sanguinea* undergo typical planktotrophic development before metamorphosing on its bryozoan prey (Page, 2007). Eyster (1981) made observations on metamorphosis and growth in the subannual, lecithotroph *Armina tigrina*. A notable comparison of growth rates in immature and mature *A. tigrina* showed that linear growth decreases as slugs devote more energy to reproduction (Eyster, 1981). More studies are needed that address the complete development of arminacean nudibranchs.

The arminacean nudibranch *Janolus fuscus* is found from Klu Bay, Alaska to the Gulf of California and is conspicuous from April to October in the boulder fields of Oregon, where they feed exclusively on arborescent bryozoans such as *Bugula pacifica*, *Tricellaria circumternata*, and *Scrupocellaria diegensis* (Sphon, 1972; Belcik, 1975; McDonald and Nybakken, 1980; Goddard, 1984, 1992, 1998; chapter II). In a thorough review of the developmental patterns of NE Pacific nudibranchs, Goddard (1984, 1992) gives background data on development of *J. fuscus*. He found that capsules contain 50-60 embryos with diameters of ~81 µm (Goddard, 1984; 1992). His study suggests that planktotrophic veligers (~138 µm) hatch after an embryonic period of 14-16 days (at 11-13°C) with a Type 1 shell and eyespots already present (Goddard, 1984; 1992). In this study I examine egg mass characteristics, embryonic timing, larval growth and duration, larval settlement and metamorphosis as well as juvenile growth through adulthood for *Janolus fuscus* from the northeast Pacific.

Materials and Methods

Site and collection

Janolus fuscus specimens were collected from two sites, North Cove (43°18'30.6N, 124°23'58.92W) and a site inside Coos Bay (43°21'32.4N, 124°18'45.36W) between Pigeon and Fossil Point, Charleston, OR, USA, during February and August 2007, April 2009, and July 2010. The unnamed site is referred to as Fossil Pt. throughout the text for convenience. Individual slugs were collected by hand and placed in separate 50-ml Falcon tubes to prevent mating and returned to the Oregon Institute of Marine Biology (OIMB), Charleston, OR, which is less than 10 km from each study site. Slugs were held in individual plastic containers (10.5x10.5x12.5 cm) with window screen mesh sides submerged in flowing seawater tables, where they were fed arborescent bryozoans *Bugula pacifica*, *Tricellaria circumternata*, and *Scrupocellaria diegensis* to satiation. Slug length was measured with calipers as slugs crawled upside down on the water surface.

Egg masses and fecundity

Fourteen slugs were paired with mates of similar size (<5 mm difference) and allowed to mate for 24 hours. Egg masses produced subsequently by each slug were collected every other day for 21 days (August 3-23, 2007) and held in coarse-filtered (5 μm) seawater changed every other day, in test tubes kept cool in seawater tables for 7 days, until veligers were visible within the egg capsules. At this time all egg masses were broken apart to free the individual capsules, then preserved in 10% formalin in seawater buffered with sodium borate to conserve shell structure. The total number of capsules in

each egg mass was determined and the number of viable embryos in 10 haphazardly chosen capsules was counted. The total number of viable embryos in each egg mass was extrapolated from the average of these 10 sub-samples.

Embryology

In April 2009 and July 2010, egg masses were collected immediately after they were laid in the lab and kept in 150-ml culture dishes in 0.45 μm filtered seawater at 11-13°C. Water was changed every other day. Egg masses (n=28) from 14 different freshly collected *J. fuscus* adults were examined in this study. Developmental stage was recorded roughly every two to five hours for the first 24 hours, then twice daily until hatching. Only early embryonic development was examined in July 2010. Embryonic development was documented using light, scanning electron, and confocal micrographs. A developmental timeline was compiled using the average time between cleavage stages or later developmental markers such as gastrulation. Developing embryos were examined and photographed using a light Olympus BX50 compound microscope (40x) and individuals were preserved for scanning electron and confocal microscopy. Embryos for SEM were preserved using 2% osmium tetroxide followed by 0.45 μm filtered seawater and freshwater rinses, dehydrated through a graded alcohol series, and critical point dried before viewing with a Tescan Vega II SEM. Embryos for confocal microscopy were fixed in 4% paraformaldehyde at room temperature, washed in 1X Phosphate Buffer Solution (PBS), then stained with Rhodamine-phalloidin (5U/ml) and propidium iodide (10μg/ml) in Phosphate Buffer Solution with Tris (PBT) to illuminate cell boundaries and nuclei, respectively (von Dassow, 2004; Maslakova et al., 2004b). Before viewing with an Olympus Fluoview FV1000 Confocal Microscope System, semi-

opaque embryos were mounted on a slide and cleared using 'Murray Clear' (2:1 mixture of benzyl benzoate to benzyl alcohol) (von Dassow, 2004).

Larval growth, competence, settlement, and metamorphosis

Once hatched, eleven batches of veligers from eight different slugs were cultured at a density of 1 larva/ml in 0.45 µm Millipore filtered seawater in 1-L glass jars. Two cultures of *Janolus fuscus* were kept stationary in a flow-through seawater tank at 11-13°C. Nine cultures were filled to the top of the jar, sealed without air bubbles with parafilm (to prevent larvae getting trapped in the surface tension) and lids. Jars were attached with large rubber bands to an adjustable-rate 16-position plankton grazing drum similar to those used in studies of invertebrate larval settlement and plankton predation rates (Checkley, 1980; Dagg and Grill, 1980; Landry, 1980; Gifford et al., 1981; Rumrill et al., 1985; Young, 1989). Jars were attached to the drum on their side, with their long axes parallel to the axis of rotation. Cultures were rotated at 2 rpm in a cold room at 11-13°C. Rotation prevents larvae from settling to the bottom of the culture dish where they are more likely to clump and/or contact bacteria and ciliates. All cultures were cleaned by reverse filtration and fed 10,000 cells/ml of 1:1 flagellates *Rhodomonas lens* and *Isochrysis galbana* every three days (Harrigan and Alkon 1978, Strathmann, 1987; Avila et al., 1997). Larval length (average of length of long axis of larval shell of two to eight veligers) was measured every 3 to 7 days for each larval culture. Measuring larval shell length involves using methyl cellulose and/or gently pressing the larva between the slide and coverslip to prevent movement. As this process decreases viability, fewer veligers were measured as cultures aged and progressively fewer individuals survived in each. As larval cultures declined (<50 larvae), larvae were switched to 150 ml culture dishes and

kept stationary in a flow-through sea water table at 11-13°C. As veligers of *J. fuscus* showed signs of competence (i.e., halted shell growth, mantle retraction from the shell, and development of a propodium), they were incubated with small colonies of the arborescent bryozoan *Bugula pacifica*, their preferred prey. Bryozoans were rinsed in filtered seawater before adding them to larval cultures to minimize contamination of the culture. Additionally I attempted to induce (n=10) competent veligers to metamorphose using 20 mM KCl in filtered seawater (Hubbard, 1988; Avila et al., 1997). Elevated concentrations of monovalent cations like K^+ in seawater probably induce settlement by affecting the electrical potential at or downstream from primary chemosensory cells and have proved effective for some opisthobranchs (Yool et al., 1986; Hubbard, 1988; Gibson, 1995; Pechenik et al., 1996; Avila et al., 1997; Hadfield et al., 2000).

Best-fit growth curves were determined for larval growth of four cultures of *J. fuscus* larvae that achieved competency using XLFit5 best curve fit design in Microsoft Excel 2007.

Juvenile and adult growth

Recruits of *Janolus fuscus* collected from Fossil Point in February 2007 were reared in the lab to examine growth of *J. fuscus* from juvenile to adulthood. Slugs were kept in stirred seawater in 2-liter glass containers at 11-13°C, fed to satiation, and their lengths measured once a week. Length was used as the measure of slug size as repeatedly blotting and measuring wet weights of slugs is damaging, particularly for small juveniles. Slug length can be converted to wet weight using a regression described in chapter II but this regression does not accurately convert length of very small *J. fuscus*

(1-3 mm) as these could not be weighed without sacrificing slugs. As many *J. fuscus* in this study were small recruits, I calculated growth from length measurements only.

Early recruits collected from the field were not all the same size (i.e. age) and were collected on different days, as it is uncommon to find numerous recruits at one time. To compare growth in these slugs across a common time frame, I plotted length by Julian date at which each length measurement was taken for each *J. fuscus*. I determined the date at which each slug was 10 mm in length from these plots. I assigned this date as time zero and calculated the number of days at which each length measurement was taken for each slug relative to this date (i.e., before 10 mm were negative days and after 10 mm were positive days) to give the same relative time axis (i.e., days from 10 mm). Best-fit growth curves were plotted on growth for 10 *J. fuscus* using XLFit5 curve fitting software. During the monitored growth period one individual began to shrink and senesce on the final two days in which length was measured. These last two days of measurements for this individual were removed from the data set. In two other instances slugs decreased slightly in length; these measurement periods were not removed as these slugs continued to increase in length in subsequent measurements.

Results

Egg masses

Janolus fuscus produced Type B egg masses (as described by Hurst, 1967), cylindrical capsule-filled cords, usually attached along one side by a jelly sheet to the plastic flow- through container or to their aborescent bryozoan food. Each *J. fuscus* adult produced 3 to 9 individual egg masses over a 21- day period in the lab. At about 21 days, *J. fuscus* had exhausted their sperm stores and begin laying partially or entirely

infertile egg masses. Individual egg masses contained as few as 5 to a maximum of 1087 capsules (average = 324 ± 23, n=69), containing 0 to 160 embryos per capsules (average = 66 ± 3, n=69). All error reports represent standard error. Capsules at the extreme ends of the egg masses had consistently fewer embryos than those in the middle of the egg mass. The estimated number of viable embryos (i.e., veligers) per egg mass ranged from 272 to 79,786 (average = 23,063 ± 2059, n=69).

Embryology

Janolus fuscus follows the typical holoblastic, spiral cleavage pattern conserved within annelids, molluscs, and nemerteans. Embryos are 80.9 ± 1.3 μm in diameter. Two polar bodies are extruded (at 2 and 5 hours, respectively), with the second polar body positioned directly below the first (Fig. 2.1A). Cleavage occurs about every 4 hours in the early embryonic stages (Fig. 2.1B-G, Table 2.1). The first two cleavages are equal and the third unequal, producing four micromeres and four macromeres at the animal and vegetal poles, respectively (Fig. 2.1C, D, E). The fourth cleavage appears asynchronous, with the macromeres dividing first to produce a transient 12-cell stage, followed by a thirteen-cell stage as one first-quartet micromere consistently divides before the other three (Fig. 2.1E). Embryos appear flattened along the animal-vegetal axis at the 32-64 cell stage, with microvilli covering first and second quartet micromeres (Fig. 2.1F). Embryos reached the blastula stage by 48 hours. A blastocoel was not visible within the blastula, suggesting *J. fuscus* has a stereoblastula. Around the onset of gastrulation, the embryo develops a slight hump at the animal pole and trochoblast cells begin to produce patches of prototrocal cilia (Fig. 2.1I). Embryos undergo gastrulation by day three (Fig. 2.1H, I, Table 2.1). Though the process of gastrulation is unknown, SEM micrographs

suggest part of the gastrulation process may occur by epiboly, with micromeres moving to the vegetal pole and partially covering the macromeres (Fig.2.1H). The resulting blastopore starts as a wide pit, subsequently deepening into the archenteron. As gastrulation progresses, the blastopore shrinks and cellular extensions cover the blastopore (Fig. 2.1J, K). I did not witness whether the blastopore closes completely before the opening of the stomadaeum at the trochophore stage. Also during gastrulation, the blastopore and pretrochal region are ventrally displaced, situating the blastopore ventrally just below the developing prototroch. The trochophore stage has a distinct prototroch with long cilia (Fig. 2.1L). A more disparate set of cilia are oriented below and within the mouth of the trochophore, though they do not form a distinct band of cilia characteristic of a true metatroch (Fig. 2.1L). As the trochophore turns into a veliger, the prototroch expands to form the velum that resides dorsolateral to the mouth (Fig. 2.1L, M, N, O, 2.2A). At the early veliger stage the shell field has developed into a saucer-shaped shell primordial that will give rise to the Type-1 larval shell (Fig. 2.1M, N, O, P, 2.2A). Trochophores and veligers were moving within egg capsules in day four and six, respectively (Fig. 2.1L, M, N, O, P). Veligers hatch with eyespots already present (Fig. 2.1O).

Table 2.1: Developmental timeline of *Janolus fuscus.* Average times from uncleaved embryo to different developmental stages. Embryological timeline, larval period to metamorphosis and early juvenile growth from *J. fuscus* reared in the laboratory at 11-13°C during February-June, 2007, April – July, 2009 and July 2010. Growth to maturity (19 mm) and 50 mm from juveniles collected in the field and reared in the lab from February through May 2007. The estimated lifespan (to 50 mm adult) combined these two data sets and assumed the average growth rate of juveniles (2.5 - 19 mm) to be (0.667 mm/day) and mature adults to be (0.821 mm/day) as calculated from juvenile and adult growth from February to May, 2007. Asterik indicates time values from one juvenile surviving past metamorphosis (day 94), and from average growth rate of juveniles from the field (0.667 mm/day) (day 90).

Stage	**Time**
Laid Embyro	0 hr.
1st Polar Body	2 hrs
2nd Polar Body	5 hrs.
2-cell	8 hrs.
4-cell	12 hrs.
8-cell	16 hrs.
16-cell	20 hrs.
32-64	23 hrs.
Blastula	48 hrs.
Gastrula	Day 3
Trochophore	Day 4
Veliger	Day 6
Hatching	Day 13
Competence	Day 51
Metamorphosis	Day 63
2.5 mm juvenile	Day 80
9.1 mm juvenile	Day 94, 90*
Mature 19mm	Day 105
50mm adult	Day 143
Lifespan	~5 months

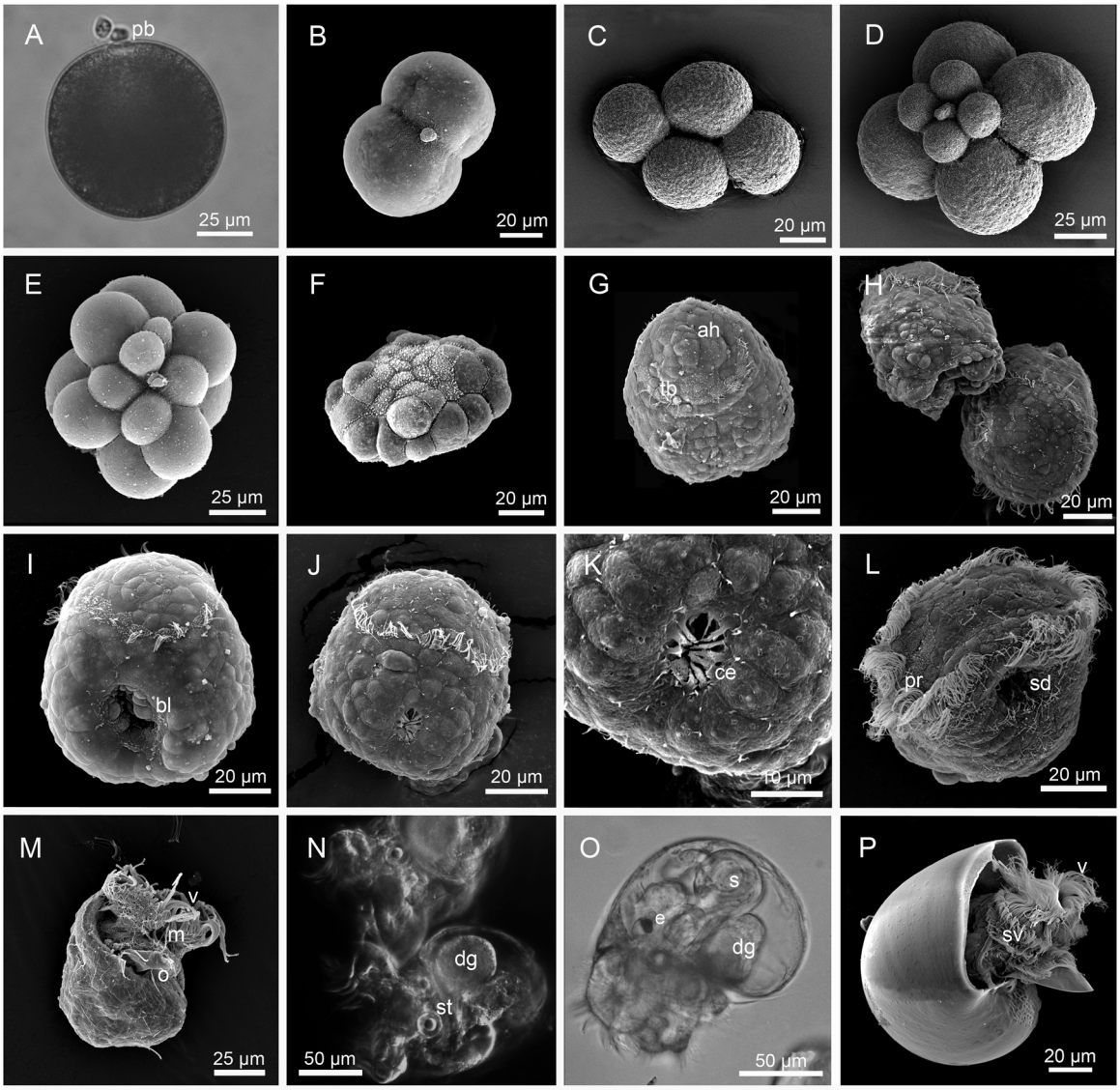


Figure 2.1: Embryonic development of *Janolus fuscus*. (A) Fertilized embryo with two polar bodies. (B) 2-cell. (C) 4-cell. (D) 8-cell. (E) 13-cell. (F) 32-64-cell with microvilli on 1st and 2nd quartet. (G) late blastula/early gastrula with apical hump and four groups of trochoblast cells. (H) early gastrulation via epiboly, prototroch present. (I) gastrula with blastopore. (J) late gastrula with cellular extensions covering blastopore. (K) same gastrula as J at higher magnification. (L) trochophore with prototroch and stomadaeum. (M) pre-hatched veliger with developing velum and operculum. (N) newly hatched veligers. (O) newly hatched veligers with eyespot. (P) newly hatched veliger. ah (apical hump), bl (blastopore), ce (cell extensions), dg (digestive gland), m (mouth), o (operculum), pb (polar body), sd (stomadaeum), st (stomach), sv (subvelum), tb (trochoblast cells with cilia), v (velum).

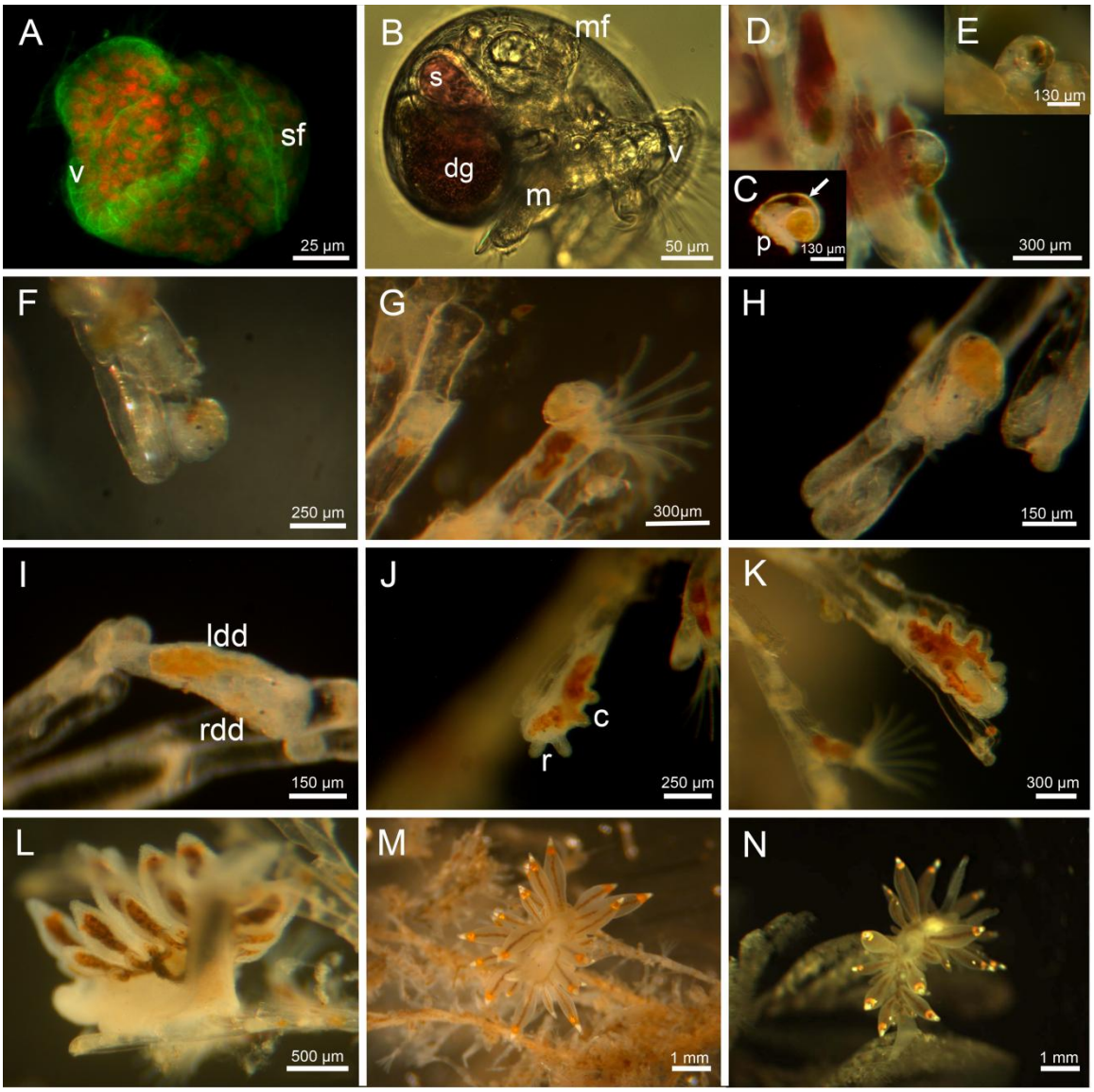


Figure 2.2: Larval development, competence, settlement, and metamorphosis in *Janolus fuscus*. (A) developing veliger stained with rhodomine phalloidin and propidium iodide. (B) pre-competent veliger with early mantle retraction from the shell aperature and enlarged metapodium with cilia. (C) competent veliger, with reduced velum, propodium, and full mantle retraction designated by the arrow. (D, E) newly settled veligers. (F) newly metamorphosed juvenile with red spot pigments and mantle bulge. (G) day 2 juvenile with extended red pigment around the mantle skirt. (H) three day old juvenile consuming lophophore of *Bugula pacifica.* (I) elongated four day old juvenile with large left and small right digestive diverticula. (J) 7 day old juvenile with developing rhinophores and cerata. (K) day 8 juvenile. (L) 17 day old juvenile with orange-tipped cerata. (M) 28 day old juvenile. (N) juvenile found on *B. pacifica* from the field. c (cerata), dg (digestive) gland, ldd (left digestive diverticula), m (metapodium), mf (mantle fold), p (propodium), r (rhinophores), rdd (right digestive diverticula), s (stomach), sf (shell field), v (velum).

Larval growth, competence, settlement, and metamorphosis

On average, veligers hatched on day 13 but time to hatching ranged from 10 to 18 days. The shell lengths of *Janolus fuscus* were 125-153.8 µm at hatching (Fig. 2.1P). Among egg masses there was a significant positive relationship between the number of days until hatching and the size of veligers released (regression analysis, $r^2 = 0.517$, F = 9.619, p = 0.013, n=11), with small veligers hatching earlier than larger veligers (Fig. 2.3). There was no significant relationship between the average length of larvae at hatching and the maximum length the larvae attained (regression analysis, $r^2 = 0.002$, F = 0.015, p = 0.906, n=11) or total days survived (regression analysis, $r^2 = 0.036$, F = 0.337, p = 0.576, n=11).

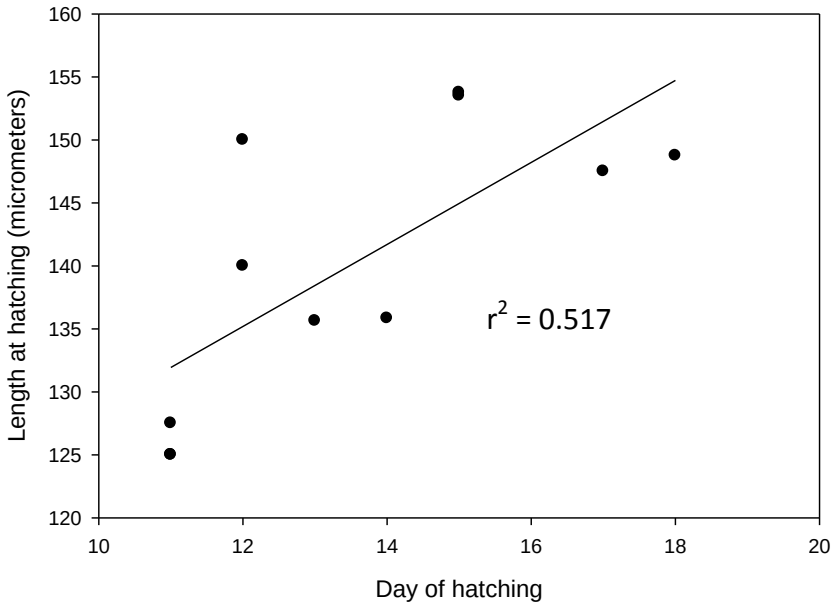


Figure 2.3: The average length of veligers of *Janolus fuscus* at hatching by the age of the egg mass.

Larvae of *Janolus fuscus* began to feed and grow immediately upon hatching. Their stomach and digestive gland became green and red as they filled with *Isochrysis galbana* and *Rhodomonas lens*, respectively. Larval growth curves vary in shape and are best fit by variations of cubic polynomial distributions (Fig. 2.4, Table 2.2). In three of four cultures of veligers that achieved competency, newly hatch veligers exhibited slower growth just after hatching, with increasing growth from 160 to 220 µm after which growth slowed and ceased as the larvae began to show early signs of competence (i.e., enlongating metapodium and slight retraction of the mantle fold) as early as 235 µm, (Fig. 2.2B, 2.4). In contrast, one larval culture experienced greater growth in newly hatched veligers (127 µm) to 160 µm, slower growth when veligers were 175 µm to 200 µm before growth increased again up to 235 µm when the few remaining larvae reached competency (Fig. 2.4D). Larvae of *J. fuscus* from cultures from four different parents (two stationary and two plankton drum cultures) reached competency (Fig. 2.2B, C). Only four veligers from each of two plankton drum cultures showed morphological signs of competency (i.e., cessation of shell growth and enlarged metapodium), on days 48 and 52 post-hatching at average lengths of 230 to 240 µm. The other seven cultures of larvae reared on the plankton drum did not survive to reach competency. No larvae reared in plankton drum cultures settled or metamorphosed.

In the two stationary cultures, veligers reached an average size of 254.0 µm and 255.5 µm, respectively and ceased growing by day 32 and 37. Large veligers (~266 µm) exhibited all signs of competence on days 36 and 41 (i.e., appearance of propodium, enlarged metapodium, and extensive retraction of the mantle fold) (Fig. 2.2C). A few

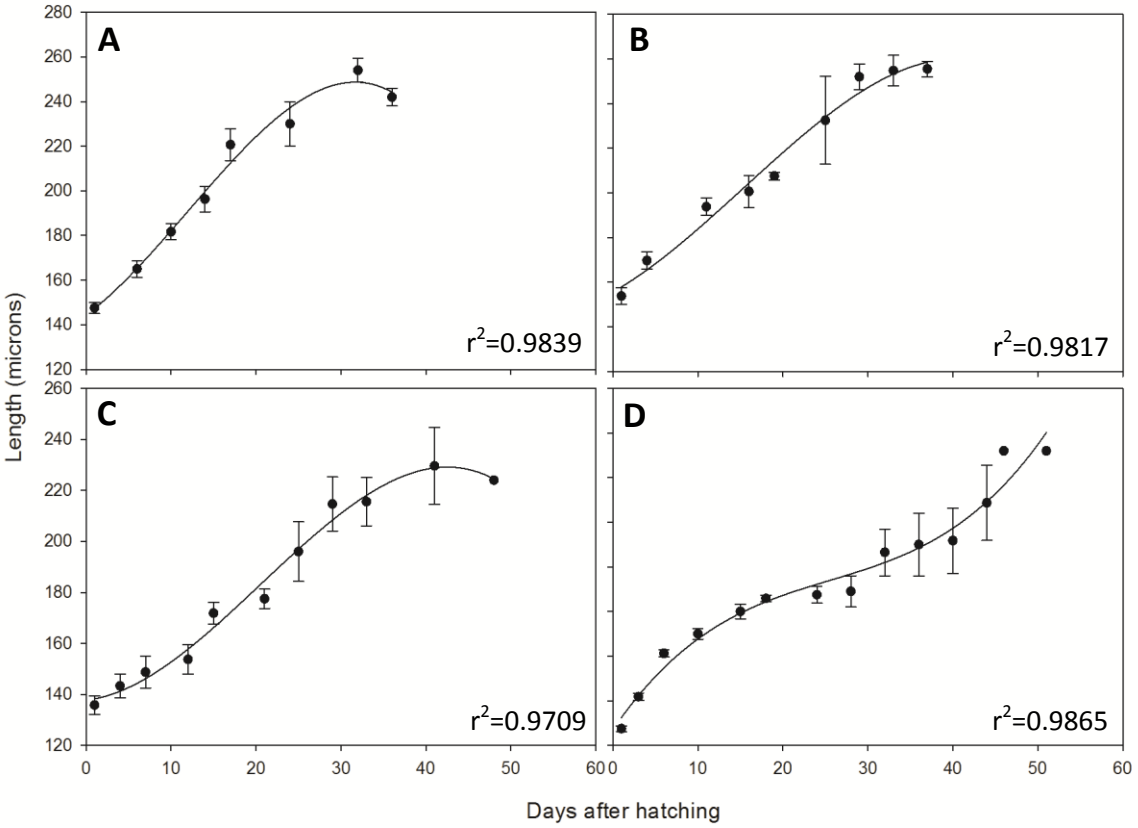


Figure 2.4: Larval growth of four cultures of *Janolus fuscus* veligers that reached competency, two stationary cultures (A) and (B) and two cultures rotated on a plankton drum (C and D). Error bars represent the standard error of 8 veligers measured from each culture, except where error bars are absent in C and D. Only one individual of the four remaining was measured in these final dates to prevent the stress of handling during the measurement process.

veligers exhibited searching behavior with intermittent swimming and crawling using their enlarged, mobile foot to feel the bottom of the culture dish (Fig. 2.2B, C). At this point cultures were small with less than 20 competent larvae per culture; 10 larvae from each of the two cultures were added to dishes with *Bugula pacifica*. Five larvae were added from each larval culture to dishes with KCl two day later to test for metamorphic competence. No larvae settled or metamorphosed in the KCl treatments. Four veligers (from one culture) incubated with *B. pacifica* settled on the bryozoan on days 40 through 51 (4 to 10 days after showing signs of competence) (Fig. 2.2D, E). Veligers initially

Table 2.2: Results of non-linear regression analyses of cubic polynomial curves applied to larval growth of four larval cultures in which veligers of *Janolus fuscus* reached morphological competence (A-D) after larval growth from April-June, 2009.

Larval Culture	Source	df	SS	MSE	F	p	r^2
A	Growth	3	344989.4	3333.7	81.27	0.0005	0.9839
	residual	4	164.1	41.0			
B	Growth	3	11187.1	3729.0	89.28	<0.0001	0.9817
	residual	5	208.84	47.8			
C	Growth	3	17252.2	5750.7	111.36	<0.0001	0.9709
	residual	10	516.4	51.6			
D	Growth	3	11904.3	3968.1	170.18	<0.0001	0.9865
	residual	7	163.2	1206.7			

crawled along the bryozoan colony, but finally settled near the distal ends of the colony and remained stationary until metamorphosis (if metamorphosis occurred) (Fig. 2.2D, E, F). Two of four settled larvae metamorphosed two and three days after settlement, on days 46 and 54 post-hatching, respectively, discarding their larval shell (Fig. 2.2F).

I made two observations regarding the behavior of *Bugula pacifica* in response to larval *Janolus fuscus*. I observed a lophophore of *B. pacifica* "capture" a competent veliger, "spit it out" and capture it a second time before the veliger was released and fell to the bottom of the culture dish. One veliger was pierced between its shell and mantle by the tip of an avicularium of *B. pacifica* as the larva crawled along the bryozoan substrate. The later veliger did not survive the attack.

In the first day after metamorphosis, juveniles were ~280 μm in length and retained a dorsal visceral bulge before tissues and organs formerly contained in the larval

shell were incorporated into the more elongate juvenile body structure around day 2 (Fig. 2.2G). Red pigment found laterally, and ventral to the eyes on both sides of competent veligers and newly metamorphosed slugs, began to extend posteriorly in a line of red dots as the juvenile slug elongated (Fig. 2.2G, H). The mantle extended over the dorsal surface of the slug and formed an obvious mantle skirt by day 2 (Fig. 2.2G). In the first two days after metamorphosis, juvenile slugs were observed repeatedly placing their mouths over the lophophores of bryozoans. This caused the lophophores to retract, but inflicted no damage. By day three, juvenile *J. fuscus* were consuming the bryozoans' lophophores (Fig. 2.2H). Only one juvenile survived past the third day following metamorphosis. This individual grew and elongated rapidly between days 3 and 4 (Fig. 2.2H, I). At this stage, the large left digestive diverticulum was visible and extended posteriorly while the small right digestive diverticulum was barely visible (Fig. 2.2I). Cerata and rhinophore primordia developed by day six. By day seven, the digestive diverticula extended the length of the slug and into the cerata (Fig. 2.2J). The distinctive orange and white pigmentation at the tips of the cerata did not develop until day 16 and 20 post-metamorphosis, respectively (Fig. 2.2L, M). This juvenile survived to a length of 9.14 mm by 31 days after metamorphosis (i.e., 94 days, post-hatching) with a growth rate of 285.2 μm per day. Growth rate between 2.46 mm to 9.14 mm was 0.557 ± 0.278 mm per day (12.9 ± 1.54% of length per day).

Juvenile and adult growth

Total growth of *Janolus fuscus* from February 2007 through April 2007 ranged from 26.84 mm to 41.62 mm. Growth per day ranged from 0.47 mm to 0.95 mm (average = 0.69 ± 0.05 mm, all reported error represents standard error). *Janolus fuscus*

grew at an average rate of 6.22 ± 0.74% per day. When mated, *J. fuscus* begin laying egg masses around 19 mm (pers. obs). *Janolus fuscus* at pre-ovipositional lengths (<19 mm) grew at an average rate of 8.79 ± 0.83% per day compared to 3.52 ± 0.75% per day when larger than 19 mm. Overall growth of *J. fuscus* from juvenile to adult is represented by a cubic polynomial curve (Fig. 2.5). Growth curves of individual *J. fuscus* vary in shape and were best fit by variations of cubic polynomial distributions (Fig. 2.6, Table 2.3). Those individuals that grew to maximum sizes (≥35 mm) during the period of observation experienced a decrease in linear growth roughly after 30 mm (Fig. 2.6B, D, G) reaching an asymptote between 35 mm and 50 mm. Other *J. fuscus* did not reach maximum lengths by the end of the observation period and did not experience decreased growth at maximum size (Fig. 2.6C, E, H, I, J). Peak growth rate (in mm/day but not percent of length per day) appears to occur from 18-20 mm in length, though the residuals of the best fit cubic polynomial curve and low regression coefficient suggest much variation in the size at which *J. fuscus* exhibit maximum growth (Fig. 2.7).

Average growth rate from a ~2 mm to 13 mm juveniles was 0.565 ± 0.041 mm per day (11.8 ± 0.7% per day), similar to the rate seen in the single post-metamorphic veliger reared from a larva. Growth from a 2 mm juvenile to a reproductively mature 19 mm slug took about 25 days. At the end of the observation period, slugs ranged in size from 30 mm to 50 mm in length. Four slugs reached maximum sizes of 45 to >50 mm at the end of the study period or in the following two weeks, laid infertile egg masses (as slugs were never mated), shrunk in length and died shortly thereafter. Six slugs were mated for a concurrent experiment. Their first egg masses were partially infertile egg masses. Subsequent egg masses were fertile and were followed shortly by slug

senescence and death. Length measurements were not taken during slug senescence. From these data I suggest growth from a 2 mm juvenile slug to 50 mm takes approximately 63 days. While slugs do survive past 50 mm (up to 57 mm), few are found above this size in nature. Slugs typically died shortly after reaching such a large size and often after laying a last set of egg masses.

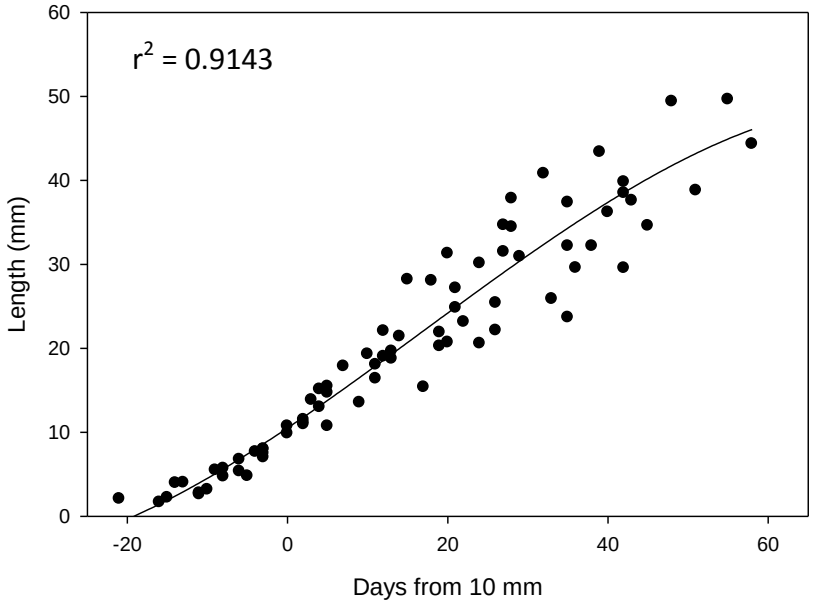


Figure 2.5: Pooled growth of ten individual *Janolus fuscus* in the lab over from February 2007 to May 2007. Length is plotted by the number of days of each measurement before or after each slug was 10 mm in length, with day zero representing the point at which slugs were 10 mm. The best fit growth curve is a cubic polynomial, with the regression coefficient displayed. Each point represents one length measurement for one *J. fuscus*.

Table 2.3: Results of non-linear regression analyses of cubic polynomial curves applied to growth data of 10 juveniles of *Janolus fuscus* reared through adulthood in the laboratory from February-May 2007.

Individual	Source	df	SS	MS	F	p	r^2
A	Growth	3	1325.7	441.9	50.09	0.0004	0.9678
	residual	5	44.1	8.8			
B	Growth	3	1765.0	588.3	72.63	<0.0001	0.9732
	residual	6	48.6	8.1			
C	Growth	3	771.4	257.1	1004.81	<0.0001	0.9995
	residual	3	0.7	0.3			
D	Growth	3	1414.3	471.4	166.23	<0.0001	0.9881
	residual	6	17.0	2.8			
E	Growth	3	749.2	249.7	97.47	0.0017	0.9898
	residual	3	7.7	2.6			
F	Growth	3	795.5	265.2	81.0	<0.0001	0.9799
	residual	5	16.4	3.3			
G	Growth	3	2014.0	671.3	137.9	<0.0001	0.9881
	residual	5	24.3	4.9			
H	Growth	3	460.6	153.5	119.58	0.0083	0.9945
	residual	2	2.6	1.3			
I	Growth	3	523.7	174.6	43.83	0.1105	0.9925
	residual	1	4.0	4.0			
J	Growth	3	486.3	162.1	68.49	0.0885	0.9952
	residual	1	2.4	2.4			

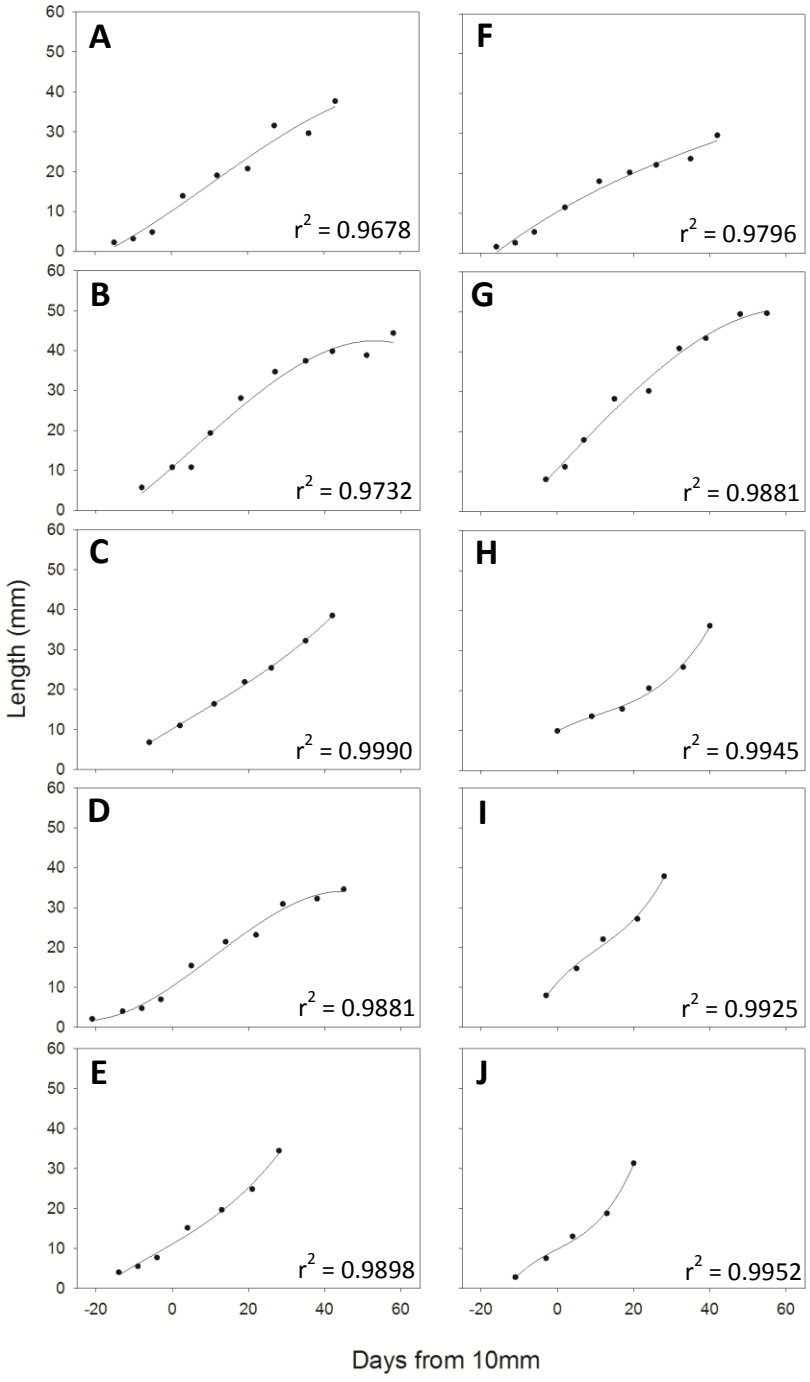


Figure 2.6: Growth of ten individual *Janolus fuscus* (A through J) in the lab from February 2007 to May 2007. Length is plotted by the number of days of each measurement before or after each slug was 10 mm in length, with day zero representing the time at which each slug was 10 mm. Best fit growth curves are plotted on each graph. All scatterplots are best fit by cubic polynomial curves, with regression coefficients displayed on each graph.

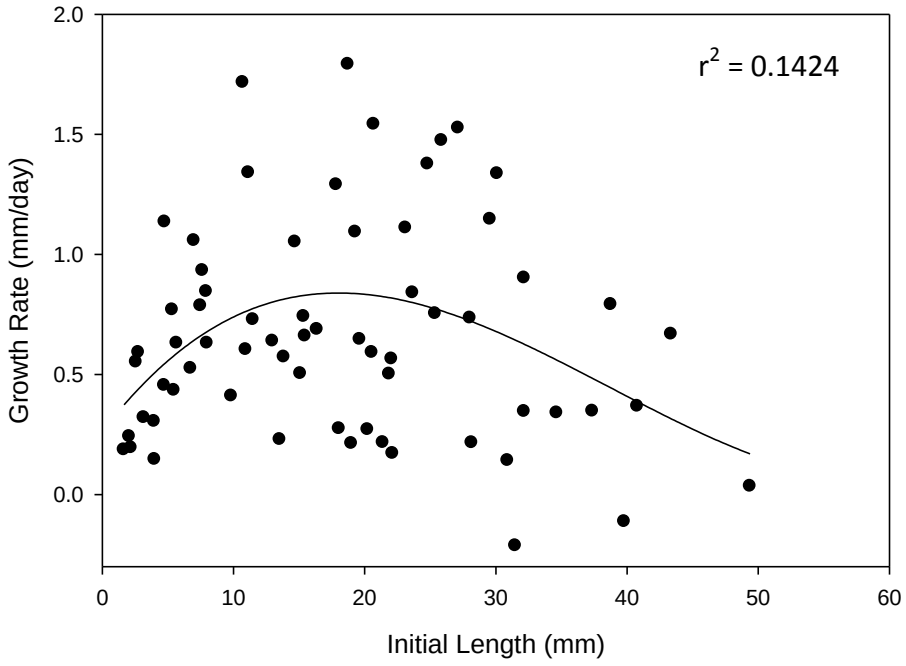


Figure 2.7: Growth rate by initial length for each measurement interval (each period of time between length measurements) of ten *Janolus fuscus* in the lab between February 2007 to April 2007. Peak growth rate occurs from 18 - 20 mm in length.

Discussion

Egg masses of *Janolus fuscus* observed in this study varied greatly in size, containing 5 to 1087 capsules, containing 0 to 160 embryos per capsule, and 272 to 79,786 embryos per egg mass. The number of embryos per capsule agrees with Goddard's (1992) observations of 50-60 embryos per capsule for *J. fuscus*, though I also observed capsules containing much larger numbers of embryos (140-160 per capsule).

As predicted, embryological development of *Janolus fuscus* follows the patterns characteristic of opisthobranchs and other invertebrates that undergo holoblastic, spiral cleavage (i.e., nemerteans, annelids, and other molluscs) (Wilson, 1892; Casteel, 1904;

Thompson, 1958). Unequal cleavage of the third cell division and asynchronous cleavage that leads to transitory stages with temporary odd cell counts (e.g., 12-cell or 13-cell), has been documented in other nudibranch species (e.g., Thompson, 1958; Williams, 1971; Biggelaar, 1996; Martinez-Pita et al., 2006). While other studies of sea slug embryology have noted 64-cell to blastulae flattened along the animal-vegetal axis of embryos, I found no accounts in the literature of the patterned accumulation of microvilli on the first and secondary quartets at the 32-64 cell stage (Chia, 1973; Biggelaar, 1996). I assume *J. fuscus* has a stereoblastula as no blastocoel is visible at this stage under transmitted light, while the developing archenteron is visible in the gastrula stage. Stereoblastulae are common in many molluscs, including opisthobranchs, particularly those that undergo gastrulation by epiboly (Williams, 1980; Verdonk and Biggelaar, 1983; Schaefer, 1997).

Opisthobranchs are known to gastrulate by a variety of methods including epiboly and emboly, or some combinations thereof (Thompson, 1958; Gohar and Soliman, 1967a, b; Williams, 1971; Soliman, 1978; Verdonk and Biggelaar, 1983; Schaefer, 1997). Though SEM micrographs of gastrulation in *J. fuscus* appear similar to those of *Crepidula fornicata* undergoing gastrulation by epiboly, I am uncertain of the origin or consequence of the cellular extensions covering the blastopore on the ventral surface of the late gastrula stage, so I cannot entirely rule out the combination of epiboly with other known methods of gastrulation (Verdonk and Biggelaar, 1983). It is possible these extensions are retraction filaments or the trailing edge of cells retreating into the archenteron, though similar observations have not been noted in other studies, to my knowledge. Blastopore closure (prior to the opening of the stomadeum in the same

location) has been documented in opisthobranchs and other molluscs (Casteel, 1904; Thompson, 1958; Verdonk and Biggelaar, 1983). While I did not witness complete blastopore closure, as few individuals were preserved between the gastrula and trochophore stage, these cell extensions may play a role in the fate of the blastopore.

Like other opisthobranchs, embryos of *Janolus fuscus* go through a trochophore-like or pre-veliger stage inside the egg capsule. The stomadeum is found ventrally just below the prominent prototroch of the trochophore-like stage of *J. fuscus*. Shorter cilia are present below and within the mouth of the trochophore, though not in a distinct metatrochal band; nor is the ciliated food groove obvious in the trochophore stage. Likewise, Martinez-Pita (2006) found only a single ring of cilia (i.e., prototroch) in the pre-veliger stage (i.e., trochophore) of two species of *Polycera.* Like other opisthobranchs, *J. fuscus* does not possess the long, cohesive apical tuft at the trochophore stage that occurs only in basal gastropods, such as the Patellogastropoda (Kempf et al., 1997; Page, 2002).

The subsequent development and morphology of the veliger are similar to that described in other studies of nudibranchs (Casteel, 1904; Thompson, 1958). However, eyespots were already visible at hatching, an unusual trait among planktotrophic nudibranchs, as the appearance of eyespots and a propodium during ontogeny is generally indicative of metamorphic competence (Chia and Koss, 1978; Todd, 1981; Hadfield and Switzer-Dunlap, 1984; Goddard, 1984; 1992). Interestingly, 10 of the 50+ northeast Pacific nudibranchs examined by Goddard (1984 and 1992) with planktotrophic develop (including *J. fuscus*) possessed eyespots at hatching. He suggests these species might have a shorter obligatory planktotrophic period than the planktotrophic species hatching

without eyespots or may be associated with species with type 2 shells which appear inflated or egg-shaped, and do not grow during larval development (Thompson, 1961; Goddard, 1984, 1992). While this hypotheses holds true for many species examined by Goddard (1984, 1992), *J. fuscus* have a type 1 shell and do not have a shorter planktonic period than most other nudibranchs with planktotrophic larvae.

The small size of uncleaved zygotes (~81 μm), large number of embryos per capsule and per egg mass, and relatively short embryonic period (10-18 days) seen in *J. fuscus* is common in nudibranch species with planktotrophic larvae (Thompson, 1967; Todd, 1981; Todd and Doyle, 1981; Hadfield and Miller, 1987; Soliman, 1991; Goddard, 1984; 1992; 2004). The embryonic period and larval size (10-18 days and 125-153 μm at hatching) of *J. fuscus* observed in our lab showed greater variation than that observed by Goddard (1992;14-16 days with a shell length of 138 μm). This is surprising as egg masses were kept at ambient seawater temperatures (11-13°C) of the NE Pacific region in both studies; these differences are unlikely to be a product of different temperature regimes. Numerous studies report high intra-specific variability in hatching times of nudibranch embryos, which may result from individual differences in the rate of development, variation in the stage of development at hatching and/or a prolonged period of fertilization and egg laying (Avila, 1997; Williams, 1980; Goddard, 2004). I did not observe great variation in the timing of early development, nor notice prolonged periods of fertilization and egg laying in *J. fuscus*, though the latter was not adequately addressed in this study. However, those larvae that hatched earlier were significantly smaller than those hatching later, suggesting some variation in the stage of development at hatching. There was no significant relationship between the length of larvae at hatching and the

number of days they survived or the maximum length achieved. As planktotrophic larvae, they are feeding and growing during this pelagic period and may reach the same lengths and survive as long as those larvae hatching later at larger sizes (Thompson, 1967; Hadfield and Switzer-Dunlap, 1984). However, the effect of time and size at hatching on larval survivorship in nature is unknown.

The growth pattern for *Janolus fuscus* larvae is like that of other planktotrophic nudibranch larvae (Perron and Turner, 1977; Chia and Koss, 1978; Hubbard, 1988) with rapid shell growth rate early in the larval period and a cessation of shell growth prior to competence. A plateau in the veliger shell growth curves several days prior to full competence and settlement may be a consequence of the retraction of the mantle fold from the shell aperature, as noted in other nudibranch studies (e.g. Kempf and Willows, 1977; Perron and Turner, 1977; Chia and Koss, 1978; Todd, 1981). Cessation of shell growth prior to competence has been observed in aeolid, dentronotid, and dorid nudibranchs such as *Doridella obscura* (Perron and Turner, 1977), *Hermissenda crassicornis* (Harrigan and Alkon, 1978), *Rostanga pulchra* (Chia and Koss, 1978) and *Onchidoris bilamellata* (Todd, 1981). The slight decrease in average shell length of *J. fuscus* cultures after the cessation of shell growth is due to dwindling larval cultures with a few large veligers (reaching competence) pooled with small veligers that never showed signs of reaching competence.

Veligers of *Janolus fuscus* showed early signs of competence (cessation of shell growth) as early as 235 μm in length and 32 days from hatching. Further signs of competence such as full retraction of the mantle from the shell and propodial development did not occur until 36 and 41 days (254-266 μm shell length). These signs

of competence are common among opisthobranch veligers and the timing of competence is comparable to other species of nudibranchs (Aeolidacea, Doridacea, and Dendronotacea) with planktotrophic development cultured at 10-15 °C such as *Hermissenda crassicornis*, *Tritonia diomedea*, *Rostanga pulchra* and *Onchidoris bilamellata* (Kempf and Willows, 1977; Chia and Koss, 1978; Harrigan and Alkon, 1978; Bickell and Chia, 1979; Todd, 1981).

Janolus fuscus was successfully reared to metamorphosis in this study. Induction of metamorphosis by a specific substratum, often the adult prey, has been documented for many opisthobranch species (Thompson, 1958; Harris, 1975; Hadfield, 1977; Kempf and Willows, 1977; Harrigan and Alkon 1978; Hadfield and Pennington, 1990; Lambert et al., 1994; Avila 1998; Trowbridge and Todd, 2001). New recruits of *J. fuscus* (1-10 mm) are commonly found on *B. pacifica* in the field (chapter II), suggesting they may provide a cue for larval settlement and metamorphosis in *J. fuscus.* Indeed, four veligers settled and two underwent metamorphosis when given the bryozoan substrate. Our results suggest that the presence of *Bugula pacifica* induced settlement and metamorphosis in competent *J. fuscus*. However, the bryozoan alone may not be the optimal cue. Chemical cues associated with conspecifics, microbial films, algae or other benthic substrata as well as artificial inducers (e.g., neurotransmitters) can act alone or in combination to induce metamorphosis in a variety of invertebrate larvae (Cameron and Hinegardner, 1974; Crisp, 1974; Baloun and Morse, 1984; Burke, 1986; Yool et al., 1986; Hadfield and Pennington, 1990; Pawlik and Hadfield, 1990; Pawlik, 1992; Pechenik et al., 1996; Avila, 1998; Trowbridge and Todd, 2001).

Studies of other opisthobranchs show high between-culture and within-culture variation in the timing of settlement and metamorphosis, suggesting veligers of some opisthobranchs do not achieve competency in a synchronous manner (e.g., Gibson, 1995; Avila, 1998). I observed much variation in the timing at which the larvae of *J. fuscus* settled, within and among clutches of veligers. It is possible that many *J. fuscus* were not competent at the point of exposure to *B. pacifica.* Since the only way to conclusively demonstrate that a larva is competent is to induce metamorphosis, it may be that only four larvae had truly reached competency. Alternatively, larvae may have been exposed to *B. pacifica* before they reached competency and exhibited some habituation to the inducer (Hadfield and Scheuer, 1985).

Additionally, *Bugula pacifica* may not act as a passive substratum for settlement of *Janolus fuscus*. I observed an avicularium stab a newly settled veliger crawling on the colony and a lophophore "take in" a swimming veliger, causing the later to retract its velum and sink to the bottom of the culture dish. Harris (1975) found veligers of *Phestilla melanobranchia* did not settle directly on the polyps (but in close proximity) of their coral prey, *Dendrophyllia elegans,* as the former were eaten if they encountered the tentacles of a whole polyp. While I do not suggest that lophophores of *B. pacifica* damage or consume veligers of *J. fuscus,* it appears that zooids may deter settlement. Also, avicularia may be a hazard that limits the success of veligers attempting to settle and metamorphose on this prey. Harmer (1909) suggests avicularia are well adapted to warding off larvae settling on bryozoans colonies. Veligers appeared to settle preferentially on the distal ends of bryozoan colonies. As avicularia are not typically found on the distal-most tips of *B. pacifica* colonies, settling *J. fuscus* may preferentially

settle on or crawl up to the distal-most autozooids before metamorphosing to avoid interactions with avicularia.

The one surviving juvenile of *Janolus fuscus* did not consume tissue of *Bugula pacifica* until day 3 after metamorphosis, though it touched, and caused the retraction of, a lophophore repeatedly on day 2. While energy reserves from the feeding larval stage may be sufficient to sustain the juvenile for two days before the onset of feeding, Hadfield (1963) and Perron and Turner (1977) suggest newly metamorphosed nudibranchs may feed on algae, sessile ciliates, benthic diatoms, and/or debris attached to their prey before they grow large enough to consume prey tissue (barnacle and bryozoan, respectively). I did not examine energy reserves in newly metamorphosed nudibranchs, nor witness them consuming alternative nutritive sources on the bryozoans to support or refute either hypothesis. The surviving juvenile slug grew rapidly to 9.1 mm in 31 days, before a failure in the seawater system caused its demise. Its growth rate from 2.5 mm to 9.1 mm (0.557 mm per day, 12.9 % of length per day), was similar to rates seen in juveniles collected from the field over this same size range (0.565 mm per day, 11.8 % per day).

New recruits of *Janolus fuscus* collected from the field grew rapidly in the laboratory. As slugs reach sexual maturity and begin laying egg masses (if mated) around 19 mm, they begin to devote more resources to reproduction. Thus, *J. fuscus* smaller than 19 mm (immature) grew at a faster average rate (8.79%) than those above 19 mm (3.52%). Folino (1993) found greater growth rates prior to maturation and oviposition in the aeolid *Cuthona nana*. Likewise, Eyster (1981) found the subannual arminacean, *Armina tigrina,* to have higher pre-ovipositional growth rates (3.4% per day)

compared to rates during early oviposition (0.6% per day). Pre-ovipositional growth rates are slightly higher in *J. fuscus* than those reported for subannual and annual nudibranchs. Harris (1975) reported growth rates of 3.4-6.8% per day and 6.8-7.6% per day in two subannual aeolids, *Phestilla melanobranchia* and *P. sibogae*, respectively. Two annual dorids, *Diaulula sandiegensis* and *Archidoris pseudoargus*, have lower pre-ovipositional growth rates (based on weight measurements) of 1.3-4.2% per day and 1.7% per day, respectively (Carefoot, 1967; Elvin, 1976; Eyster, 1981). Growth rate based on length and wet weight measurements can be compared, particularly in immature nudibranchs, as length and wet weight have been shown to be tightly correlated (Clark, 1975; Eyster and Stancyk, 1981). Length may not be the best measure of true growth in invertebrates as it can suggest a decrease in growth rate while the animal continues to grow in mass or volume. However, weighing very small juvenile slugs is difficult and requires sacrificing those individuals, and so was not a realistic method for this study. The curvilinear regression of length to weight I developed for *J. fuscus* from extensive field surveys (chapter II), is inaccurate for predicting weight of new recruits <7 mm for the same reason as above (i.e., I did not wish to sacrifice small individuals to establish weight measures during surveys).

I recognize that growth of *Janolus fuscus* in the lab, where they are fed to satiation and not allowed to mate, may give artificially high growth rates or an extended longevity compared to their counterparts in the field, where energy is required for finding food and mates, copulating, and laying egg masses. Additionally, nudibranchs in their natural environment typically experience degrowth, a cessation or decrease in size during and following oviposition (Todd and Havenhand, 1988). I observed instances in which *J.*

fuscus did not grow or decreased slightly in size, but as *J. fuscus* were not laying egg masses during the experiment, they likely did not experience degrowth until the end of their life when they laid infertile egg masses, shrunk and died, or for those that were mated for a subsequent experiment laid fertile egg masses before senescence and death. *Janolus fuscus* grown in the lab did not exceed the maximum size of those found in the field (~57 mm) before dying, though finding such large individuals is rare. Thompson (1961b) and Eyster (1981) found that non-reproductive nudibranchs died at the same time as those that did reproduce, suggesting the lack of reproduction does not increase longevity. If this is true for *J. fuscus*, the estimated lifespan should be a good estimate for this nudibranch in its natural habitat.

A 2 mm juvenile can grow to sexual maturity (~19 mm) in ~25 days and may reach a length of ~50 mm in 63 days. Combined with the previous times for embryonic development and larval and early juvenile growth, this suggests an egg to egg development period (i.e., generation time) of roughly 105 days and growth to maximum size before death of ~143 or roughly five months. This suggests *Janolus fuscus* is a subannual species. These data are supported by concurrent field surveys of two populations of *J. fuscus*, which show evidence of repeated recruitment and multiple, overlapping generations, characteristic of subannual populations of nudibranchs (Miller, 1962; chapter II). A combination of developmental timeline with the demographic patterns seen in the field, yield a broader picture of the natural history and reproductive ecology of this arminacean nudibranch.

Bridge II

In the previous two chapters (II and III) I found that *Janolus fuscus* is a subannual nudibranch with a five-month lifespan. Its peak densities are correlated with peaks in prey abundance at two intertidal sites. In chapter IV, I examine changes in the prevalence and intensity of *Ismaila belciki* in relation to host density in the same field surveys described in chapter II. Through these field studies and host dissections, I determine the distribution of *I. belciki* in the host population and their position in the host body. I describe site specificity and the potential role of intraspecific competition within the host in this endoparasite.

CHAPTER IV

PREVALENCE AND DISTRIBUTION OF AN ENDOPARASITIC COPEPOD, *ISMAILA BELCIKI,* IN TWO POPULATIONS OF ITS NUDIBRANCH HOST, *JANOLUS FUSCUS,* ALONG THE OREGON COAST

1. Introduction

The life history of a host and its parasite are intricately entwined. Numerous studies have described synchrony in the timing of the life cycle of both partners that enable a parasite to find and infect hosts, establish themselves in a host population and change with that host population over time (Lewis et al., 2002; Roberts and Janovy, 2009). This has lead to the discovery of elaborate adaptations in morphology and ecology as well as the evolution of complex life cycles in many parasites to optimize their chances of finding a host (Lewis et al., 2002; Rhodes, 2004; Roberts and Janovy, 2009). Establishing in a host population is a formidable challenge even for parasites of long-lived hosts such a helminth parasite of vertebrates and invertebrates (Anderson and May, 1979; May and Anderson, 1979; Kuris, 1980, Lafferty and Gaines, 1995). For those parasites inhabiting short-lived hosts with ephemeral populations that live less than a year, the challenge a parasite faces in rapidly finding and establishing itself in the host population is daunting (Anderson and May, 1979; May and Anderson, 1979). The endoparasitic copepods of opisthobranch gastropods face such a challenge.

Opisthobranchs are short-lived, typically surviving for a year or less (Todd, 1981). Also, many opisthobranch populations exhibit rapid and complete disappearance or reappearance of their intertidal populations, a pattern that makes them temporally unstable and difficult to study (Thompson, 1958, 1961, 1966; Potts, 1970). As a result, basic studies on the change in the prevalence and intensity of these parasites over time and their distribution within the host population have not been conducted for this group of endoparasitic copepods.

Copepods from the family Splanchnotrophidae (Order Poecilostomatoida) are large endoparasites that reside within the hemocoels of opisthobranch gastropods. Of the five splanchnotrophid genera (*Splanchnotrophus*, *Lomanoticola*, *Arthurius*, *Ceratosomicola* and *Ismaila*), *Ismaila* is the most speciose, with eleven described host-specific species. Many studies have examined the morphology and systematics of these parasites (Bergh, 1868; Belcik, 1965, 1981; Ho, 1981, 1987a; Jensen, 1987; Huys 2001; Haumayr and Schrödl, 2003) but information on their biology is limited to occasional observations and anecdotal notes of sex ratios, position and orientation within the host, and host condition (Belcik, 1965, 1981; Jensen, 1987; Haumayr and Schrödl, 2003). The ecology of these parasites, including temporal patterns of prevalence or intensity of infection over time and the impact of the parasite on host survivorship or reproduction remain mostly unknown (Schrödl, 1997, 2002). The paucity of ecological studies may be attributed in part to the ephemeral life-histories of the opisthobranch hosts and the rarity of finding stable populations that support splanchnotrophid copepods.

Ismaila belciki (renamed by Ho, 1987a) was first described as *I. montrosa* (Belcik, 1965) in Coos Bay, OR in *Janolus fuscus* (Nudibranchia, Zephyrinidae).

Prevalence (percent of the host population that is infected) of *I. belciki* was high (62%) during June-July 1963, but no quantitative information on the number or size of the host, *Janolus fuscus* or intensity of infections (the number of parasites per host) was recorded. Belcik (1965) did note that "it was not unusual to remove as many as five females and four males from a single host." Additionally, he mentioned that female copepods favored positions anterior or posterior to the host digestive gland, with males residing near females or wrapped in female appendages (Belcik, 1965).

Janolus fuscus is found from Klu Bay, AK to the Gulf of California and is conspicuous from April to October in the boulder fields of North Cove and Fossil Point near Coos Bay, OR, where it feeds exclusively on arborescent bryozoans such as *Bugula pacifica* and *Tricellaria circumternata* (Sowell, 1949; Gosliner, 1982; Goddard, 1984, 1998; chapter II). As the population of *J. fuscus* is present predictably at these sites from spring through fall and *I. belciki* are also present during this time, I had the opportunity to examine the ecology of this opisthobranch and its copepod in more detail. I was able investigate how the prevalence, intensity, and distribution (frequency of different parasite intensities) of *I. belciki* change in the host population over time. At the host level, factors such as site specificity (location, orientation and attachment) within the nudibranch host were recorded, as well as evidence of intraspecific competition of copepod parasites within the host body.

Previous studies suggest that most splanchnotrophid copepods maintain low prevalence within the host population (Jensen, 1987; Schrödl, 2002; Marshall and Hayward, 2006). However, high prevalence values with large variation have also been reported for a few splanchnotrophid species from temperate regions (Belcik, 1965;

Schrödl, 2002; Haumayr and Schrodl, 2003). While great inter- and intraspecific variation has been observed in the intensity and sex ratios of splanchnotrophids in their opisthobranch hosts (Hancock and Norman, 1863; Ho, 1981; Schrödl, 2002; Haumayr and Schrödl, 2003; Marshall and Hayward, 2006; Salmen et al. 2008a, 2008b), no studies have examined the distribution of intensity or size distribution of infected and uninfected individuals in a host population with splanchnotrophid parasites. The uniform distribution of one female and one male found in *Splanchnotrophus willemi* and the extremely high intensity (425 copepods) of *Ismaila occulta* in one *Dendronotus iris*, imply that splanchnotrophid copepod distributions may range from uniform to highly aggregated in their host populations (Ho, 1981; Marshall and Hayward, 2006).

Parasite distribution within a host population may be classified according to the variance/mean ratio of parasite abundance as: 1) regular (or underdispersed), where mean parasite abundance>variance 2) random, where mean = variance and 3) aggregated (or overdispersed), where mean<variance. Additionally a uniform distribution, in which all hosts have exactly the same number of parasites, is an extreme case of underdispersion (Crofton, 1971; Anderson and Gordon, 1982; Shaw and Dobson, 1995). Factors that may affect the distribution of a parasite within the host population include birth and death rates of the host and parasite, density-dependent limitations of parasite infection or success, infection rate and heterogeneity in exposure to infective stages, host susceptibility, immunological responses, and parasite pathology (Anderson and May, 1979; May and Anderson, 1979; Anderson and Gordon, 1982). The additive or opposing roles of these factors and the relative magnitude and variation of each effect will determine how the parasite is distributed within the host population. The distribution of a

parasite within its host population often varies with the type of parasite. Most macroparasites (including crustaceans) have an aggregated distribution within the host population, with the majority of hosts being uninfected or with low intensity of infection and a few hosts with high intensities supporting most of the parasite population (Anderson and Gordon, 1982; Anderson, 1986; Shaw and Dobson, 1995; Karvonen et al., 2006; Barson et al., 2008; Webb, 2008; Dippenaar et al., 2009). In contrast, parasitoids and parasitic castrators (i.e., parasites that cause mortality or reproductive death, respectively) are often underdispersed or uniformly distributed within the host population (Reinhard, 1956; McDermott, 1991; Glenner et al., 2003; Fogelman et al., 2009; Lafferty and Kuris, 2009). Parasitic castrators may have an underdispersed distribution in the host population as a product of their large size relative to the host (3% to 50% of host mass), as high intensities of infection with such large parasites would likely increase host morbidity (Lafferty and Kuris, 2009). At such large sizes, parasitic castrators typically optimize space and occupy specific sites in the host. Thus, parasitic castrators often exhibit high site specificity and intraspecific competition within the host, compared to typical parasites (Lafferty and Kuris, 2009). Understanding the distribution of parasitic copepods in the host nudibranch population can provide insight into the factors that may influence the observed dispersion patterns. For example if hosts suffer from parasite-induced mortality or the parasites experience intraspecific competition within the host, one may expect to see an underdispersed parasite distribution.

Density-dependent processes, such as intraspecific competition, may decrease size and/or fecundity of parasites in high intensity infections. Such interactions may limit the number of parasites that may inhabit a single host and lead to underdispersion of a

parasite within the host population (Croll et al., 1982; Keymer, 1982; Bush and Lotz, 2000; Heins et al., 2002; Lagrue and Poulin, 2008). The "crowding effect," is an inverse relationship between parasite size and number that has been well-documented in adult and larval cestodes and (more rarely) other helminth endoparasites (Read, 1951; Krupp, 1961; Jones and Tan, 1971; Roberts, 2000; Bush and Lotz, 2000; Heins et al., 2002). While this relationship has not been proposed or explored in splanchnotrophids, their typically large size relative to their host and apparent site specificity (Ho, 1981, Schrödl, 1997; Haumayr and Schrödl, 2003; Marshall and Hayward, 2006; Salmen et al., 2008a) suggest that space competition may occur. The body cavity, appendages, and lateral processes of female splanchonotrophids are often filled with gonad (Belcik, 1981, Salmen et al., 2008a), indicating that size has a direct effect on fecundity. This suggests that even at low intensities female copepods compete for host space, which could limit their size and fecundity.

The position and orientation of splanchnotrophids in the hemocoel of their opisthobranch hosts show little intraspecific variation, but differ for each host/parasite system. Some parasite species occur anterior and others posterior to the kidney and pericardium of the host, with parasite lateral processes wrapped around the host's central organs and/or ovotestis. Female copepods can have several different orientations within the host. For example, the body axis of a female copepod may be perpendicular to the body axis of the host, with her head oriented toward the host foot or the body axis of the female copepod may be parallel to that of the host, with the head pointed toward the host's anterior or posterior end. From these positions, the female's posterior (urosome) typically penetrates the dorsal or lateral host integument and produces egg masses outside

of the host's body (Hancock and Norman, 1863; Schrödl, 2002; Haumayr and Schrödl, 2003; Marshall and Hayward, 2006; Salmen et al., 2008a). A few studies suggest that site specificity of female splanchnotrophids may be related to optimal egg position outside the host. For example, egg masses positioned near the host gills may benefit from increased structural support, water flow and aeration, whereas those associated with the "digitiform outgrowths" (such as the cerata or papillae of certain opisthobranch families), may be camouflaged and protected from predators (Hancock and Norman, 1863; Hecht, 1893; Jensen, 1987; Marshall and Hayward, 2006).

I examined the change in prevalence (percentage of host population infected) and intensity (number of parasites per host) of *Ismaila belciki* within two populations of *Janolus fuscus* on the Oregon coast. Seasonal intertidal surveys and dissections were conducted over four years to document the distribution of *I. belciki* in the host population. The potential for intraspecific competition in *I. belciki* and the influence of this interaction on the parasite's distribution is discussed. Position, orientation and attachment of *I. belciki* within the host were recorded to identify potential benefits of site specificity to the parasite. These features were then compared to those of typical parasites and parasitic castrators, to put the association of *I. belciki* with its host in a broader context.

2. Methods

2.1. Study sites

North Cove (43°18'30.6N, 124°23'58.92W) is a large intertidal area on the outer coast of Oregon containing a mixture of sandstone shelves, outcrops, and boulders of various sizes. Surveys were conducted in the inner boulder field, as this is the most

protected area of the cove and contained the majority of *Janolus fuscus*. The inner boulder field is composed of fissured and pocketed bedrock and boulders varying in size from 0.25 to 0.5m in diameter as described by Goddard (1984). *Janolus fuscus* were found in large tidepools, and under and among boulders that support two of their arborscent bryozoan prey species, the epiphytic *Tricellaria circumternata* and, more rarely, *Bugula pacifica* (Soule et al., 2007). While more protected than many other coves on the outer coast, North Cove is more exposed than Fossil Point, which is located within Coos Bay, approximately 2 km from the entrance. The second site is an unnamed point (43°21'32.4N, 124°18'45.36W) midway between Fossil Point and Pigeon Point, Charleston, OR, USA. It is referred to as Fossil Pt. for convenience below but is not Fossil Pt. proper. This site is a large mudflat interspersed with broad sandstone shelves, which contain fields of boulders and cobbles (0.25-2.5 m in diameter). Surveys were conducted in a narrow (12 m wide) field of boulders and cobbles running perpendicular to the axis of the bay and just south of the most prominent sandstone shelf. *Janolus fuscus* were found in small tidepools and around and under undercut boulders that support primarily *Bugula pacifica* with occasional alternative bryozoan prey, *Scrupocellaria diegensis* (Soule et al., 2007).

2.2. Field surveys and collections

Populations of *Janolus fuscus* were surveyed for host density and prevalence of *Ismaila belciki* over 23 months between August 2005 and December 2009; seven times between August 2005 and April 2007, and every other month from July 2007 to December 2009. All *J. fuscus* were maintained and processed at the Oregon Institute of Marine Biology, Charleston, Oregon.

To determine the population density of *Janolus fuscus*, every individual was collected along five haphazardly placed 30 m x 2 m and 30 m x 1 m belt transects through the low intertidal boulder fields of North Cove and Fossil Point, respectively. The difference in transect widths for the two sites were chosen because Fossil Pt. had a greater abundance of *J. fuscus* that was spatially concentrated in a smaller area of suitable boulder habitat than at North Cove. Slug distributions at both sites were extremely patchy, which led to high variability in abundance of *J. fuscus* among transects. Thus all five transects were pooled into a single value for abundance and density estimates at each site and date. All slugs were collected by hand and placed in separate 50-ml Falcon tubes to prevent mating and aggressive interactions and returned to the Oregon Institute of Marine Biology. Approximately 500 cm^3 of representative arborescent bryozoans, *Bugula pacifica, Tricellaria circumternata,* and/or *Scrupocellaria diegensis* were also collected at each site, returned in seawater to the lab and examined under a dissecting microscope for newly recruited *J. fuscus*.

2.3. Nudibranch maintenance and parasite prevalence, intensity, and site specificity

In the laboratory, *Janolus fuscus* were kept in seawater tables in separate plastic 10.5x10.5x12.5 cm flow-through containers and fed to satiation with *Bugula pacifica, Tricellaria circumternata* or *Scrupocellaria diegensis* every few days. Nudibranch lengths were measured with calipers as individuals crawled upside down on the water surface. Nudibranchs were gently blotted on a paper towel before recording a wet-weight measurement; however, measuring weights of very small individuals was difficult and impacted their condition, so length was used as the primary measurement of host size. Infection status was determined by external examination of the host's transparent

mantle. Because the dwarf male parasites were too small to visualize using this method, only female parasites were enumerated in this analysis. For each nudibranch, infection intensity and position of the parasite within the host body was documented. Prevalence of female *I. belciki* in the host population was recorded for each site and date. These data were used to document the following: 1) density of the host population, 2) percentage of the host population infected (parasite prevalence), 3) number of female parasites per host (infection intensity) and 4) site specificity within the host.

Dissections were conducted in addition to the external examination to determine the number of male *I. belciki* associated with each female and the position and weight of each copepod. Approximately equal numbers of infected and uninfected *J. fuscus* were haphazardly subsampled from the August 2005, 2007, 2008, 2009 and October 2008 collections. Individuals were anesthetized in 7.5% $MgCl_2$ and preserved in 10% formalin for dissection. Dissections were conducted under a Unitron LWZ dissecting microscope (4.5x). The weight of the host and each parasite was recorded to four decimal places on a Mettler Toledo At4600 Delta Range digital balance after gently blotting on a paper towel. For infected individuals the sex, orientation, point of attachment, and position within the host were noted. These data were used to examine the intensity of infections, sex ratios, and site specificity of male and female *I. belciki*, and to calculate parasite indices (PI), where PI=(mass of Parasite/(mass of Host + mass of Parasite))x100. Only apprently healthy individuals (i.e., actively crawling with all cerata) were used in the analyses as dying animals lose cerata and shrink while dying.

2.4. Statistical analyses

2.4.1. Host density and parasite prevalence

To examine how parasite prevalence differs by site and season at the host population level, I examined variation in three measures of parasite occurrence: 1) prevalence, 2) number of infected *Janolus fuscus*, and 3) number of female copepods (number of infecting events) for each site and date, using three single-factor ANCOVAs with site as a random factor and host density and average length as covariates. Three additional single-factor ANCOVAs were run for each site with season as a main factor and host density and average length as covariates. Prevalence data were arcsine transformed to meet General Linear Model assumptions of normality and homogeneity of variances. To examine how parasite prevalence differs by site and season at the individual host level, I used three single-factor generalized linear models (GzdLM) with a binomial distribution and logit transformation. Each individual was designated as infected or uninfected with a 1 or 0, respectively. Site and season were fixed factors and individual host length was a covariate in the models. Host density was a poor covariate in these analyses and was not included in the models as all individuals in a given collection period would have the same host density value. Seasons were designated as winter (January-March), spring (April-June), summer (July-September), and fall (October-December) in accordance with general patterns in occurrences of *J. fuscus*. Winter samples were removed from analyses of parasite prevalence by season, because hosts and/or parasites were absent or scarce during the winter months. Dates when hosts were absent were not included in the analyses but low host densities (<0.01 individuals

m^{-2}) were included as they did not affect analysis outcomes. Bonferroni pairwise comparisons of season were used if a significant difference was found ($\alpha = 0.05$).

To determine if the length of infected *Janolus fuscus* differed from those of uninfected individuals in the host population, I compared lengths from each survey collection at both sites between infected and uninfected individuals in separate single-factor ANCOVAs, with infection status as a fixed factor and host density as the covariate. North Cove lengths were $\log_{10}$ transformed to meet assumptions of normality and homogeneity of variances for GLM. Fossil Pt. lengths were untransformed since transformations did not alleviate heteroscedasticity.

To determine whether particular size classes of *Janolus fuscus* were more likely to be infected, parasite prevalence was calculated for slugs from three size classes: 0-15 mm, 16-30 mm, and 31-45 mm for each collection date and site. Slugs longer than 45 mm were too rare to include in these analyses. Prevalence data were analyzed with two (one for each site), single factor ANOVAs, with size class as a fixed factor and each collection date used as a replicate. These data did not meet normality and homoscedasticity assumptions for GLM despite transformation attempts.

2.4.2. Intensity and distribution of infection

During host surveys the intensity of infection by only female *Ismaila belciki* was recorded, because male copepods were not visible externally. During subsample dissections, the intensity (number of parasites per host) of both male and female *I. belciki* was recorded. Measures of mean parasite intensity (average number of parasites per infected host) followed those described in Bush et al. (1997) and Shaw and Dobson (1995). Mean abundance was defined as the total number of *I. belciki* in the sample of *J.*

fuscus divided by the total number of hosts examined (including both infected and uninfected slugs).

Mean parasite intensity and mean abundance were calculated for each collection period (except when hosts were absent). Additionally, overall mean infection intensity and mean abundance were calculated across sites and collection dates to combine the rare cases where infection was greater than one female parasite per host. To describe the distribution of *Ismaila belciki* in their host population, three measures of parasite dispersion were calculated from both survey and dissection data. Mean abundance ($\bar{X}$) and variance in copepod numbers per host were used to calculate the variance to mean ratio (VMR = $\sigma^2/\bar{X}$), a measure of the degree of aggregation of a parasite within the host population (Gregory and Woolhouse, 1993; Rhode et al., 1995; Shaw and Dobson, 1995; Bush et al., 1997; Opara and Fagbemi, 2008; Roberts and Janovy, 2009). The coefficient of variation was calculated as described in Gregory and Woolhouse (1993) as CV = σ (100 /$\overline{X}$). Macroparasites are typically aggregated within their host population and are characterized by a negative binomial distribution (a unimodal distribution defined by a positive exponent, k, and the mean abundance of parasites ($\overline{X}$)). K varies inversely with the degree of overdispersion and is calculated k= $\overline{X}^2$ / (σ^2-$\overline{X}$) (Fisher, 1941; Crofton, 1971; May and Anderson, 1979; Shaw and Dobson, 1995). K values under one indicate overdispersion (aggregation) while values above one indicate underdispersion of the parasite in the host population

To examine how intensity of *Ismaila belciki* in the host population varies with host size, mean intensity and mean abundance of female *I. belciki* were calculated for each 5mm size class for every collection date. Intensity was combined over site and date

as low sample sizes during many collection dates would not give an accurate indication of parasite distribution by host size. Weights of hosts infected with one, two, or three or more copepods (including females and males) were compared from dissection data in a single factor ANOVA with $\log_{10}$ transformed host weight to meet the assumptions of normality and homogeneity of variances assumptions.

2.4.3. Copepod mass and the crowding effect

Calculations of two parasite indices (PI) were completed using the formula PI=P/(P+H), in which P is the weight of the copepod parasite and H is the mass of the host (Arme and Owen, 1967; Heins et al., 2002). Total PI was calculated using the total weight of all copepods in each host. The second index, Female PI, was calculated using the heaviest female copepod in each host (LoBue and Bell, 1993; Heins et al., 2002). All dissection data were pooled across dates. To test for a possible crowding effect, I examined whether parasite index decreases with increased intensity, by running two, single-factor ANOVAs, one for each parasite index, to examine how total and female copepod indices vary with single and double infections. Hosts with more than two female copepods were not common enough to include in analyses. Additionally, female copepod mass was calculated for each host with one female copepod and compared to the mean female copepod mass of slugs with two female copepod with a single factor ANCOVA, with host weight as a covariate. To assess whether double infections by two female copepods residing in the same position within the host incurs a negative impact on one or both copepods, the weight of both female *Ismaila belciki* from these double infections were compared using a pair-wise t-test. As samples were collected from the

field, I could not control the time of infection. Therefore, copepod weights per host may reflect both crowding effects and the time since infection.

Regression analyses were conducted to determine whether parasite mass (total and female only) correlated positively with host mass. Host mass was $\log_{10}$ transformed to meet the assumptions of linear regression.

2.4.4. Positional preference in the host

The positions of female *Ismaila belciki* within the host were noted by external examinations of nudibranchs collected from the field, and positions of male and female copepods were noted during dissections. Positions of copepods were noted as "anterior" if located just anterior to the host pericardium and posterior to the buccal mass; or "posterior" if they were located between the pericardium and the anus. For each collection date and site, the percentage of hosts with single or double infections of female copepods in each position was tallied. Infection intensities above two were not frequent enough to include in the analyses. Male infections were not visible externally and therefore were not included in this data set. The two most frequent positions for single and double infections were included in the analyses. For single infections, a single female located in the anterior position was most common; all other positions were more rare and thus, lumped together in a "single other" category. Double infections with one anterior and one posterior female were most common, with all other combinations of double infections being rare and therefore lumped into a "double other" category. A single-factor ANOVA pre-test revealed no significant variation in position percentages between collection sites ($F_{17,2}$= 0.182, p=0.676); thus, sites were pooled. I then reanalyzed the data using a single-factor ANOVA with parasite position as a fixed factor

and each collection date as a replicate. If significant differences were found, I ran a Bonferroni pairwise comparison. Assumptions of normal distribution and homogeneous variance were not met in this analysis even after arcsine transformation because single infections with one anterior individual were much more common than other sites. However, as these data had the same number of replicates, the moderate violation of homogeneous variance can be ignored as balanced ANOVA designs have small bias in the P value (Box, 1954a, b).

To see if the same trend in positional preference was seen when hosts were dissected, I calculated the percentage of dissected *Janolus fuscus* with single and double infections for the four most frequent positions found (single anterior, single other, double infections with one anterior and one posterior female, and double infections with two anterior females). To explore whether site within the host impacts parasite fitness, the weight of female *Ismaila belciki* from double infections with one anterior and one posterior individual were compared with a pairwise t-test.

3. Results

3.1. Host density and parasite prevalence

Host population density was consistently greater at Fossil Point (mean density = 0.253 ± 0.04 individuals m^{-2}) than at North Cove (mean density = 0.057 ± 0.017 individuals m^{-2}) (Fig. 3.1). Prevalence of *Ismaila belciki* was also higher and more consistent throughout the year at Fossil Pt. than at North Cove (Fig. 3.1). Prevalence of *I. belciki* ranged from 0 to 36% (average = 9.52 ± 3.57%) and 0 to 81% (average = 31.67 ± 5.39%) at North Cove and Fossil Pt., respectively (Fig. 3.1). At both sites, *I. belciki*

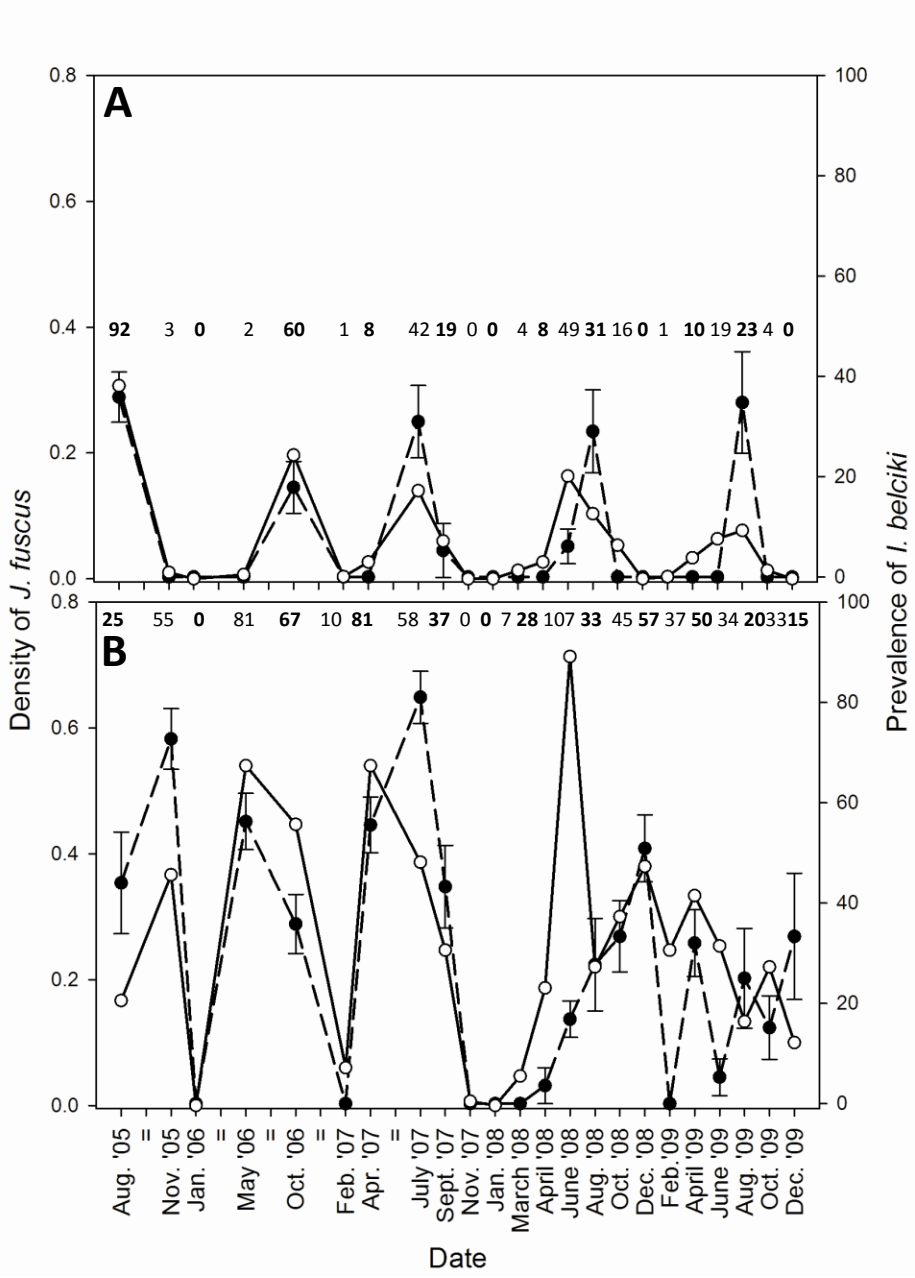


Fig. 3.1: Density of *Janolus fuscus* and prevalence of *Ismaila belciki* by 23 collection dates from August 2005 to December 2009 at (A) North Cove and (B) Fossil Point. Solid lines with open circles and dashed lines with closed circles represent density of *J. fuscus* and prevalence of *I. belciki*, respectively. During dates of host absence, prevalence of *I. belciki* is automatically graphed as zero. Surveys were conducted every other month from July 2007 to December 2009. Collection dates before July 2007 were not evenly distributed in time and the dashed line represents temporal intervals greater than two months. Error bars represent standard error. Sample sizes are displayed above data points.

was present in host populations with densities of 0.1 individuals m^{-2} and greater, though a single infected slug was found at a density as low as 0.06 individuals m^{-2} at North Cove (Fig. 3.1, 3.2).

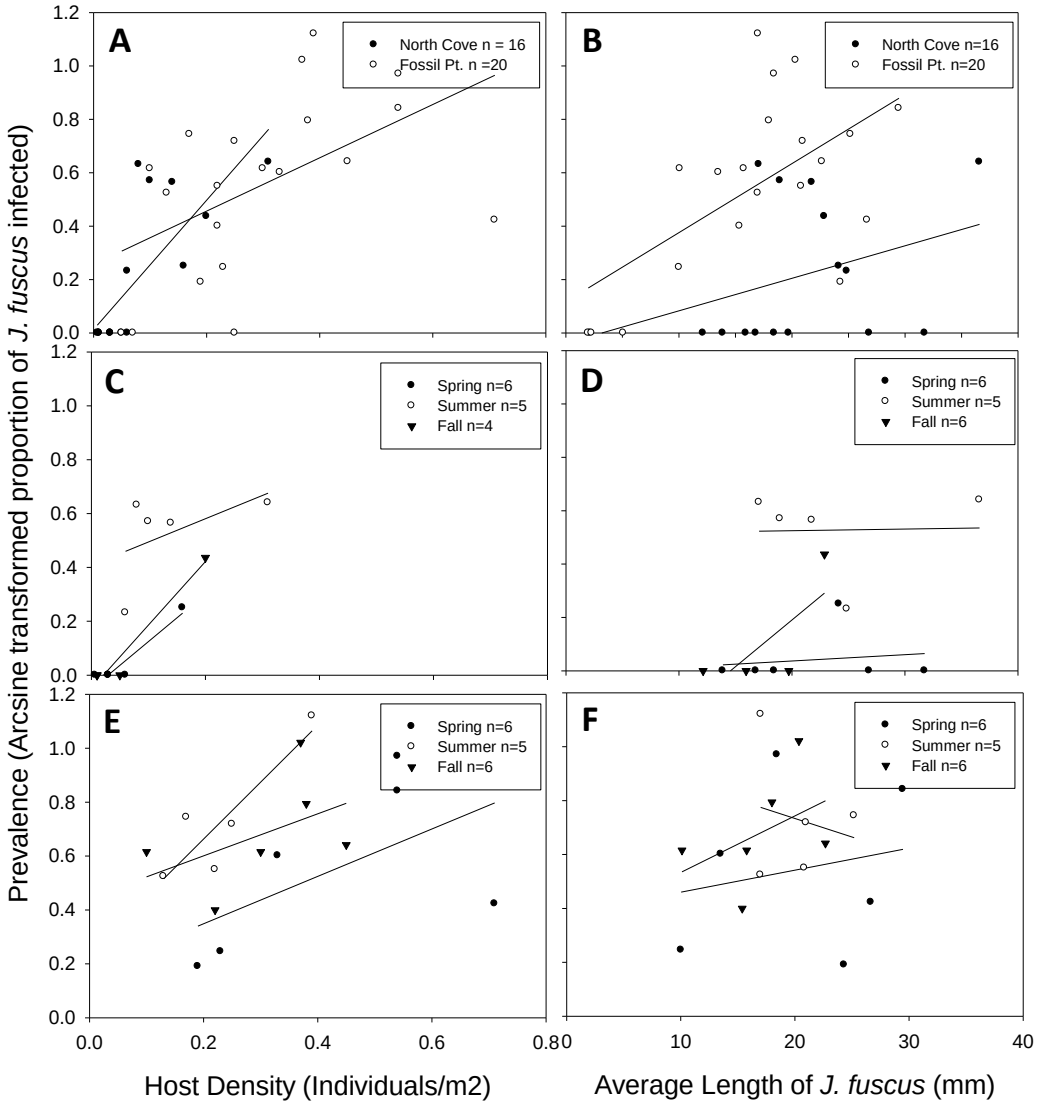


Fig. 3.2: Prevalence of *Ismaila belciki* by host density and average host length. Prevalence of *Ismaila belciki* by host density (A) at North Cove and Fossil Pt.; (C) at North Cove divided by season; and (E) at Fossil Pt. divided by season. Prevalence of *I. belciki* by average length of *Janolus fuscus* at (B) North Cove and Fossil Pt.; (D) at North Cove divided by season; and (F) at Fossil Pt. divided by season. Number of collection dates for each site and season are displayed on each graph. Winter collection dates are not included in season analyses.

At population level, all three measures of parasite occurrence (prevalence, number of infected *J. fuscus* and number of female copepods) increased significantly with host density (Fig. 3.2A, C, E, Table 3.1). Average host length was not a significant covariate in most analyses (Fig. 3.2B, D, F, Table 3.1). When the residuals from the three regressions of host density, one with each measure of parasite occurrence, were examined in ANCOVAs with site or season, these main effects and average host length did not significantly explain the remaining variation in any of the models.

Measures of parasite occurrence did not differ significantly between North Cove and Fossil Pt. (Fig. 3.2, Table 3.1), probably because of variation in host density. Slopes of host density by measures of parasite occurrence were similar at both sites. There was a significant season x host density and season x average host length interaction for all measures of parasite occurrence at North Cove (Fig. 3.2, Table 3.1). The increase in measures of parasite occurrence with host density and average host length was more rapid during fall than spring and summer at this site (Fig. 3.2B). There was no significant difference in any measures of parasite occurrence by season at Fossil Pt. On average, the numbers of infected *Janolus fuscus* (spring 21.17 ± 8.05, summer 17.6 ± 7.56, fall 19.67 ± 5.69) and the numbers of female copepods (spring 25.17 ± 10.24, summer 21.80 ± 8.22, fall 21.5 ± 6.81) were highest in spring but prevalence was highest in summer (spring 0.55 ± 0.13, summer 0.73 ± 0.11, fall 0.68 ± 0.09) at Fossil Pt.

Table 3.1: ANCOVA analyses of three measures of parasite occurrence: arcsine transformed prevalence, number of infected *Janolus fuscus*, and number of female copepods by collection site (A) and season (B) at North Cove and Fossil Pt. with host density and average host length as covariates for each analysis. Interactions with p>0.100 were removed from the model.

A	Arcsine Prevalence		Number of Infected *J. fuscus*		Number of Female Copepods	
Factor	F	p	F	p	F	p
Site	2.30	0.14	0.09	0.76	0.02	0.80
Density [a]	5.80	0.02	24.66	<0.0001	18.90	<0.0001
Length [b]	2.70	0.11	0.80	0.38	1.43	0.24

	Arcsine Prevalence				Number of Infected *J. fuscus*				Number of Female Copepods			
B	North Cove		Fossil Pt.		North Cove		Fossil Pt.		North Cove		Fossil Pt.	
Factor	F	p	F	p	F	p	F	p	F	p	F	p
Season	53.24	<0.0001	3.43	0.07	3.71	0.09	1.11	0.36	6.64	0.03	0.71	0.51
Density [a]	117.62	<0.0001	6.63	0.02	248.0	<0.0001	11.17	0.01	12.35	0.01	7.01	0.02
Length [b]	6.05	0.05	0.53	0.48	0.46	0.53	0.15	0.71	0.44	0.53	0.03	0.87
Season x Density	4.8	0.06			64.5	<0.0001			309.6	<0.0001		
Season x Length	22.56	<0.001			8.68	0.02			73.0	<0.0001		

[a]Host Density
[b]Host Length

At the level of the individual host, parasite prevalence increased significantly with individual host length in all analyses (Table 3.2). Prevalence was significantly higher at Fossil Pt. than North Cove (Table 3.2A). As seen at the population level, there was a significant interaction between season and slug length at North Cove (Table 3.2B), as prevalence increased more rapidly with host length during fall than spring and summer at this site. At Fossil Point, prevalence was lower in spring than summer (Bonferroni pair-wise comparison $p<0.001$) and fall ($p<0.001$), but did not differ between summer and fall ($p=0.926$).

Infected individuals were significantly larger than uninfected individuals at North Cove and Fossil Pt. (Fig. 3.3, Table 3.3A). Host density was a significant covariate at both sites (Table 3.3A). Residuals from the linear relationship between host density and length were analyzed against infection status with two ANOVAs. Infection status explained a significant part of the variation in host length at both North Cove and Fossil Pt. (Table 3.3B). Prevalence of *Ismaila belciki* was highest (38-50%) in intermediate to large slugs (11-55 mm), while very small (<10mm) and very large slugs (>55 mm) had lower prevalence of *I. belciki* (Fig. 3.4). At North Cove, parasite prevalence did not differ significantly according to host length ($F_{24,3}= 0.285$, $p=0.755$) (Fig. 3.5). At Fossil Pt. however, prevalence varied significantly by length ($F_{50,3}=7.73$, $p=0.001$); *J. fuscus* in the smallest size class (0-15 mm) were significantly less likely to be infected than other size classes (Fig. 3.5).

Table 3.2: Results of generalized linear model analyses examining the effect of site and season (fixed factors) and host density (covariate) on prevalence of *I. belciki*. Wald Chi-Square values (X^2) and p-values are listed (α=0.05) for all main factors and covariates. Interactions with p>0.100 were removed from the model.

A Factor	X^2	df	p
Site	60.7	1	<0.001
Length [a]	58.1	1	<0.001
Model [b]	110.6	2	<0.001
GOF [c]	852.0	847	<0.001

B	North Cove			Fossil Pt.		
Factor	X^2	df	p	X^2	df	p
Season	11.9	2	0.003	32.5	2	<0.001
Length [a]	11.7	1	0.001	52.4	1	<0.001
Season x Length	7.3	2	0.027			
Model [b]	61.3	5	<0.001	116.6	3	<0.001
GOF [c]	381	336	<0.001	632.3	651	<0.001

[a]Host Length
[b]Whole Model
[c]Goodness of Fit

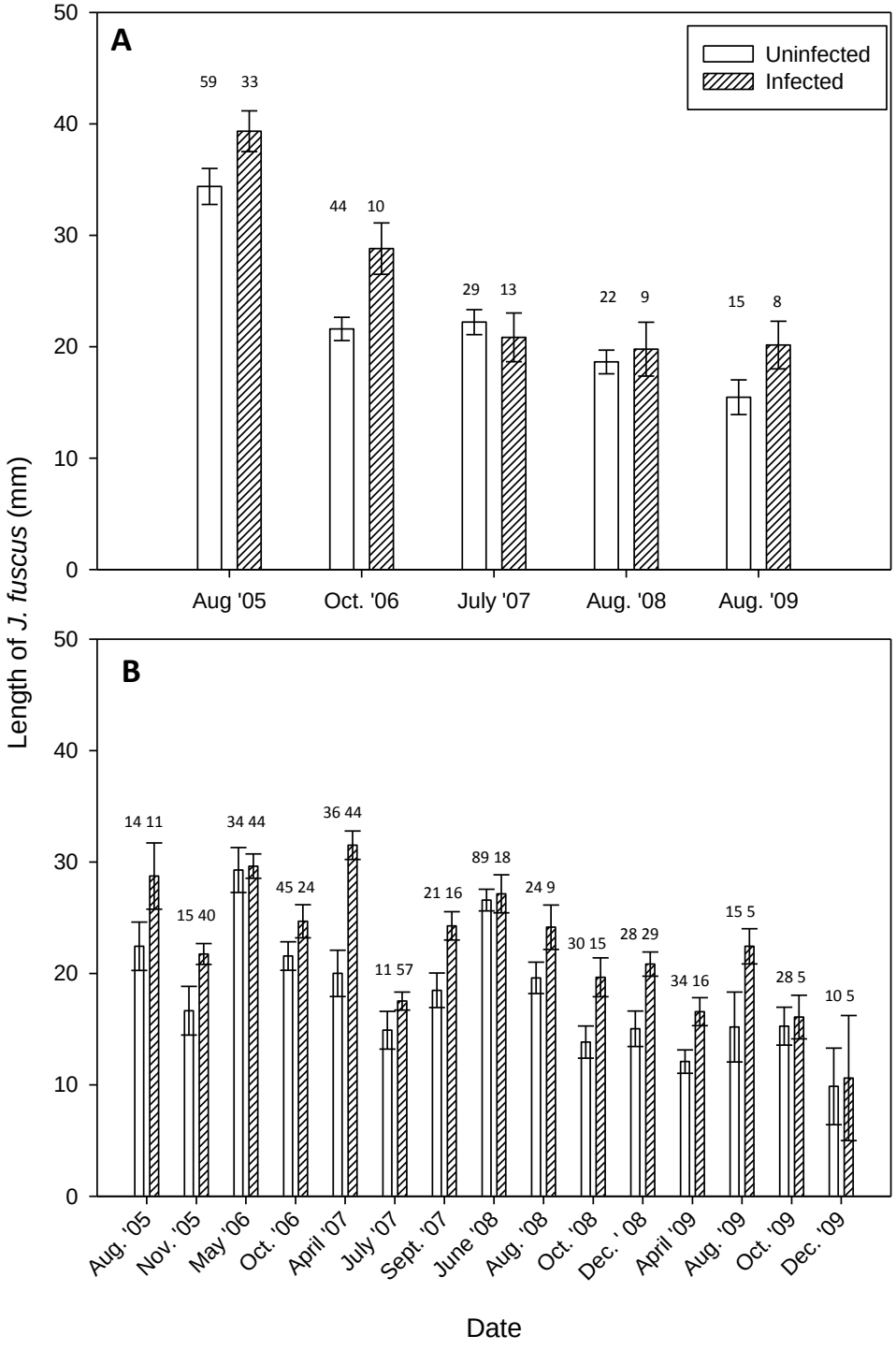
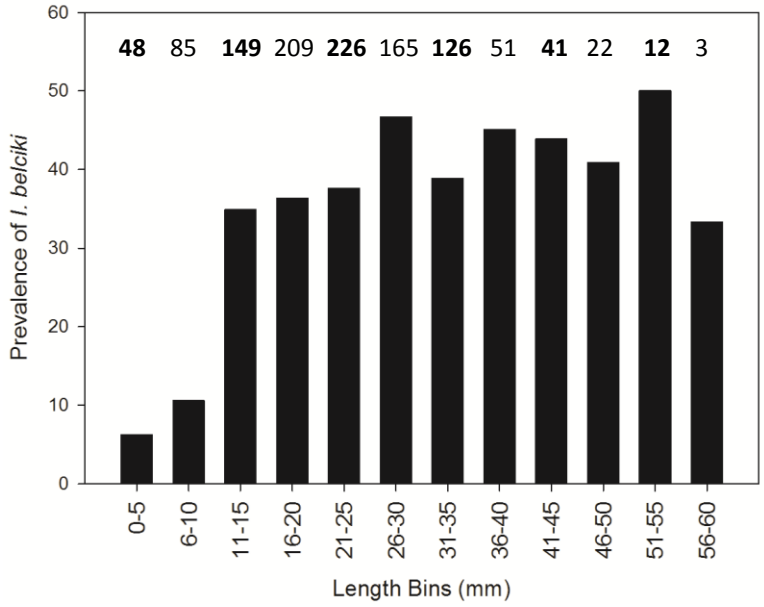


Fig. 3.3: Length of infected versus uninfected *Janolus fuscus* from (A) North Cove and (B) Fossil Point during collection periods with comparable numbers of infected and uninfected *J. fuscus*. Sample sizes are listed above each bar. Error bars represent standard error.

Table 3.3: Results of general linear models examining the (A) effect of infection status (i.e. uninfected or infected) on host length with host population density as a covariate (B) ANOVA results of the effect of infection status on the residuals of host length by host density at North Cove and Fossil Pt., respectively.

A		North Cove					Fossil Pt.				
Analysis	Factor	SS	df	MS	F	p	SS	df	MS	F	p
GLM	Status[a]	0.231	1	0.231	8.9	0.003	3274.1	1	3274	40.7	<0.0001
	Density[b]	3.321	1	3.321	128.6	<0.0001	11094.4	1	11094	137.8	<0.0001
	Error	6.221	241	0.026			61112.5	759	80.52		
B											
Residual	Status[a]	0.230	1	0.230	9.0	0.003	3269.9	1	3270	40.7	<0.0001
	Error	6.222	244	0.026			61116.7	760	80.42		

[a]Infection Status
[b]Host Density



Fi g. 3.4: Prevalence of *Ismaila belciki* in 5 mm length bins from North Cove and Fossil Point combined from all collection dates in which *I. belciki* were present in the population. Sample size of hosts are listed above bars.

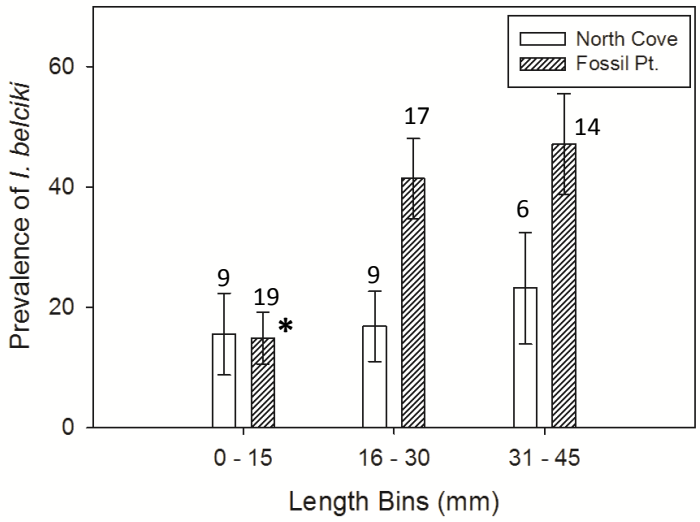


Fig. 3.5: Prevalence of *Ismaila belciki* by three size categories at North Cove and Fossil Point. There was no significant difference in prevalence by length category for North Cove ($F_{24,3}$= 0.285, p=0.755). Fossil Point collections show prevalence as significantly lower in the 0-15 mm length category than all others ($F_{50,3}$=7.73, p=0.001). Error bars represent standard error and the asterisk a significant difference. Sample sizes (with each collection site and date considered a replicate) are given above each bar.

3.2. Intensity of infection

A total of 76 parasitized *Janolus fuscus* were dissected and the majority of these (78.6%) were infected with a single female parasite; of these, females with no male (35.5% of hosts) were most common, followed by infections with one (27.3%) or more (15.8%) male *Ismaila belciki* (Fig. 3.6A). Infections with two females were less common (14.5% of hosts); of these 7.9% were found with one or more males and 6.6% without any males (Fig. 3.6A). Infections with single male parasites or > 2 female parasites were rare, accounting for 5.3% and 1.3% respectively of the host population (Fig. 3.6A). The percentage of each of these sex ratios was used to estimate the true intensity of infection of slugs examined superficially from surveys of the host population (see below) (Fig. 3.6, Table 3.4).

Uninfected *Janolus fuscus* and those with a single female copepod comprised 67% and 30% of the surveyed host population (Fig. 3.6B). Double and triple infections of female copepods comprised 2.4% and 0.6%, respectively (Fig. 3.6B). Mean intensity (=1.1 ± 0.02) and abundance (=0.4 ± 0.02) of only female *Ismaila belciki* were low (Fig. 3.6B, Table 3.4). Mean female copepod intensity ranged from 1 to 1.3 at both North Cove and Fossil Pt. over the four year sampling period (Fig. 3.7A, B). Measuring intensity of only female copepods underestimates the true parasite load per host. To get a better idea of the distribution of female and male copepods in the host population, I applied the calculated percentage of single and double female infections with single, multiple or no males from dissections (n = 76, see above) to the survey data. For each site and date of collection with infected slugs I knew the number of female copepods for each host, then estimated the percentage of those hosts with single and double female

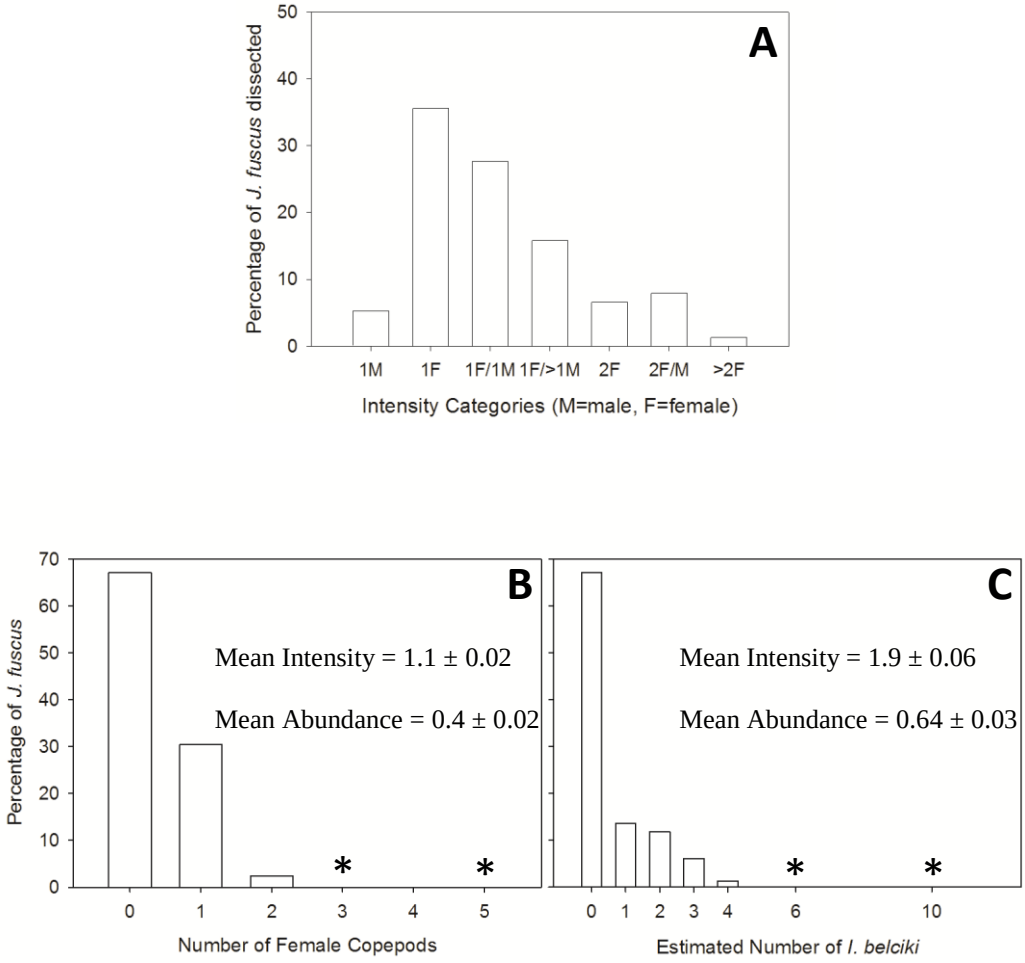


Fig. 3.6: Percentage of *Janolus fuscus* (A) dissections with different sex ratios (n=76 infected slugs) (B) with different intensities of female *Ismaila belciki* from North Cove and Fossil Point (combined) pooled for every collection period from November 2005 to December 2009 when hosts were present in the population; and (C) with the total estimated number of *I. belciki* per host estimated from superficial examination of *J. fuscus* from the same surveys with sex ratios from dissections applied to female intensities (total n=1151 with n=383 infected individuals). Values of mean intensity (average intensity of infection in infected *J. fuscus*) and mean abundance (average intensity of infection in infected and uninfected *J. fuscus*) of *I. belciki* are shown in B and C. 1M (single male), 1F (single female), 1F/1M (one female and one male), 1F/>1M (one female and 2 or 3 males), 2F (two females no males), 2F/M (two females and ≥1 males), >2F (more than two females). Asterisks indicate copepod intensities found in <<1% of hosts examined and are, therefore, not visible on the graph.

Table 3.4: Measures of the distribution of *Ismaila belciki* in two populations of *Janolus fuscus* combined. (A) Column 1: Number of female and male *I. belciki* per host. Column 2: Number of *J. fuscus* from field surveys (i.e., external examination) with 0-11 *female I. belciki*. Column 3: Number of *J. fuscus* from field surveys with 0-11 *I. belciki* (i.e., number of female copepods from column 2 plus the number of male copepods estimated from dissections). Column 4: Number of *J. fuscus* dissected that had 0-11 *I. belciki* (male and female). (B) Measures of intensity of *I. belciki* calculated from (A). As uninfected *J. fuscus* (i.e., number of copepods=0) were undersampled for dissections, only mean intensity was calculated in column 4. Mean intensity = $\bar{X}$I (i.e., average number of copepods/infected host) and mean abundance = $\bar{X}$A (i.e., average number of copepods/slug, infected and uninfected slugs included). All other measures use data from mean abundance. Variance (σ^2), standard deviation (σ), variance to mean ratio (VMR), coefficient of variation (COV%), and exponent of the negative binomial distribution (k).

A	**Number of *Janolus fuscus***		
Number of Copepods	**Field Surveys (superficial examination)**	**Field Surveys (estimated from dissections)**	**Dissections**
0	768	768	47
1	348	156	31
2	28	135	24
3	6	70	13
4	0	15	4
5	1	0	3
6	0	6	0
7	0	0	0
8	0	0	0
9	0	0	0
10	0	1	0
11	0	0	1
B	**Calculations of Intensity**		
Measure of Intensity	**Field Surveys (superficial examination)**	**Field Surveys (estimated from dissections)**	**Dissections**
$\bar{X}$**I**	1.1	1.9	2.1
$\bar{X}$**A**	0.4	0.6	
σ^2	0.3	1.2	
σ	0.6	1.1	
VMR	0.9	1.9	
COV (%)	155%	171%	
k	-3.4	0.7	

copepods that had single, multiple, or no males associated with them (as suggested from percentages calculated in dissections) (Fig. 3.6A,C, Table 3.4). These are referred to as 'estimated intensities' below for the surveyed host population. From these estimated total infection intensities of *I. belciki*, I calculated the overall mean intensity (=1.9 ± 0.06 copepods/host), mean abundance (=0.64 ± 0.03 copepods/host), and measures of *I. belciki* distribution in the natural populations for both sites and all collection periods combined (Fig. 3.6C, 3.7C, D, 3.8C, D, Table 3.4). Mean estimated total intensity of *I. belciki* ranged from 1.1 to 2.1 at North Cove and 1.5 to 2.3 at Fossil Pt., mirroring patterns in parasite prevalence at both sites (Fig. 3.7C, D). Some collection dates had very low prevalence (Fig. 3.7). As sample size of infected individuals decreases, the sample mean parasite abundances and intensities as well as their associated variances and the measures of parasite aggregation are likely underestimated and should be viewed with caution (Gregory and Woolhouse, 1993; Anderson and Gordon, 1997).

Mean intensities of female *Ismaila belciki* were highest in the 20-45 mm size classes of *Janolus fuscus* (Fig. 3.9A). Infected *J. fuscus* in 0-15 mm and 50-60 mm size classes harbored only one female *I. belciki* in all specimens examined (Fig. 3.9A). Mean abundance of female copepods was lowest in the 0-10 mm and >40 mm size classes, with intermediate size classes increasing steadily from 15 to 40 mm lengths (Fig. 3.9B). However, small sample sizes of infected individuals in the largest size classes probably lead to underestimates of intensity (Gregory and Woolhouse, 1993). Intensity of infection did not differ by host weight ($F_{43,3}$ =0.044, p=0.957) (Fig. 3.10).

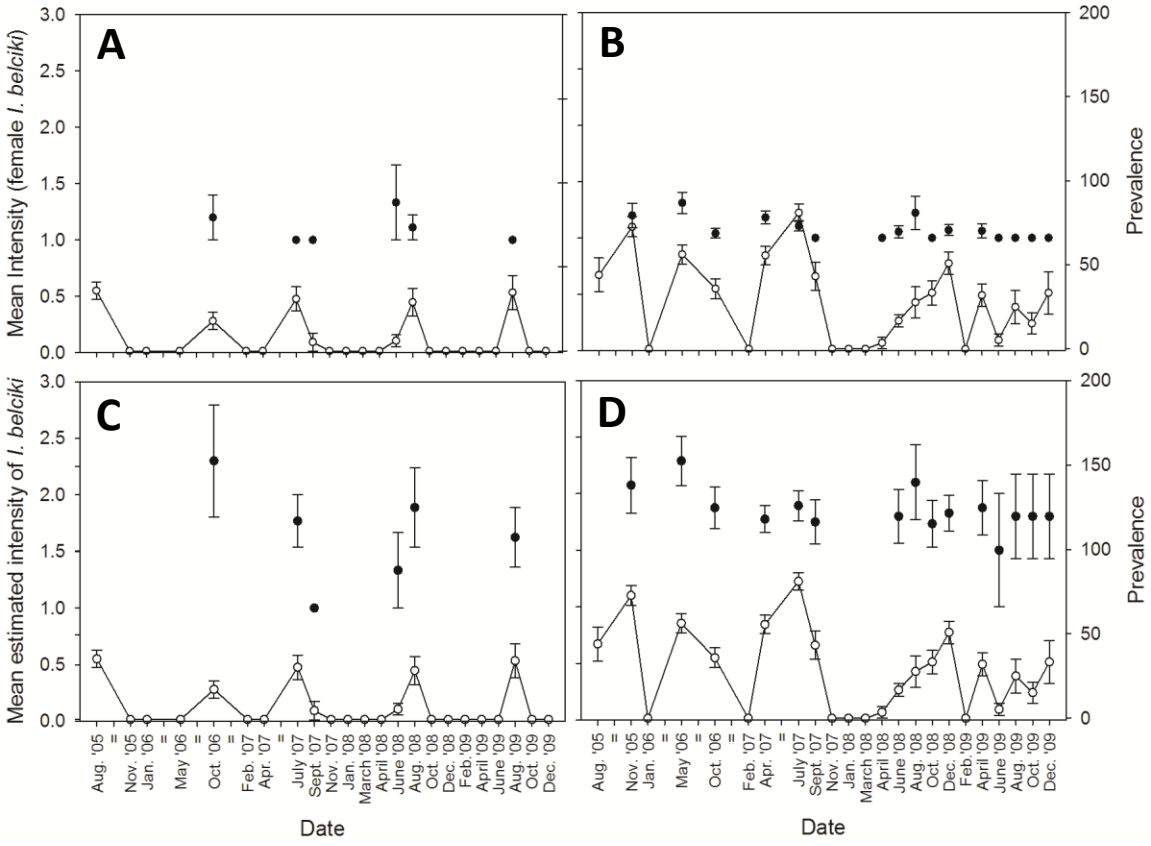


Fig. 3.7: Mean intensity (average number of copepods/ infected host) (closed circles without lines) and prevalence (percent of hosts infected) (open circles connected by lines) of *Ismaila belciki* by date of collection. Mean intensity of female copepods at (A) North Cove and (B) Fossil Pt. from superficial examinations of slugs from field surveys from November 2005 to December 2009 when hosts were present in the population. Mean intensity of all copepods at (C) North Cove and (D) Fossil Pt., estimated from the percentage of single and double female infections with zero, one, or two males per female from dissections of slug subsamples. Dates without plots of mean intensity had no hosts or no *I. belciki* present in the host population. Collection dates before July 2007 were not evenly distributed in time and dashed lines on the x-axis represent temporal intervals greater than two months. Error bars represent standard error.

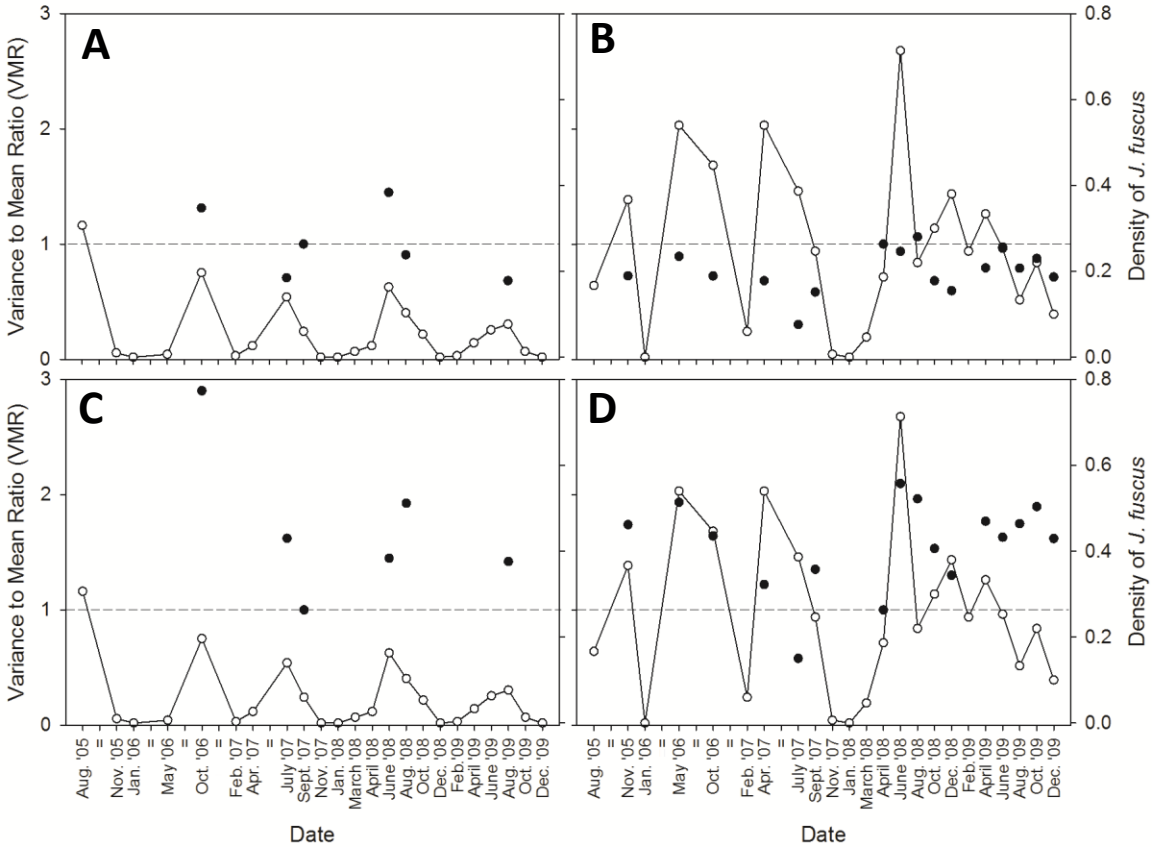


Fig. 3.8: Variance to mean ratios (VMR) of the abundance of *Ismaila belciki* (i.e., number of copepods/host in infected and uninfected slugs) (unconnected closed circles) and host density (open circle/solid line) by date of collection. VMRs of female copepods at (A) North Cove and (B) Fossil Pt. from superficial examinations of slugs from field surveys from November 2005 to December 2009. VMR of all copepods at (C) North Cove and (D) Fossil Pt., estimated from sex ratios of dissected specimens. The dashed horizontal line represents VMR=1 (variance = mean abundance, where the parasite is randomly distributed in the host population). VMR values <1 and >1, suggest regular and aggregated distributions, respectively, of parasites in the host population. Collection dates before July 2007 were not evenly distributed in time and dashed lines on the x-axis represent temporal intervals greater than two months.

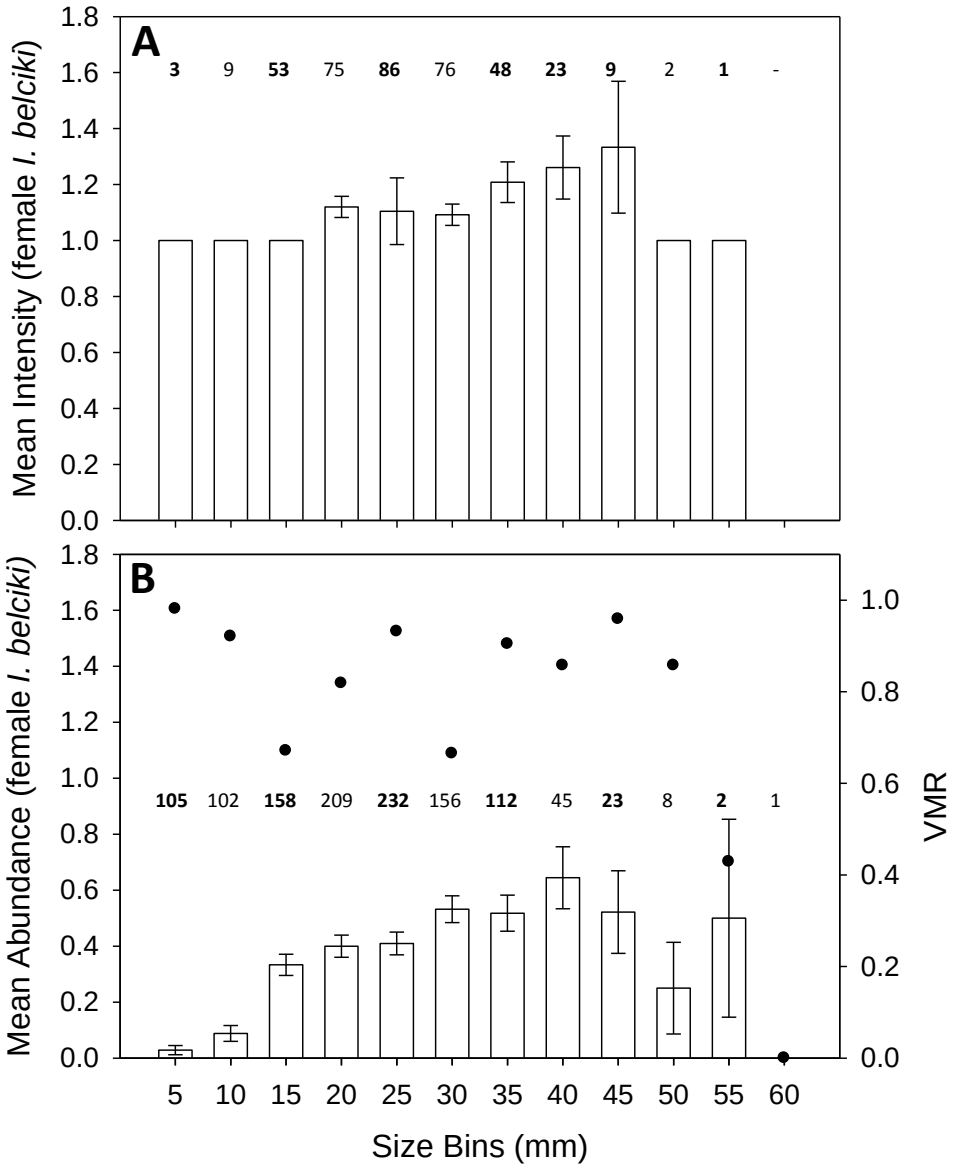


Fig. 3.9: Intensity measures (bars) of female copepods only by 5 mm size bins of *Janolus fuscus* (A) Mean intensity (i.e., average number of female *Ismaila belciki*/host, infected slugs only) and (B) mean abundance (i.e., average number of female *I. belciki*/slug, infected and uninfected slugs) and variance to mean ratio (closed circles) from North Cove and Fossil Point (combined) pooled over every collection period from November 2005 to December 2009 when hosts were present in the population. Sample sizes of infected (A) and all (B) hosts are displayed above each graph. Error bars represent standard error.

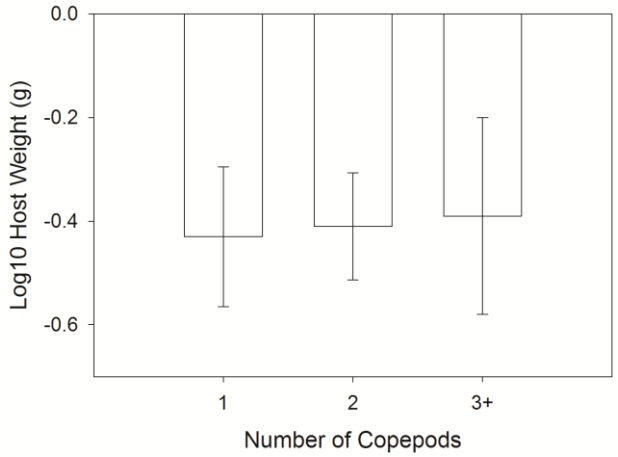


Fig. 3.10: Intensity of infection (number of *Ismaila belciki* per host) by $\log_{10}$ host weight. Larger hosts did not harbor significantly more parasites ($F_{43,3}$ =0.044, p=0.957).

3.3. Distribution of infection

Variance to mean ratios (VMR) for only female *Ismaila belciki* in the host surveys ranged from 0.7 to 1.4 at North Cove and from 0.3 to 1 at Fossil Pt., with an overall (sites combined) value of 0.9, suggesting the female parasite population may be underdispersed within the host population (Fig. 3.8 A, B, Table 3.4). In contrast, the coefficient of variation was over 100% (155%) indicating aggregation and the k value under one (-3.4), suggesting a negative binomial distribution indicative of parasite overdispersion in the host population (Table 3.4). Likewise, the overall (both sites) VMR (1.9), CV (171%), and k value (0.72) of total estimated parasite abundance (male and female *I. belciki*) suggest a weakly aggregated distribution within the host population (Table 3.4). VMR for estimated total intensities ranged from 1.5 to 2.9 at North Cove and 0.5 to 2.1 at Fossil Pt., with *I. belciki* being overdispersed (i.e VMR>1) in the host population at all dates when parasites were present except July 2007 (Fig. 3.8C, D).

3.4. Copepod mass and the crowding effect

Total parasite index ranged from 0.6 to 6.0% (average = 2.1 ± 0.3%). Female *Ismaila belciki* were much larger (6.4 ± 0.52 mg) than their male counterparts (0.42 ± 0.06 mg). Average parasite index was 1.7% (ranging from 0.5% to 4.5%) for female copepods versus 0.12% for male copepods. Regression analyses revealed a significant positive correlation between $\log_{10}$ host mass and both total copepod mass (r^2=0.231, slope=0.746, p=0.002) and single female copepod mass (r^2=0.218, slope=0.671, p=0.002) (Fig. 3.11A, B). The largest female index was greater for females of single (2.0 ± 0.19%) versus double (1.1 ± 0.31%) infections, though not significantly ($F_{38,2}$=3.14, p=0.085) (Fig. 3.12A). Total Parasite Index was not significantly different ($F_{38,2}$=0.099, p=0.755) in hosts with intensities of one (2.16% ± 0.22) or two (2.35% ± 0.64) female *I. belciki* and associated males (Fig. 3.12B). Average female copepod mass was not significantly different in slugs with single (7.0 ± 0.5 mg) versus double infections (5.0 ± 1.2 mg) ($F_{38,2}$=2.36, p=0.133) (Fig. 3.12C). Average female copepod mass varied positively with host mass (F=6.96, p=0.012). There was a significant difference in the weight of two female *I. belciki* occupying the same anterior position in the host (larger individual =8.6 ± 1.9 mg and smaller individuals = 2.7 ± 0.7 mg) (mean difference = 5.9 ± 1.6 mg, t=3.74, p=0.020, n=5) (Fig. 3.12D).

3.5. Orientation, attachment, and site specificity

Female *Ismaila belciki* in the anterior position of the host were in the hemocoel between the buccal mass and pericardial cavity (Fig. 3.13A, B). Females in this site were commonly oriented with the body axes perpendicular to that of their host, with heads located ventrally and urosomes dorsally, often protruding through the dorsal mantle of

the host (Fig. 3.13A, B). In this position a female's head and mouth was often attached to the ventral anterior-most ovotestis while she gripped the central mass and secondary sexual organs with her lateral processes (Fig. 3.13C, D, 3.14A). The orientation of females in the posterior position was variable but usually involved them lying with body axes parallel to that of ther hosts and gripping the host ovotestis. Sixty percent of females (including anterior and posterior individuals) and 5.3% of males had cephalic structures attached to the host ovotestis (Fig. 3.13D, 3.14A, B, C). Copepods were easily

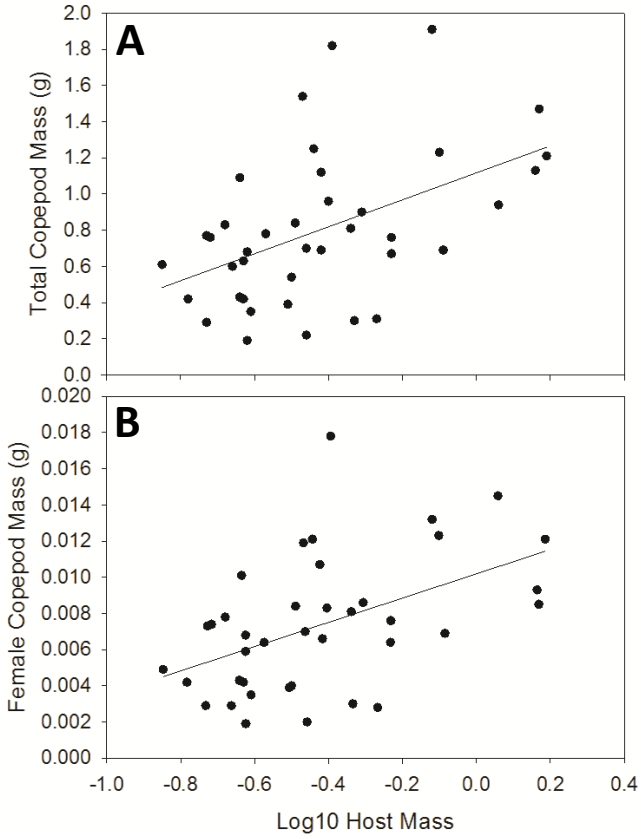


Fig. 3.11: Regression analyses of $\log_{10}$ host mass by (A) total copepod mass (r^2=0.231, slope=0.746, p=0.002) and (B) single female copepod mass (r^2=0.218, slope=0.671, p=0.002).

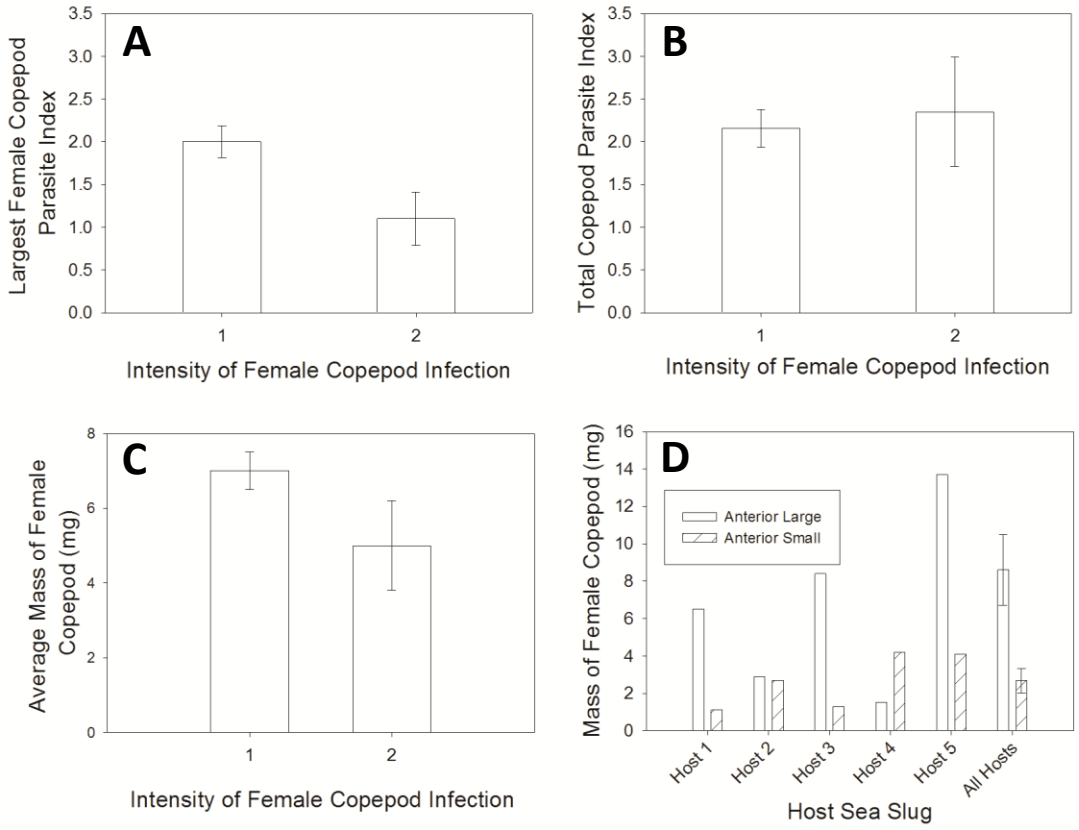


Fig. 3.12: Parasite indices and mass by intensity of infection and slug host. (A) The largest female copepod parasite index in single and double infections; (B) total copepod index in single and double infections of *Ismaila belciki*. (C) Mass of single female copepods (n=33) and the average mass of two female copepods (n=5). (D) Paired comparisons of two female copepods, one small and one large, both residing in the anterior site of the host hemocoel. Error bars represent standard error.

removed from the host's ovotestis and caused no detectable tissue damage, only slight indentions. Males were often (44.7%) associated with female copepods, lying "embraced" within her legs and lateral processes, frequently with genital pores in contact (Fig. 3.14D).

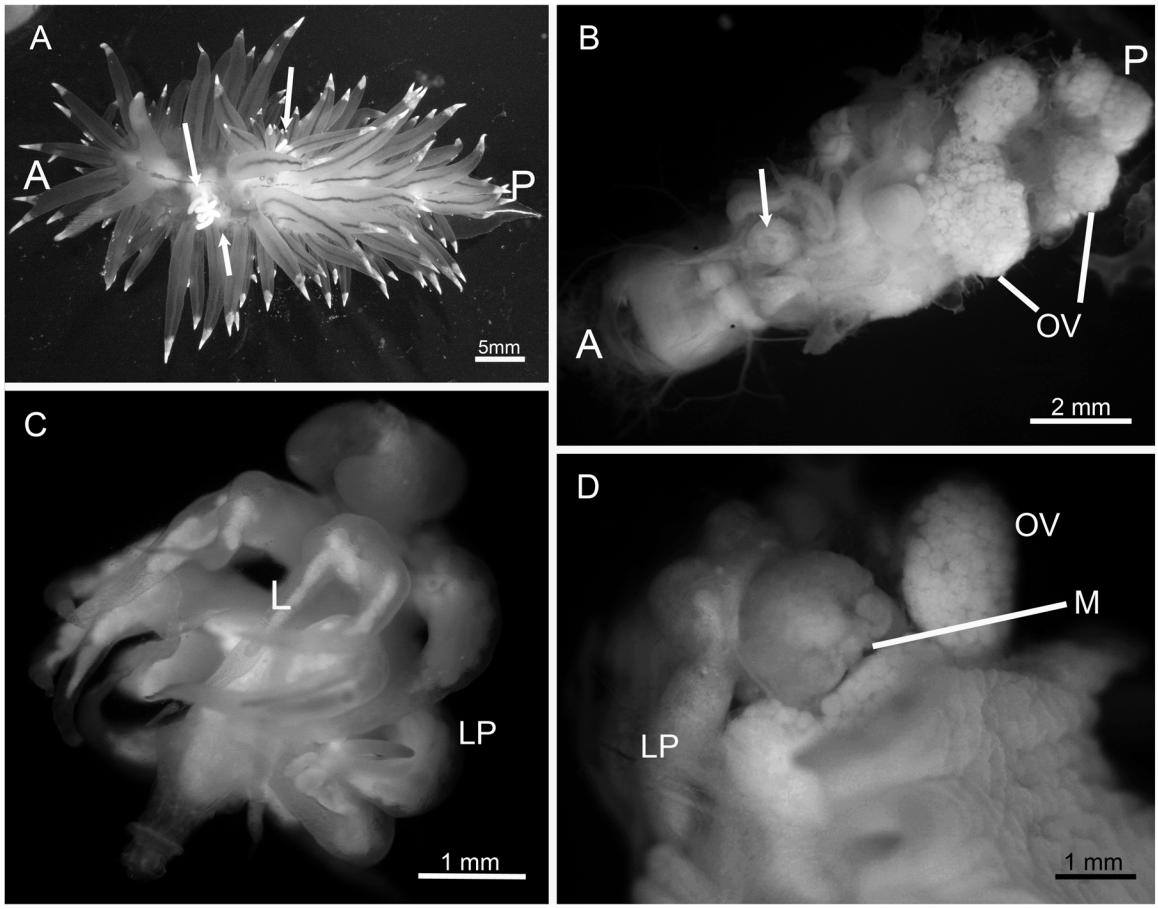


Fig. 3.13: Light micrographs of dissections of *Janolus fuscus* and female *Ismaila belciki*. (A) *J. fuscus* infected with three female *I. belciki*, two in the anterior position and one in a lateral position, indicated by white arrows. (B) Dorsal view of a whole dissection of *J. fuscus* showing inverted orientation of female *I. belciki* relative to host. Arrow points to the urosome (posterior) of the female copepod. (C) Female *I. belciki* with appendages and lateral processes filled with gonad. (D) Female *I. belciki* with mouth attached to anterior, ventral host ovotestis and ventral appendages and lateral processes gripping host ovotestis and secondary sexual organs. A (anterior), L (legs), LP (lateral processes), M (female copepod mouthparts), P (posterior), OV (ovotestis).

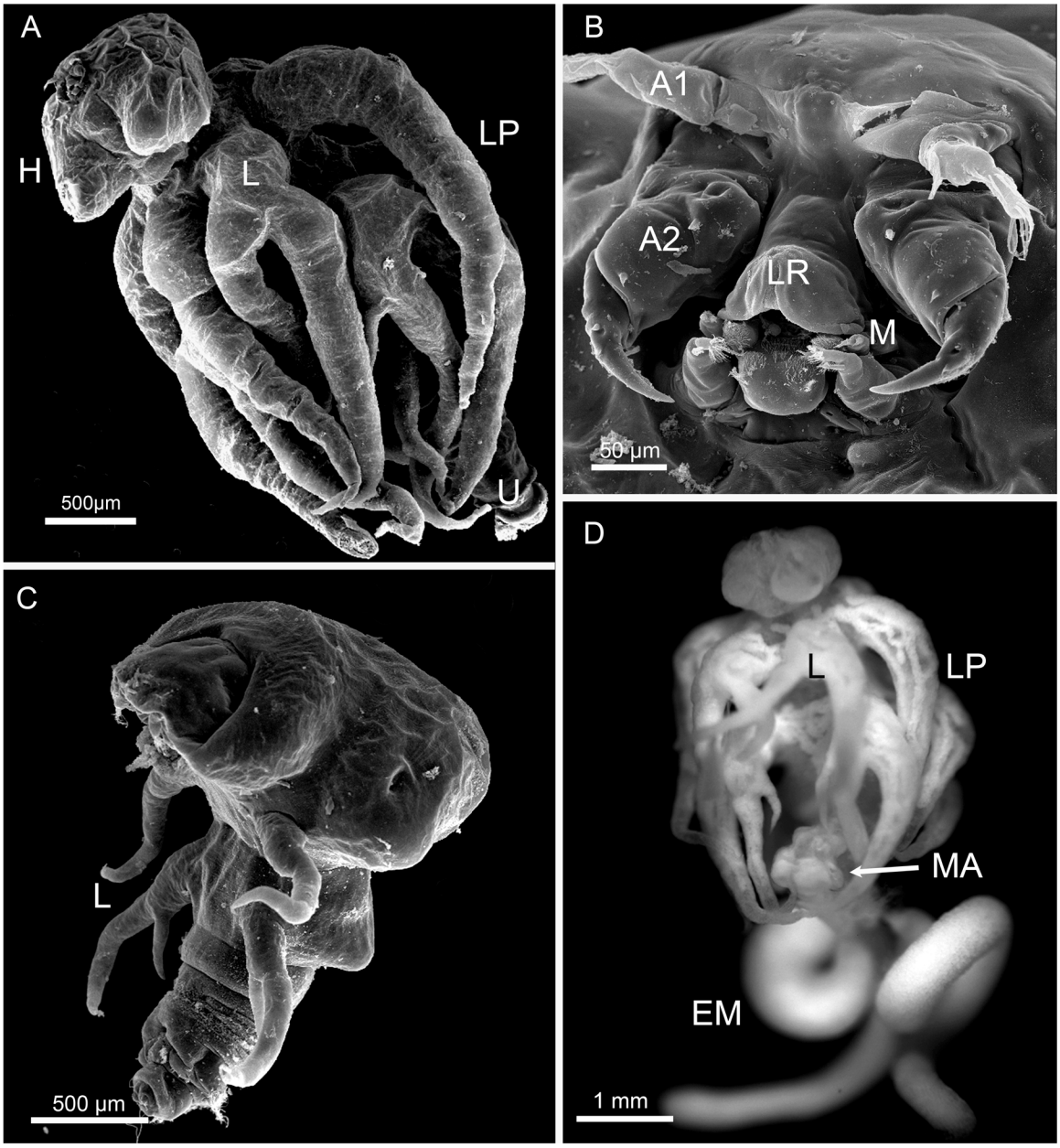


Fig. 3.14: Scanning electron and light micrographs of female and male *Ismaila belciki*. (A) SEM of female *I. belciki*; (B) SEM image of same female's cephalic structures; (C) SEM image of juvenile male *I. belciki*; (D) Male *I. belciki* (arrow) in "embrace" of female. Note the female's gonad-filled appendages, lateral processes and two egg masses. A1 (first antenna), A2 (second antenna), EM (egg masses), H (head), L (legs), LP (lateral processes), LR (labrum), M (mandible), MA (male copepod), U (urosome).

Janolus fuscus collected from field surveys showed that the majority of infections were single anterior females ($F_{48,4}$=599.05, p<0.0001) (Bonferroni post-hoc p<0.05) (Fig. 3.15). Likewise, in dissections of slugs infected with female *Ismaila belciki*, 78.3% had a single infecting female in the anterior position and 4.3% of hosts had a single female in the posterior position (n=69) (Fig. 3.16A). Double infections with a) one anterior and one posterior female and b) two anterior females were each found in 8.7% of hosts (Fig. 3.15). In hosts with a double infection with a female in the anterior and posterior sites, hosts had equal numbers of larger anterior females and posterior females (Fig. 3.16B). A paired t-test showed no significant difference in mass of female *I. belciki* that occupied an anterior versus posterior position within the host (t=0.238, p=0.827, n=4) (Fig. 3.16B).

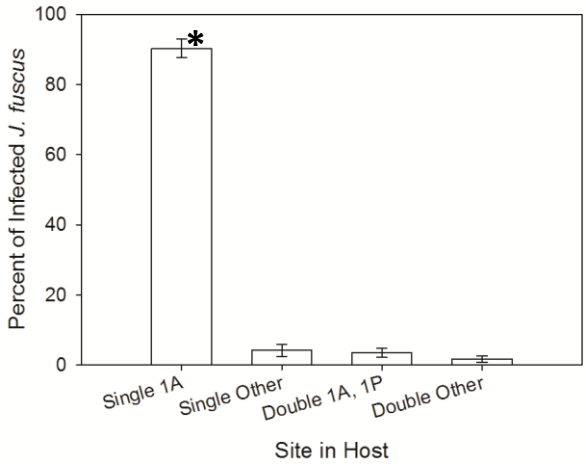


Fig. 3.15: Site specificity of female *Ismaila belciki* in single and double infections from field surveys of slugs. Sites were combined and each collection date a replicate (n=12) of the percent of infected individuals residing in the four most common sites in the host: Single 1A (a single female located in the anterior hemocoel of the host just anterior to the host pericardium), Single Other (a single female located in any position other than the anterior position within the host), Double 1A, 1P (two females, one in the anterior position, one in the posterior position (just posterior of the pericardium)), Double other (two females in any combination of positions other than one anterior and one posterior). Error bars represent standard error.

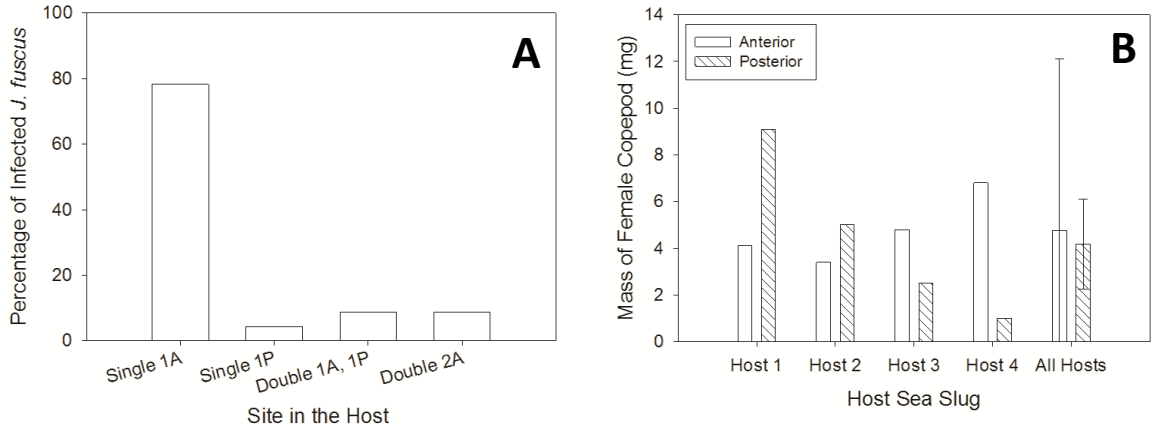


Fig. 3.16: Site specificity and mass of female *Ismaila belciki* dissected from host slugs (A) The percentage of infected *J. fuscus* with a female *I. belciki* residing in the four most common sites in the host: Single 1A (a single female located in the anterior hemocoel of the host (just anterior to the host pericardium)), Single 1P (a single female in the posterior position (just posterior of the pericardium) within the host), Double 1A, 1P (two females, one in the anterior position, one in the posterior position), Double 2A (two females in the anterior position) (n=69). (B) Paired t-test comparison of female copepod mass in anterior versus posterior individuals in four hosts with double infections. Error bars represent standard error.

4. Discussion

Prevalence of *Ismaila belciki* ranged from 0-81% within the host population. At North Cove and Fossil Pt., *I. belciki* were always present when host population densities were above 0.1 slugs/m^2, with occasional infections at 0.06 slugs/m^2. This suggests that *I. belciki* may require a host density threshold between 0.05-0.1 slugs/m^2 before they are able to successfully establish and be maintained within the host population. The existence of a critical host density, below which the parasite population cannot be maintained, is a key feature in any host/parasite relationship (Anderson and May, 1979; May and Anderson, 1978, 1979; Blower and Roughgarden, 1987, 1989). At the host population level, host density was a significant covariate in all analyses, suggesting that

variation in measures of parasite occurrence at different sites and during different seasons may be attributed, in part, to differences in density of the host population. For example greater overall prevalence at Fossil Pt. compared to North Cove is likely a function of consistently greater host density at the former site. Concurrent work suggests that these differences in host density at these two sites and by season (spring through fall) and the resulting changes in prevalence of *I. becliki*, may be attributed to differences in abundance of the arborescent bryozoan prey of *J. fuscus*, namely *Bugula pacifica* and *Tricellaria circumternata* (chapter II). However, the absence of *J. fuscus* during winter is better explained by winter storm effects, not prey abundance (chapter II). Unlike parasites infecting long-lived hosts with stable populations, *Ismaila belciki* must establish itself in a population of short-lived hosts that are absent (as adults) from the intertidal populations every winter before returning as small recruits that grow into the adult host populations by spring. This suggests that the life cycle of *I. belciki* tracks that of the host, disappearing in winter and rapidly re-establishing itself within the host population in spring. Two measures of parasite abundance, the number of infected *J. fuscus* and the total number of female copepods, coincide with peak densities of the host at Fossil Pt. and North Cove, in spring and summer, respectively. However, at Fossil Pt. prevalence of *I. belciki* is higher (on average) in summer and fall than spring. This suggests that, once established, the parasite population infects a greater percentage of the population over time, even if the number of total copepod and infected individuals in the population is lower as there are fewer hosts overall. These data suggest *I. belciki* is able to rapidly establish and accumulate in the host population over time, even in such short-lived hosts.

Few studies have examined spatial and temporal variation in prevalence of a splanchnotrophid species, with two notable exceptions (Hecht, 1895; Schrödl, 2002). Schrödl (2002) found little variation in prevalence of *Ismaila* species across years and seasons in central Chile. Although most host populations were quite stable, Schrödl (2002) only found the nudibranch *Okenia luna* and its parasite *Ismalia androphila* in the south during summer. Hecht (1895) observed that the prevalence of a *Splanchnotrophus* species differed by year and was seasonally frequent in the nudibranch *Aeolidia papillosa* from Europe. While different seasons were not examined, Belciki (1965) found 62% of *J. fuscus* from Coos Bay, Oregon were infected with *I. belciki* during June and July at Fossil Pt., North Cove, and the Charleston boat basin. These studies suggest that stable populations of opisthobranch hosts along the central Chilean Coast and Fossil Pt. (in the current study) may not show significant seasonal effects (apart from their absence in winter in Oregon), but that host populations that are ephemeral or have very low densities with a single seasonal peak in abundance, such as *Aeolidia papillosa* in Europe and *J. fuscus* at North Cove, OR, only support the parasite population during a limited season or a few months throughout the year.

Host density was much lower at both sites during 2009 than previous years. While differences in biotic (e.g., prey abundance) or abiotic factors (e.g., oceanographic conditions) during this year may be the cause of this decline in the host and parasite population, my continuual sampling of these slug populations from 2005-2008, may be a contributing factor. Continual sampling of the population of *J. fuscus* or its prey may have led to a decline in the host population density. This could cause a subsequent decline in parasite prevalence and intensity of infections. While it is possible that I

reduced population sizes, I think my impact of the population was relatively low because I returned slugs to their respective populations unless needed for experiments or dissections. Bryozoans were only collected from sites during surveys and the volume collected was minimal (500 ml) compared to the overall bryozoan population at each site.

Infected *Janolus fuscus* are larger than their uninfected counterparts. While larger size classes are more likely to be infected, only 35-50% of intermediate and large size classes are infected. Additionally, I found a few infected recruits/juveniles (<5 mm). Possible explanations for these patterns include: 1) infection rate is similar over time, and small individuals are less likely to be infected only as a product of less exposure time to infective larval stages of *I. belciki*; 2) larger slugs are more likely to be infected (as a product of being larger targets to infective stages or more susceptible to infection); 3) *I. belciki* may cause high mortality of smaller (i.e. younger) hosts or 4) *I. belciki* may cause increased growth in *J. fuscus* (Baudoin, 1975). While I found infected individuals in all size classes, nothing is known about the infection process by splanchnotrophid copepods or changes in host immunity based on size (age), though increased resistance with size is common in intermediate host snails facing larval trematode infection (reviewed in Lim and Heyneman, 1972). This would not lead to larger infected individuals in the population as seen in this study. Likewise, very small (<5 mm) infected slugs are quite rare and their survivorship has not been compared to uninfected slugs of similar size to determine if mortality is higher in young hosts.

The fourth alternative, parasite-induced growth in infected hosts, has been extensively examined in parasitic castrator systems such as larval digenean trematodes on intermediate gastropod hosts and crustacean castrators (e.g., epicarid isopods or

rhizocephalan barnacles) on crustacean hosts (Rothschild 1936, 1941; Baudoin, 1975; O'Brien and Van Wyk, 1985; Sorensen and Minchella, 2001; Hechinger, 2010). While *Ismaila belciki* is not a complete castrator like its Chilean congener *I. damnosa* (Schrödl, 1997), the former does cause a significant decrease in the reproductive output of its host (chapter V). Additionally, the mass of *I. belciki* is positively correlated with host mass and a single female copepod may occupy 0.5% to over 4% of the of host mass, characteristics shared with true castrators (Kuris, 1974; Lafferty and Kuris, 2002). Parasitic castrators may cause an increase in host growth, termed "gigantism" or reduce mortality without affecting growth by cutting off host reproduction, allowing the host to redirect energy to growth and/or survival (Rothschild 1936, 1941; Kuris, 1974; Baudoin, 1975; O'Brien and Van Wyk, 1985; Sorensen and Minchella, 2001; Hechinger, 2010). Such castrator systems show highest prevalence (80-100%) in the largest size classes of host populations (Rothschild, 1941; Sousa, 1983; Hechinger, 2010). In contrast, only 33-50% of *J. fuscus* in the largest size classes were infected. This may be a result of the short lifespan (~5 months) during which *J. fuscus* is exposed to infective larval stages compared to many of the relatively longer lifespans of some hosts housing parasitic castrators (Rothschild, 1936, 1941; Sousa, 1983; Sorensen and Minchella, 2001; Hechinger, 2010). However, concurrent studies suggest that patterns in growth rates differ between infected and uninfected slugs. In contrast to uninfected slugs in which growth rate decreases with size, the opposite is true for infected slugs (chapter V). Additionally, *I. belciki* may cause decreased survivorship in larger (older) *J. fuscus* (chapter V). This combination could explain why infected individuals are larger than

uninfected counterparts during most collection dates and why prevalence is not extremely high (80-100%) in the largest size classes of the host population.

In contrast to Belcik's (1965) report that "it was not unusual to remove as many as five females and four males (*Ismaila belciki*) from a single host," I found hosts with intensities above two female copepods to be extremely rare, only 1.3% of 76 infected individuals dissected and 0.5% of over 1100 *J. fuscus* examined superficially over the four year sampling period. One explanation for this inconsistency may be the sites of collection. While he did collect some copepods from both Fossil Pt. and North Cove, the majority of copepods collected by Belcik (1965) were from the small boat basin in Charleston, OR. As the number of infected or uninfected hosts was not recorded, I cannot make inferences about the distribution of parasites in the population during Belcik's study. The present study examined the Fossil Pt. and North Cove populations of *J. fuscus* but rarely collected hosts from the docks, where hosts tended to be less available throughout the year. However, those few hosts collected from the docks often had double infections.

While female *Ismaila belciki* were underdispersed (VMR=0.9), overall copepod (i.e., male and female) dispersion was weakly aggregated (VMR=1.9) in the host population. This overall variance to mean ratio of parasite abundance was above one (1.9), which is lower than VMRs reported for many macroparasites (with VMRs ranging below one to over 1000), but more dispersed than the distributions characteristic of some parasitic castrator species (Kuris, 1974; McDermott, 1991; Shaw and Dobson, 1995; Rohde et al., 1995; Karvonen et al., 2006; Dippenaar et al., 2009; Lafferty and Kuris, 2009; Monello and Gompper, 2010). The coefficient of variance and k values of both

female only and male and female copepods also suggest that *I. belciki* are overdispersed in the host population. While the vast majority of *J. fuscus* were uninfected or had low intensities, frequency by intensity curves (Fig. 3.6) do not have the right-skewed tail with extremely high intensities that are indicative of aggregated distributions of many macroparasite species in their host population (Fisher, 1941; Crofton, 1971; Shaw and Dobson, 1995; Karvonen et al., 2006; Dippenaar et al., 2009). Instead, I see an abrupt absence of intensities above six total copepods, with rare individuals (n=2) supporting as many as 11 *I. belciki* (with 5-6 females). Crofton (1971) suggests this "truncated" binomial distribution may be a result of parasite-induced host mortality at high intensities of infection. Other factors that may lead to underdispersion of a parasite species are density-dependent processes such as intraspecific competition for limited resources in the host or an immunological response of the host that increases disproportionately faster than the parasite intensity (Pennychuick, 1971; Anderson and Gordon, 1982; Croll et al., 1982; Shaw and Dobson, 1995). Factors that may oppose these effects and lead to overdispersion include gregarious settlement of infective larvae and heterogeneity of susceptibility and/or immunological response among hosts (Crofton, 1971; Pennychuick, 1971; Anderson and Gordon, 1982; Shaw and Dobson, 1995).

Identifying the processes responsible for creating patterns of parasite distribution in the host population is difficult and thus discussed with caution here. Anderson and Gordon (1982) suggested that parasite-induced host mortality would cause a convex age-intensity curve, with peak mean intensity of infection in intermediate aged hosts and a decline in mean intensity and the variance to mean ratio in the oldest age classes as a product of rapid host mortality with greater intensity of infection. Infected *J. fuscus* may

experience decreased survivorship compared to uninfected slugs, but as double female infections of *I. belciki* were rare, I could not compare mortality rates in hosts with varying intensities of infection (chapter V). A mean intensity-by-size plot (Fig. 3.9) shows larger individuals with lower mean intensities than hosts of intermediate sizes, but VMRs do not decrease except in the very largest size class (Fig. 3.9). Additionally, there was no significant relationship between intensity of infection and host size to suggest larger individuals have lower intensity of infection. Finally, small sample sizes of the largest size classes make interpretation of these patterns difficult and unreliable (Anderson and Gordon, 1982).

There is weak evidence for a crowding effect (i.e., density-dependent process) that could limit overdispersion in *Ismaila belciki*. Though not significant, single female *I. belciki* had a higher parasite index and mass than the largest female or average mass of two females in double infections, an indication that crowding may limit growth under these conditions. If there were no limitations to parasite growth, I would expect the total parasite index for double female infections to be double the total parasite index for single female infections (with variation due to difference in number of male copepods and the timing of infection). Although I found the total copepod index in double infections to be larger on average than that of single infections, it was not double. Additionally, there was a significant difference in the mass of two female *I. belciki* occupying the same anterior position within the host. Theses analyses were conducted with very few (n=5) samples of hosts with double female infections, limiting my statistical power. Intensities above two were too infrequent to analyze, but I would expect crowding to be even stronger in rare host specimens with higher intensities of infection. While the causative

mechanism for this potential crowding effect is unknown, studies examining this phenomenon in cestodes list exploitative competition, interference competition, and host immune response as potential explanations (Bush and Lotz, 2000; Robert, 2000; Heins et al., 2002). In my study, differences in timing of infection may also impact parasite size within the host as I could not control for time of infection. There is a significant positive linear relationship between host and female parasite mass, suggesting *I. belciki* and their hosts grow together. Therefore, a female infecting earlier would be larger than subsequent infecting females.

Nothing, to my knowledge, is known of the life cycle of splanchnotrophid copepods between the third planktotrophic naupliar stage and the second copepodid stage found within the host (Belcik, 1965; Dudley, in Belcik, 1965; Ho, 1987b; chapter VI). Thus, the process by which the infective larval stage identifies an uninfected or already infected host (containing a potential mate) and their propensity to gregariously settle on host slugs is unknown. Studies on ectoparasitic copepods infecting teleost fish suggest that infective copepodid stages respond to depth, light, and salinity cues as well as mechanical stimuli that cause them to aggregate in areas where host fish are found (Boxshall, 2005; reviewed in Mordue and Birkett, 2009). Additionally, they respond to chemical cues that aid in both host and mate location (Richie et al., 1996; Mordue and Birkett, 2009). For example, sex pheromones released by virgin female sea lice (*Lepeophtheirus salmonis*) are detected by sensory receptors on the first antennae of males and used to locate females (Mordue and Birkett, 2009). Identifying hosts already infected with a conspecific may be more difficult in endoparasitic copepods, but the protrusion of the female urosome (posterior) through the host integument could function

in attracting potential mates as well as producing external egg masses. While *I. belciki* may respond to chemosensory cues that could lead to aggregation in the host population, the high percentage of unmated females found in dissections suggests a limiting factor.

One potential consequence of underdispersed or weakly aggregated parasite populations is potential trouble in finding a mate (May, 1977; Croll et al., 1982; Shaw and Dobson, 1995). The probability that a parasite is mated depends on the sexual habits of the species and the distribution of the parasite in the host population (May, 1977; Croll et al., 1982). For dioecious, polygamous macroparasites (specifically helminthes) May (1977) plotted mean intensity by the probability of finding a mate for parasites with negative binomial (i.e., aggregated) and Poisson (i.e., random) distributions. For a mean intensity of 1.1-1.9, as found in *Ismaila belciki*, he suggests the probability of being mated in an aggregated versus random parasite population ranged from roughly 0.7-0.8 and 0.5-0.65, respectively (May, 1977; Croll et al., 1982). Only 52% of female *I. belciki* in dissections had a mate. While substantial differences likely exist between crustacean parasites and helminthes for which the model was designed, the fact that *I. belciki* are weakly aggregated in the host population may, in part, account for this lower probability of being mated. In this study, single female infections were the most common type of infection and hosts with a solo male did occur. As expected, solo females do not produce egg masses (chapter V). As the fitness of female (or male) *I. belciki* is zero without a mate, the low probability of finding a mate may be a serious limiting factor in the reproductive success of *I. belciki* within some *J. fuscus* populations. In contrast, I know of no studies of other splanchnotrophids that report frequent solo infections; all females had at least one (and up to 12) potential mate(s) in a host (Hancock and Norman, 1863;

Schrödl, 2002; Haumayr and Schrödl, 2003; Marshall and Hayward, 2006). However, Marshall and Hayward (2006) found a fixed sex ratio of 1:1 of *Splanchnotrophus willemi* in the host; they suggested an underlying unknown mechanism which prevents infection, or the maturing, of additional males when there is already a mature male present. The evolution of these different reproductive strategies among parasite groups presents intriguing possibilities for future research.

As reported by Belcik (1965), females of *Ismaila belciki* were typically found anteriorly, behind the host ganglia and anterior to the pericardium and, more rarely, posterior of the pericardium in the host hemocoel. Often females of *I. belciki* were oriented with the body axes perpendicular to that of their hosts with urosomes penetrating the dorsal surface of hosts and producing two egg masses. Site specificity of parasites within the host is unlikely to arise by chance, but rather as an evolutionary adaptation and a likely product of co-evolution with their host (Salmen, 2008a; Janovy, 2009). Hecht (1893) and Jensen (1987) note that splanchnotrophids are associated with opisthobranchs that have dorsal or lateral projections, like cerata or papillae, as these "outgrowths" may provide camouflage for the copepod egg masses against potential predators (e.g., fish and crabs) (Salmen, 2008a). *Janolus fuscus* have cerata that may act in this way. These egg masses are not within or associated with the gill circle and thus, are unlikely positioned for increased aeration as suggested by Hancock and Norman (1863). However, the common dorsal, anterior position of the egg masses is in an area with low density of cerata, which may allow for greater water flow and fewer boundary layers to aid in dispersing newly-hatched nauplii.

Females found in the anterior position in the host are not significantly larger than posterior counterparts in the same host, suggesting that anterior females are not in a more advantageous position for attaining nutrition from the host. While, 60% of female *Ismaila belciki* had their cephalic appendages attached to host gonad, they were easily removed with no evidence of tissue damage. This supports other studies that propose that splanchnotrophids are hemolymph suckers, not oophagous or tissue consumers (Haumayr and Schrödl 2003; Salmen et al., 2008a). Thus, access to host ovotestis or other organs may not impact site specificity seen in *I. belciki*. Alternatively, *I. belciki* may prefer this anterior position between the host's buccal mass and pericardium, as it provides more free space than anywhere else within the host hemocoel. Coevolution in a parasite and its host typically leads a parasite to optimize the space available within the host to maximize its own growth while minimizing damage to the host, thus increasing the parasite's longevity and reproductive success (Salmen et al., 2008a; Roberts and Janovy, 2009). Schrödl (1997) found that *I. damnosa* completely castrated its host, the nudibranch *Flabellina* sp. 1 and caused increased mortality. He interpreted gonad destruction (or atrophy) in the small hosts (10-25mm) as space competition, not active tissue feeding (Schrödl, 1997; Haumayr and Schrödl, 2003). Female *I. damnosa* are large (up to 3mm) relative to *Flabellina* sp. 1 (average 10-18mm) and are located posterior to the pericardium where they were likely to compete for space typically filled with host ovotestis (Schrödl, 1997). In contrast, female *I. belciki* may have evolved a preference for the more open anterior position in the host to minimize the impact on host organs and ovotestis, increasing the hosts, and therefore its own, survival and reproductive fitness.

Male *Ismaila belciki* were typically associated (44%) with females but could be found on host ovotestes (5%) or freely in the host hemocoel. This suggests that the position of males in the host is primarily related to mate position but they exhibit a freedom of movement throughout the host body reported in other male splanchnotrophid copepods (Hancock and Norman, 1863; Marshall and Hayward, 2006; Salmen et al., 2008a). Marshall and Hayward (2006) and Salmen et al. (2008a) did not find males of *S. willemi* and *Ceratosomicola* spp., respectively, always closely associated with female copepods in most host specimens. In these hosts, the sex ratio of female to male copepods was almost, if not always, 1:1 (Marshall and Hayward, 2006; and Salmen et al., 2008a). The authors hypothesize that without competition among males for paternity there may be no need for the close association of the male to the female copepod, except during sperm transfer. In contrast, every host containing an *Ismaila* species from Chile contained at least one male associated with a female (Haumayr and Schrödl, 2003). As sex ratios are more variable in Chilean *Ismaila* species and in *I. belciki* from Oregon, competition for female mates may play a more substantial role in controlling the duration of male and female association.

Many aspects of the life history of *Ismaila belciki* described above and in concurrent studies suggest that this species is a rare intermediate between a true parasitic castrator and a typical parasite, as defined by Lafferty and Kuris (2002, 2009). Female *I. belciki* are large relative to their host, with a parasite index ranging from 0.5% to over 4%, values above that reported for typical parasites but on the low end of those reported for parasitic castrators (Kuris, 1974; Kuris and Lafferty, 2000; Lafferty and Kuris, 2002, 2009). Similar to parasitic castrators, total copepod mass and female copepod mass are

positively correlated with host size, suggesting the parasite grows with the host, a trait less common in typical parasites (Lafferty and Kuris, 2002, 2009). Additionally, female *I. belciki* exhibit high site specificity with some evidence for intraspecific competition within the host, both traits that would lead to the underdispersed distribution of female *I. belciki* (but weakly aggregated distribution of male and female copepods) seen in the host population. Parasitic castrators are often highly site specific within the host, leading to intense intra- and interspecific competition (Kuris and Lafferty, 1994; Lafferty and Kuris, 2009). While typical parasites are often aggregated (i.e., overdispersed) in the host population, parasitic castrators are often underdispersed or have uniform distributions in the host population. Most importantly, concurrent studies show that *I. belciki* significantly decreases, but does not entirely eliminate, host reproduction (chapter V).

5. Conclusion

Prevalence of *Ismaila belciki* varied by site and season and increased with the density of *Janolus fuscus*. Intensities of infection generally followed peaks in prevalence and host density. *Ismaila belciki* is weakly aggregated in the host population. There is some evidence that intraspecific competition (i.e., crowding effect) is at play within the host, potentially leading to underdispersion of female parasites. *Ismaila belciki* are highly site specific, occupying an anterior position within the host. The cephalic appendages of female copepods are often attached but cause no tissue damage to the host gonad, supporting the hypothesis that *Ismaila* spp. may be hemolymph suckers. These observations, combined with concurrent studies of the impact of *I. belciki* on growth, survivorship, and reproduction of *J. fuscus* suggest, *I. belciki* may be a rare intermediate between a parasitic castrator and a typical parasite.

6. Bridge III

In chapter IV, I found that *Ismaila belciki* exhibit many characteristics similar to parasitic castrators. These copepods are weakly aggregated in two intertidal host populations; infections with more than two female copepods were rare. Female copepods are large relative to the host and their mass is positively correlated with their host's mass. Additionally, female *I. belciki* are highly site specific, and there is some evidence of intraspecific competition within the host. However, to be a true castrator a single parasite (mating pair or genotype) must be able to completely block host reproduction (Kuris, 1974; Kuris and Lafferty, 2000; Lafferty and Kuris, 2002, 2009). The only study to examine the impact of a splanchnotrophid copepod on its opisthobranch host, found the copepod caused decreased survival and complete castration (Schrodl, 1997). In chapter V, I examine the impact of infection with *I. belciki* on the reproduction, growth, and survivorship of *J. fuscus* and compare these findings to parasitic castrators and typical parasites.

CHAPTER V

IMPACTS OF AN ENDOPARASITIC COPEPOD, *ISMAILA BELCIKI*, ON THE REPRODUCTION, GROWTH, AND SURVIVORSHIP OF ITS NUDIBRANCH HOST, *JANOLUS FUSCUS*

1. Introduction

Models on the evolution of virulence in parasites predict that a parasite should balance its need for longevity with the costs of increasing host mortality (i.e., host consumption), as early death of a host reduces parasite lifespan and therefore reproductive duration (May and Anderson, 1979; Ebert, 2004; Lafferty and Kuris, 2009). Parasitic castration (the complete elimination of host reproduction by a single parasite) (Lafferty and Kuris, 2009), is an alternative strategy to deal with the virulence tradeoff. By preventing host reproduction, the parasite frees energy normally allocated to host reproduction without damaging host somatic tissue, thereby prolonging life span of both the host and parasite (Kuris, 1974; Gorbushin and Levakin, 1999; Arnott, 2000; Ebert, 2004; Lafferty and Kuris, 2009; Hechinger, 2010). Diverting energy away from reproduction also may lead to increased somatic growth and gigantism in infected hosts (Rothschild, 1936, 1941; Baudoin, 1975; Minchella et al., 1985; Ebert et al., 2004; Hechinger, 2010). Castration is predicted as a consumer strategy in parasites of long-lived hosts that invest heavily in reproduction (e.g., some invertebrates and small fish),

enabling the castrator to reap future reproductive benefits of a potentially prolonged life with an ample energy source (Hurd, 2001; Ebert, 2004, Lafferty and Kuris, 2009).

Characteristic traits of parasitic castrators differ in their ecological and evolutionary consequences from typical parasites (Kuris, 1974; Kuris and Lafferty, 2000; Lafferty and Kuris, 2002, 2009). First, reduction in host fecundity is intensity-independent, achieved by a single parasite (mated pair or genotype) that typically ranges in size from 3% to 50% of the mass of its host (Kuris, 1974; Kuris and Lafferty, 2000; Lafferty and Kuris, 2002, 2009). Second, host and parasite sizes are often positively correlated, suggesting the pair grow in parallel. Due to their large sizes, castrators may exhibit strong site specificity and intra- or inter-specific competition within the host (Read, 1951; Kuris and Lafferty, 1994; Roberts, 2000; Lagrue and Poulin, 2008; Lafferty and Kuris, 2009). This in turn can lead to an underdispersed, sometimes nearly uniform, distribution of the parasite in the host population, with each individual host harboring very few parasites or mated pairs (Kuris, 1974, 1980; McDermott, 1991; Glenner et al., 2003; Fogelman et al., 2009; Lafferty and Kuris, 2009). In contrast, the impact of a typical non-castrating parasite on host growth, survivorship, and/or reproduction is often intensity-dependent (i.e., host morbidity increases with intensity of infection) (Lafferty and Kuris, 2002). A single typical parasite is often orders of magnitude smaller than its host (with an upper limit of 1% of the host size); parasite size is rarely positively correlated with host size (Lafferty and Kuris, 2002, 2009). While many typical parasites show preferred positions within their hosts and experience intraspecific competition, their relatively small sizes may make this effect less intense than in castrating species (Kuris, 1974; Lafferty and Kuris, 2009). Finally, typical parasites are almost always aggregated

in the host population, with the majority of hosts being uninfected or with low intensity of infection and a few hosts supporting most of the parasite population (Anderson and Gordon, 1982; Anderson, 1986; Shaw and Dobson, 1995; Karvonen et al., 2006; Barson et al., 2008; Webb, 2008; Dippenaar et al., 2009).

Copepods from the family Splanchnotrophidae (Order Poecilostomatoida) are large endoparasites that inhabit the main body cavity and/or cerata of opisthobranch gastropods worldwide. *Ismaila* is the most speciose of the five described splanchnotrophids, with eleven described host-specific species. While extensive studies have addressed the morphology and systematics of splanchnotrophids (Hancock and Norman, 1863; Bergh, 1868; Belcik, 1965, 1981; Ho, 1981, 1987; Jensen, 1987; Huys, 2001; Haumayr and Schrödl, 2003), information on the effects of these parasites on host condition vary significantly and are typically based on dissected specimens (Bergh, 1868; Jensen, 1987, 1990; Haumayr and Schrödl, 2003; Marshall and Hayward, 2006; Salmen et al., 2008a,b) with one notable exception (Schrödl, 1997), which was an experimental study. Reports suggest that some splanchnotrophid species inflict no obvious internal damage to their hosts (Jensen, 1990; Haumayr and Schrödl, 2003; Salmen et al., 2008a, b), while others cause atrophy or damage of the host's gonad (Bergh, 1968; Jensen, 1987; Schrödl, 1997; Haumayr and Schrödl, 2003; Marshall and Hayward, 2006). Still other species may decrease the host's ability or motivation to copulate (Jensen, 1990). Jensen (1987) suggested that somatic growth was not impaired in a sacoglossan, *Ercolania funerea*, infected with *Ismaila monstrosa*. Only one experimental study has addressed the impact of an *Ismaila* species (or any splanchnotrophid) on host survivorship and fecundity. Schrödl (1997) found that a Chilean nudibranch, *Flabellina* sp., infected with

I. damnosa experienced greater mortality and produced no egg masses, suggesting total castration. The mechanism for gonad destruction is unknown for splanchnotrophids, but authors suggest *I. damnosa* displace (but do not consume) host gonad (Schrödl, 1997, 2002; Haumayr and Schrödl, 2003). Such variable impacts on host fitness within one family of parasites raises questions of how this endoparasitic copepod/opisthobranch system compares with those of typical parasites, parasitic castrators and their hosts.

In and near Coos Bay, Oregon, up to 81% of the population of the arminacean nudibranch *Janolus fuscus* can be infected with *Ismaila belciki*, an obligate endoparasite (chapter IV). This species was first described as *I. monstrosa* (Belcik 1965, 1981), then re-described by Ho (1987), who gave it the current name. *Ismaila belciki* shares many characteristics with castrating parasites. Female *I. belciki* are large relative to their host (averaging 1.7% of host mass), with a single female copepod occupying 0.5 to 6.0% of the host mass along with 0-2 males (averaging 0.12% of host mass) (chapter IV). Parasite mass is positively correlated with host mass (chapter IV). Female copepods exhibit high site specificity, preferring an inverted position in the host hemocoel just anterior to the host pericardium (chapter IV). In this position female copepods are typically found with their cephalic appendages attached to the host's anterior-most ovotestis, ventrad to the pericardium and secondary sexual organs. There is some evidence of intraspecific competition among female *I. belciki* which may lead to the observed underdispersed distribution of female *I. belciki* in the host population (chapter IV). In other words, infections involving more than two female parasites in a single host are rare. Like other nudibranchs, *J. fuscus* grow rapidly, are hermaphroditic, devote much energy to reproduction, and reproduce repeatedly throughout their short lifetime

(~5 months in this species) (Goddard, 1992, 2004; chapter III). While some life-history traits of the *I. belciki-J. fuscus* system are reminiscent of both parasitic castrators and typical parasites, the effect of infection on host fitness is unknown.

In this study I examine the impacts of *Ismaila belciki* on the reproductive ecology, growth, and survival of *Janolus fuscus* in the lab. I assess the reproductive loss for hosts with parasites that do and do not reproduce, to estimate the energy diverted from host to parasite fecundity. Finally, I discuss common characteristics of this host/parasite system with both typical parasites and parasitic castrators.

2. Materials and Methods

2.1. Study site and collection

Janolus fuscus were collected from two sites, North Cove (43°18'30.6N, 124°23'58.92W) of Cape Arago, an exposed site on the outer Oregon coast, and a small boulder field just east of Fossil Point (43°21'32.4N, 124°18'45.36W), a protected site inside Coos Bay, near Charleston, Oregon, USA. The later site is referred to as Fossil Pt. for convenience below. Collections were made in February, July, and August 2007, October, 2008, and August, 2009. All laboratory experiments were conducted at the Oregon Institute of Marine Biology, Charleston, Oregon. Upon collection, each nudibranch was placed in an individual 50-ml Falcon tube to prevent mating, and transported to the Oregon Institute of Marine Biology within two hours of collection. *Janolus fuscus* were held in individual plastic containers (dimensions: 10.5x10.5x12.5cm) with 1 mm mesh on all four sides. The containers rested in flow-through seawater tables. Nudibranchs were fed the arborescent bryozoans *Bugula*

pacifica, Tricellaria circumternata, or *Scrupocellaria diegensis* every two to three days to satiation.

Prior to all experiments, initial slug length was measured with calipers as the slug crawled upside down on the water surface. Infection status was determined by external examination of the slug's transparent mantle, revealing large female *Ismaila belicki* but not dwarf males.

2.2. Host reproduction: sperm production and transfer

Preliminary observations and video footage indicated that infected nudibranchs appear to have normal copulation behavior (pers. obs.). To determine whether infected *Janolus fuscus* still produce and transfer sperm to their mates, I collected 10 uninfected juvenile *J. fuscus* (1.66 - 9.85 mm) at Fossil Point in February, 2007 and reared these individuals in isolation as described above. By May 2007, these individuals were fully mature but had never mated. Though *J. fuscus* is a hermaphrodite that exhibits reciprocal copulation and therefore simultaneous insemination, I will define terms about sexuality to facilitate descriptions in the present paper. "Female" slugs are those from which egg masses were collected. "Mates" are those hermaphroditic slugs that donated sperm to the "females." "Female infection" refer to the infection status (i.e., infected or uninfected) of the slugs from which egg masses were collected and "mate infection" as the infection status of their mate (i.e., male role or sperm donor). However, all slugs assume both female and mate (i.e., male) roles in experiments.

Eight virgin female *Janolus fuscus* were paired with similar sized mates recently collected from the field. Four of these lab-reared virgin females were paired with four uninfected field mates and four with four infected field mates. Preliminary size analyses

were conducted to ensure no significant difference in the length of female slugs between the two treatments ($F_{8,2}$=0.008, p=0.933). Each pair was allowed a two-hour monitored mating period, during which all pairs mated. Slugs were then separated for 12 days and fed *Bugula pacifica*. Egg masses laid by slugs were collected every other day and placed in individual test tubes with regularly changed coarse-filtered (5 μm mesh) seawater in a flow- through tank for seven days until veligers were visible within the egg capsules. All egg masses were examined under a dissecting scope to determine viability. Wet weights were recorded in grams to four decimal places on a Mettler Toledo AT4600 Delta Range digital balance after blotting on a paper towel. The cumulative mass of all eggs produced over the 12-day experiment was calculated for each female slug. Total egg mass wet weights were analyzed using a single factor, ANCOVA with mate infection as a fixed factor and female slug length as a covariate. One slug died in each treatment during the experiment, leaving a sample size of n=3 for the analysis.

2.3. Host reproduction: egg production and viability

Uninfected and infected *Janolus fuscus* were collected from North Cove and Fossil Point on August, 2007 and maintained at OIMB as described above. The length of each slug was recorded on the day of collection. An *a priori* single factor ANOVA showed no difference in initial length between infected and uninfected *J. fuscus* ($F_{53,2}$=0.165, p=0.686). Only infected individuals with a single female copepod were used in this experiment. Eight uninfected and 16 infected *J. fuscus* (females) were paired with uninfected *J. fuscus* mates of similar sizes (<5 mm difference) and allowed to copulate at will for 24 hours. From previous observations, *J. fuscus* pairs mate one or more times within a two hour period. After the 24 hour mating period, pairs were

separated and kept in flow-through containers for three weeks. Individuals that died within the first 16 days of the experiment were not included in the analyses. Egg masses produced by each slug were collected every other day for 21 days and held in regularly changed filtered seawater in test tubes kept at 11-13°C in seawater tables for seven days, by which time veligers were visible within the egg capsules. Veligers are still considered embryos until hatching and are referred to as such below. At this time the masses were wet-weighed, all egg masses were broken apart, freeing capsules, and preserved in 10% formalin in seawater buffered with sodium borate. The total number of capsules in each egg mass was determined, the number of viable embryos in 10 haphazardly chosen capsules was counted, and the total number of embryos in each egg mass was extrapolated from the average of these 10 sub-samples. I calculated the total combined egg mass wet weights, number of capsules, number of embryos produced by each slug over the experimental period. Each measure of reproductive output was compared with a two factor, one-tailed MANCOVA with "female infection" and "mate infection" as fixed factors and "female slug length" as a covariate. Significant differences were compared with posthoc Bonferroni pair-wise comparisons for all general linear model analyses throughout the paper.

2.4. Parasite-host reproduction

To determine the linear relationship between egg mass weight and the number of capsules and estimated number of embryos for *Janolus fusucs* and egg sac wet weight and the number of embryos produced for *Ismaila belciki*, I collected egg masses produced by 46 nudibranchs and 16 copepods (inside hosts) over 45 days during November and December 2008. Nudibranch egg mass collection, maintenance, preservation, weighing,

and counting were conducted as described in the "Host Reproduction" experiment (above). Copepod egg sacs were gently removed from female copepods using fine forceps approximately every eight days. Egg masses were preserved in 10% formalin in seawater buffered with sodium borate. Egg sacs were wet-weighed, and the number of viable embryos counted in one of the two egg sacs. The number of viable embryos was multiplied by two to get the total number of viable embryos. Two regression analyses were conducted to determine the linear relationship of egg mass wet weight to the number of capsules and to the estimated number of embryos per egg mass produced by *J. fuscus*. Each egg mass produced was considered a replicate. These linear relationships were used to estimate the number of egg capsules and viable embryos from egg mass wet weight of *J. fuscus* in the following experiment. A single regression was conducted to determine the linear relationship of egg sac wet weight and the number of viable embryos produced per pair of egg sacs by *I. belciki*. Again, each pairs of egg sacs was considered a replicate. This linear relationship was used to estimate the number of viable embryos from egg sac wet weight in the experiment below.

To address the potential reallocation of energy (in the form of reproductive output) from slug fecundity to copepod fecundity, I ran another, almost identical egg mass experiment as described above in the "Host Reproduction" experiment but, in addition, collected and analyzed the copepod egg sacs. On August 8-9, 2009, uninfected and infected *Janolus fuscus* were collected from North Cove, Fossil Pt. and the Charleston small boat basin. Methods for collecting, holding, feeding, and measuring length and weight of *J. fuscus* and their egg masses were the same as described above. The experiment lasted 30 days, from August 10 to September 9, 2009. *Janolus fuscus* are

only able to store sperm from a single copulation period for 21 days (Wolf, unpublished data). Thus I ran a second twenty-four hour mating period (see experiment above) on August 29th with the same pairs as before to replenish sperm stores. Each pair was observed to mate at least once during a two-hour observation period. Each copepod egg sac was monitored while still attached to the female copepod. As the yolk reserves are utilized by the developing embryos, they become more transparent, revealing the nauplius within and confirming viability. When the nauplii were visible, at approximately 12 days, copepod egg sacs were gently removed using fine forceps. Wet weights of copepod egg sacs were recorded after gently blotting on a paper towel. Egg sacs were preserved the same way as slug egg masses in 10% formalin in seawater buffered with sodium borate. Only slugs infected with a single female copepod were used in this experiment. An *a priori* single factor ANOVA showed no difference in length of infected and uninfected slugs ($F_{29,2}$=0.781, p=0.385). Slugs that died within the first 25 days of the experiment were not included in these analyses.

Each measure of host reproductive output (RO) was compared using a two factor, one-tailed MANCOVA with female infection and mate infection as fixed factors and female length as a covariate. Additionally, these measures of slug reproductive output were compared in slugs with no copepods, slugs with productive female copepods (i.e., that produced egg sacs), and slugs with non-productive female copepods (i.e., that did not produce eggs sacs) using a single factor, one-tailed MANCOVA with slug length as a covariate. Mate infection was not included in the analysis, as there were not enough samples of all possible combinations of female and mate infection. To estimate the cost of parasite reproduction to host reproductive output, I compared the average percent

difference in reproductive output ((RO uninfected slugs – RO infected slugs) / RO of uninfected slugs) X 100) of hosts harboring a productive female copepod and hosts with a non-productive female copepod relative to uninfected slugs. This gives an estimation of the energy usurped from host fecundity to augment parasite reproduction. The propagated error for proportional difference estimations were calculated with Taylor's (1982) equations for propagation of uncertainties.

Regression analyses quantified the relationship of parasite reproductive output to host reproductive output. Measures of host and parasite reproductive output were standardized by host and parasite weight. As some hosts died or senesced at the end of the experiment, weight was calculated from initial host length using a significant cubic polynomial equation ($(y=0.224-0.038x+0.003x^2-(8.5^{-6})\ x^3)$ ($r^2=0.794$, $p<0.0001$) describing the length by weight curve derived in chapter II. Female parasite mass was determined from dissections following the experiment. Parasite index (PI) for female copepods was calculated as PI = (parasite mass / (host mass + parasite mass)) x 100. Additionally, two regression analyses were conducted to examine the relationship of host mass to parasite mass and parasite index.

To compare reproductive output between the two experiments "Host Reproduction" vs. "Parasite-Host Reproduction," total measures of host reproductive output were divided by experimental duration, 21 and 30 days respectively, and means compared in a three-factor MANCOVA with trial, female infection, and mate infection as fixed factors and with host length as a covariate.

2.5. Host reproduction: gonadosomatic index

Janolus fuscus were collected from August 2007 to September 2009 from North Cove and Fossil Point. Slugs were anesthetized in 7.5% $MgCl_2$ and preserved in 10% formalin for dissection. Dissections were conducted under a Unitron dissecting microscope. The mass of the host, host ovotestis, and each copepod was recorded to four decimal places on a Mettler Toledo At4600 Delta Range digital balance after gently blotting on a paper towel. Gonadosomatic index (GSI) was calculated for each slug as (gonad mass / total host mass) x 100. GSI was compared across infection status and by collection date with a two-factor ANCOVA, with infection status and collection date as fixed factors and host weight as a covariate. Gonadosomatic index and host weight were $\log_{10}$ transformed for these analyses to increase normality and homogeneity of variances. A second test for the effect of double infection was done by pooling samples of slugs infected by zero, one, or two female copepods across date of collection. Samples were pooled because GSI did not differ by date of collection and there were very low sample sizes of slugs infected by two female copepods.

2.6. Growth

Janolus fuscus from July, 2007, and October, 2008, were used to determine the impact of infection on growth. Initial slug length was measured with calipers as the slug crawled upside down on the water surface. Slug length was converted to wet weight using the regression described in chapter II.

Growth rates were measured across infection status in two trials. In the July 2007 trial, only those infected and uninfected individuals with initial lengths less than 24 mm were used for the experiment with no lower limit. In the October 2008 trial, no

individuals smaller than 13 mm were used with no upper length limit. These limits ensured that there was no significant difference in the initial lengths (or weights) of infected and uninfected individuals for either experimental period, July ($F_{22,2}$= 1.048, p=0.318) and October ($F_{30,2}$= 1.903, p=0.179), respectively from a single factor ANOVA with infection status as a fixed factor. Lengths were measured once a week for 25 (three weeks in 2007) and 47 (four weeks in 2008) days for these two growth periods, respectively. The growth rate, in millimeters per day, for infected and uninfected individuals was calculated from the maximum length (i.e., not final length) minus the initial lengths. Maximum lengths and therefore growth rate (opposed to overall growth for the same period of time) were used because *Janolus fuscus* shrink as part of their senescence before dying, giving slugs different time periods to maximum growth. However, individuals that died during the experiment were not included in these analyses. I compared growth rates by initial length with a general linear model two-factor ANCOVA, with trial and infection status as fixed factors and initial length as a covariate in SPSS 14.0. Identical analyses were run using the regressed weight as the measure of growth and initial weight as the covariate. Initial lengths and weights were significantly different (length, $F_{52,2}$=19.87, p<0.0001, weight, $F_{52,2}$=11.7, p=0.001) between trials, with initial lengths and weights of July 2007 slugs being significantly smaller (15.0 mm ± 0.844, 0.22 g ± 0.110) than those in October 2008 (23.26 mm ±4.25, 0.71 g ± 0.095).

2.7. Survivorship

Slug mortality was recorded every other day during two trials, the growth monitoring trial (trial 1, July, 2007) and the Host Reproduction experiment from August,

2007 lasting 25 and 21 days, respectively. Death was defined as the time when a slug ceased crawling, lost cerata, and did not respond to tactile stimulus. I employed a Kaplan-Meier survival analysis to test whether mortality rate differed between infected and uninfected individuals using JMP 8 statistical software.

3. Results

3.1. Host reproduction: sperm production and transfer

Both infected and uninfected *Janolus fuscus* mated during copulation experiments with no evident differences in mating ability or behavior. Viable egg masses were produced by female slugs mated with infected and uninfected mates. There was no significant difference in total wet weight of egg masses produced by females inseminated by infected or uninfected mates (0.661 g ± 0.330 and 0.594 g ± 0.330, respectively ($F_{6,2}$ = 0.020, p = 0.896).

3.2. Host reproduction: egg production and viability

Reproductive output did not differ between uninfected female slugs with infected or uninfected mates, regardless of the infection status of the female (Fig. 4.1A-C, Table 4.1). Host length had a significant impact on egg mass weight and number of embryos but not the number of capsules (Table 4.1). Longer slugs produced larger masses and more embryos. However, female infection affected all measures of reproductive output (Fig. 4.1A-C, Table 4.1). On average, infected females produced 35.2% ± 15.1 smaller egg masses, 38.3% ± 13.4 fewer capsules, and 34.4% ± 15.9 fewer embryos compared to uninfected females.

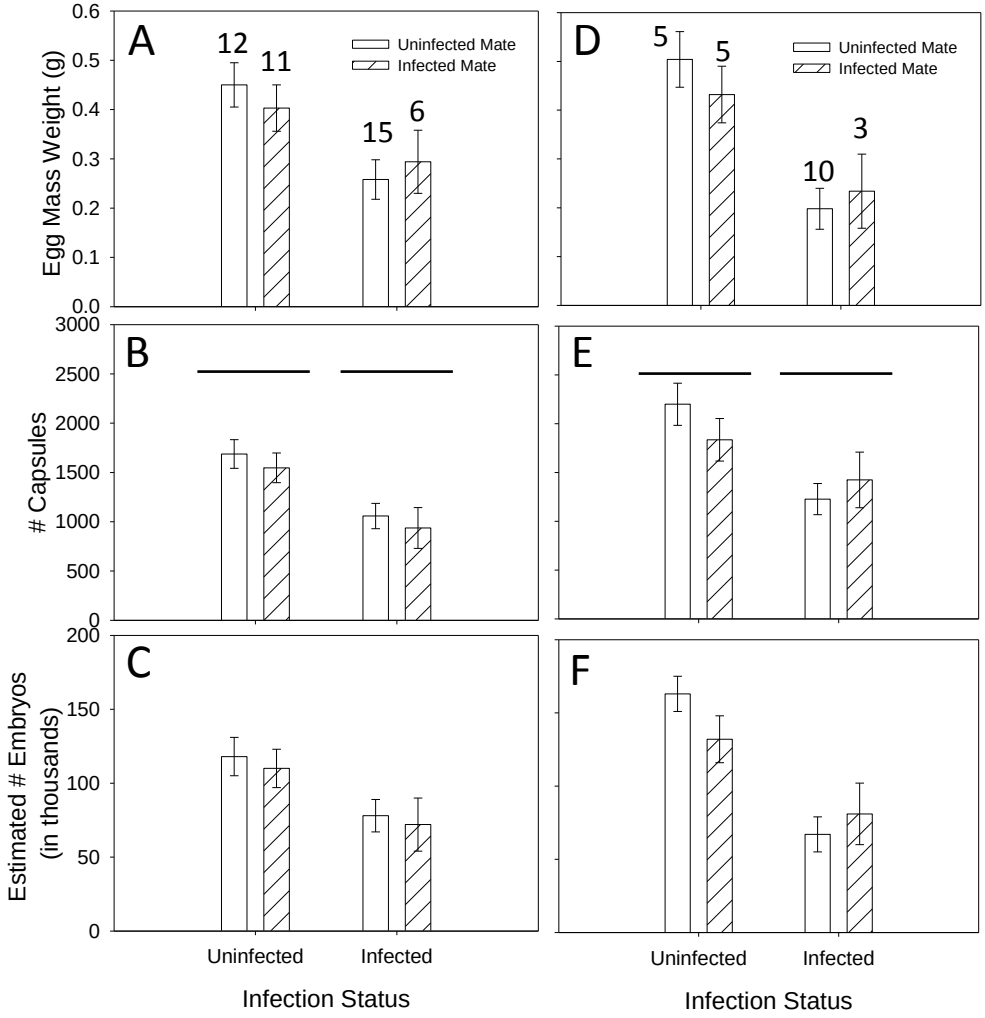


Fig. 4.1: Three measures of reproductive output (RO) (A, D) egg mass wet weight; (B, E) number of egg capsules; (C, F) estimated number of embryos of uninfected and infected *Janolus fuscus* with uninfected and infected mates produced during (A-C) the "Host Reproduction" experiment from August 3-23, 2007 (21 days) and (D-F) the "Host-Parasite Reproduction" experiment from August 10-September 9, 2009 (30 days). Mean measures of reproductive output adjusted for length covariate. Significant differences are represented by solid lines above bars in graphs (B and E). Numbers above bars are sample sizes for all three graphs. Error bars represent standard error.

Table 4.1: Host Reproduction: Egg Mass Production and Viability. Results of a one-tailed two factor MANCOVA with host length as a covariate testing impact of female infection and mate infection on three measures of slug total reproductive output ((i.e., Egg Mass Wet Weight (EM Weight), Number of Egg Capsules (Capsules), Estimated Number of Embryos (Embryos)) over 21 days in August 2007.

Factor	RO measure	df	SS	MS	F	p
Host	E M Weight	1	0.168	0.168	7.063	0.0055
Length	Capsules	1	3.459×10^{6}	3.459×10^{6}	1.392	0.1225
	Embryos	1	5.684×10^{9}	5.684×10^{9}	3.01	0.0455
Female	E M Weight	1	0.211	0.211	8.866	0.0025
Infection	Capsules	1	3.562×10^{6}	3.562×10^{6}	14.334	0.0005
	Embryos	1	1.421×10^{10}	1.421×10^{10}	7.526	0.0045
Mate	E M Weight	1	<0.0001	<0.0001	0.011	0.458
Infection	Capsules	1	1.652×10^{5}	1.652×10^{5}	0.665	0.21
	Embryos	1	4.733×10^{8}	4.733×10^{8}	0.251	0.3095
F x M [a]	E M Weight	1	0.017	0.017	0.695	0.203
	Capsules	1	910.304	910.304	0.004	0.476
	Embryos	1	8.279×10^{6}	8.279×10^{6}	0.004	0.474
Error	E M Weight	39	0.926	0.024		
	Capsules	39	9.692×10^{6}	2.485×10^{5}		
	Embryos	39	7.364×10^{10}	1.888×10^{9}		

[a] Interaction of female infection and mate infection

3.3. Parasite-host reproduction

The linear relationship of egg mass wet weight and the number of capsules and viable number of embryos produced by *Janolus fuscus* were estimated using linear equations ($y=1864x +187$, $r^2=0.644$, $p<0.0001$) and ($y=290{,}024x +2{,}391$, $r^2=0.605$, $p<0.0001$) (Fig. 4.2A, B). Likewise, the linear relationship of egg sac wet weight and the number of viable embryos ($y=5{,}369{,}771x +12{,}878$ $r^2=0.557$, $p<0.0001$) produced by

Ismaila belciki was used to estimate the number of embryos for a given egg sac weight (Fig. 4.2C).

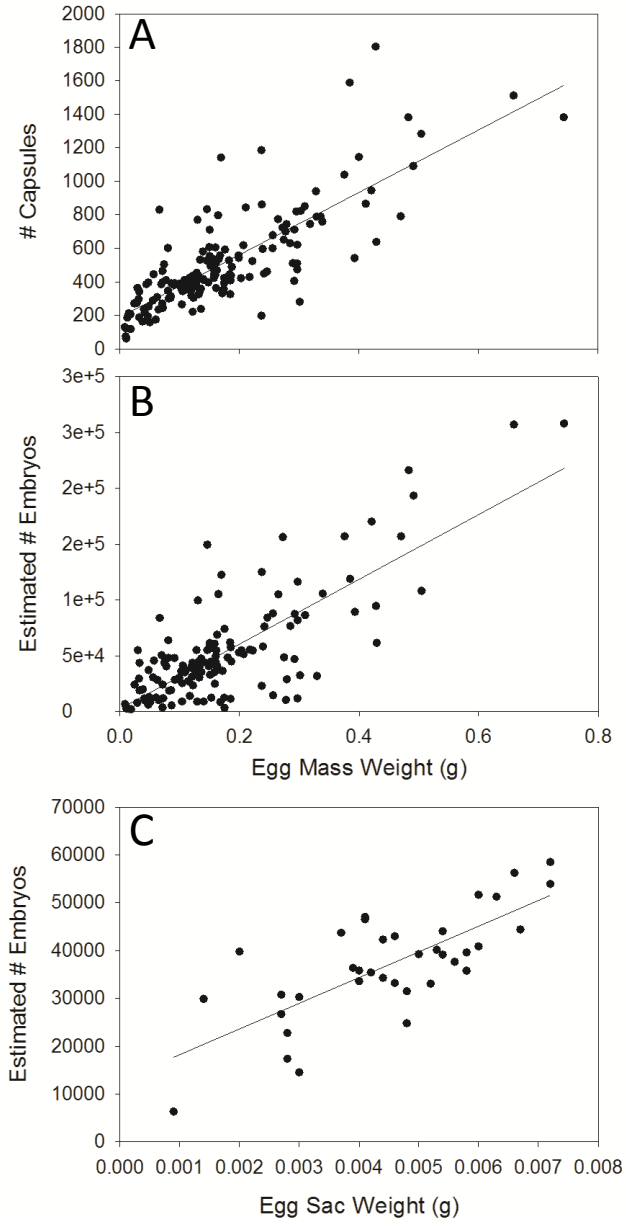


Fig. 4.2: Regression analyses of linear relationship of (A) egg mass wet weight to number of capsules produced by *Janolus fuscus* (y=1864x +187, r^2=0.644, p<0.0001); (B) egg mass wet weight to estimated number of embryos of *J. fuscus* and (y=290,024x +2,391, r^2=0.605, p<0.0001); and (C) egg sac wet weight by number of embryos of *Ismaila belciki* (y=5,369,771x +12,878 r^2=0.557, p<0.0001).

In an experiment designed to examine the cost of parasite reproduction on host reproductive output, uninfected slugs produced significantly larger egg masses, more egg capsules, and more embryos than infected slugs (Fig. 4.1D-F, Table 4.2). On average infected slugs produced 53.8% ± 23.8 smaller egg masses, 34.2% ± 15.7 fewer number capsules, and 49.5% ± 19.9 fewer viable embryos, relative to uninfected slugs.

Table 4.2: Host-Parasite Reproduction. Results of one-tailed two factor MANCOVA with host length as a covariate testing impact of female infection and mate infection on three measures of slug reproductive output ((i.e., Egg Mass Wet Weight (EM Weight), Number of Egg Capsules (Capsules), Estimated Number of Embryos (Embryos)) over 30 days from August 10-September 9, 2009.

Factor	RO measure	df	SS	MS	F	p
Host	E M Weight	1	0.160	0.160	9.792	0.003
Length	Capsules	1	4.281×10^{6}	4.281×10^{6}	18.482	<0.0001
	Embryos	1	1.942×10^{10}	1.942×10^{10}	14.810	0.0005
Female	E M Weight	1	0.303	0.303	18.521	<0.0001
Infection	Capsules	1	2.268×10^{6}	2.268×10^{6}	9.791	0.0003
	Embryos	1	2.526×10^{10}	2.526×10^{10}	19.264	<0.0001
Mate	E M Weight	1	0.001	0.001	0.087	0.3855
Infection	Capsules	1	3.143×10^{4}	3.143×10^{4}	0.136	0.3585
	Embryos	1	3.138×10^{8}	3.138×10^{8}	0.239	0.3155
F x M [a]	E M Weight	1	0.014	0.017	0.828	0.1875
	Capsules	1	3.621×10^{5}	910.304	1.563	0.1135
	Embryos	1	2.285×10^{9}	8.279×10^{6}	1.743	0.1015
Error	E M Weight	18	0.294	0.016		
	Capsules	18	4.169×10^{6}	2.316×10^{5}		
	Embryos	18	2.360×10^{10}	1.311×10^{9}		

[a] Interaction of female infection and mate infection

Mate infection did not affect reproductive output (Table 4.2). There were no significant interactions between female infection and mate infection (Table 4.2). There was, however, an indication that infected mates reduced the reproductive output of their uninfected female partners, but these differences were not significant. Host length had a significant impact on all measures of slug reproductive output. Egg mass wet weight, number of egg capsules and estimated number of embryos increased with slug length (Table 4.2).

Daily reproductive output (i.e., egg mass wet weight / day) differed significantly between the two host-fecundity experiments (August 2007 and August 2009) (Fig. 4.3, Table 4.3). Mean egg mass wet weight was significantly greater in the August 2007 (egg mass 15.93 ± 0.98 mg) than the August 2009 experiment (egg mass 12.13 ± 1.38 mg) (Table 4.3). There were no significant interactions between trial and any other factors (i.e., female infection and mate infection). There was no significant difference in slug length between the two experiments (2007 = 27.0 ± 0.65 mm, 2008 = 25.01 ± 0.86 mm).

Reproductive output differed between slugs with productive copepods, non-productive copepods, and no copepods (Fig. 4.4A-C, Table 4.4). In subsequent dissections I found that non-productive copepods all lacked a mate (i.e., hosts did not have any male copepods) but were mature (i.e., large, with gonad filling the body cavity). Slugs with productive copepods produced smaller egg masses, fewer capsules, and fewer embryos than uninfected slugs (Bonferroni post-hoc comparison, $p<0.0001$, $p=0.010$, $p<0.0001$, respectively) and slugs infected with a non-productive copepod ($p=0.011$, $p=0.047$, $p=0.012$) (Fig. 4.4A-C, Table 4.4). Reproductive output did not differ between uninfected slugs and slugs with non-productive copepods ($p=0.110$, $p=0.500$, $p=0.205$)

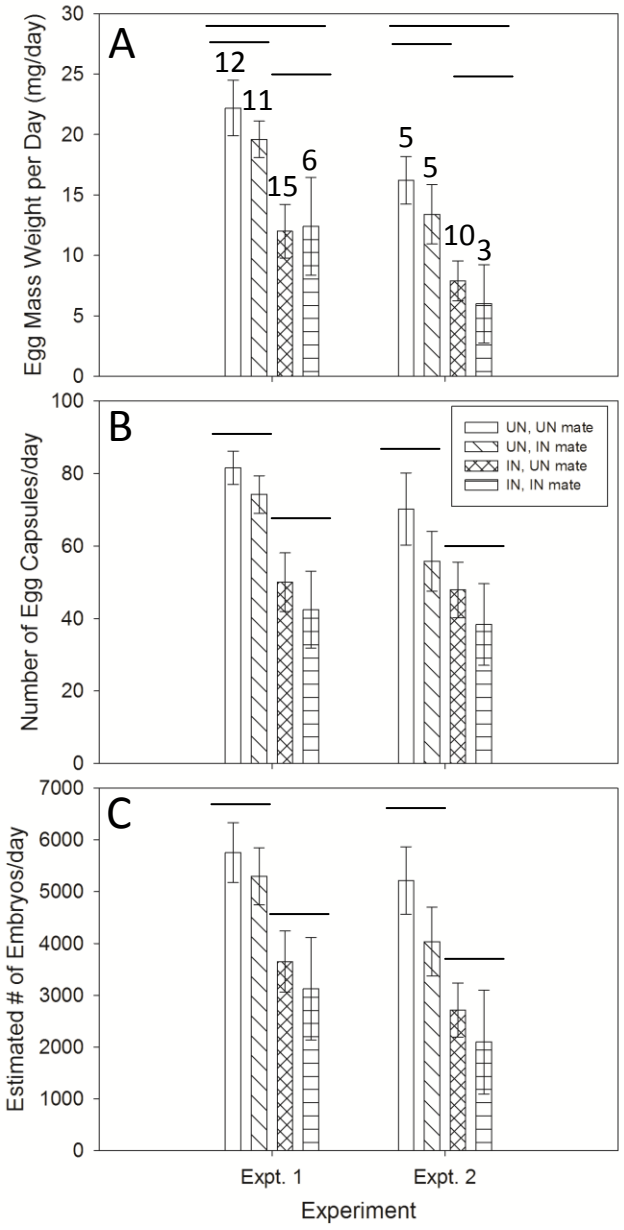


Fig. 4.3: Three measures of reproductive output (A) egg mass wet weight; (B) number of egg capsules; and (C) estimated number of embryos produced **per day** by uninfected and infected *J. fuscus* with uninfected and infected mates in two experiments, the "Host Reproduction" experiment (Experiment 1) from August 3-23, 2007 (21 days) and the "Host-Parasite Reproduction" experiment (Experiment 2) from August 10-September 9, 2009 (30 days). Numbers above bars in graph (A) represent sample sizes for all graphs. Solid lines above graphs represent significant differences. Error bars represent standard error.

Table 4.3: Comparison of Host Reproduction and Host-Parasite Reproduction Experiments. Two-tailed three-factor MANCOVA with host length as a covariate to compare the 2007 and 2009 reproduction experiments and the impact of female infection and mate infection on three measures of slug reproductive output **per day** (i.e., Egg Mass Wet Weight (EM Weight), Number of Egg Capsules (Capsules), Estimated Number of Embryos (Embryos)).

Factor	RO measure	df	SS	MS	F	p
Expt.	E M Weight	1	2.034	2.034	5.064	0.028
	Capsules	1	254.345	254.345	0.539	0.466
	Embryos	1	4.780×10^6	4.780×10^6	1.465	0.231
Host Length	E M Weight	1	5.310	5.310	13.220	0.001
	Capsules	1	3535.444	3535.444	7.490	0.008
	Embryos	1	2.641×10^7	2.641×10^7	8.094	0.006
Female Infection	E M Weight	1	9.968	9.968	24.819	<0.0001
	Capsules	1	1.060×10^4	1.060×10^4	22.456	<0.0001
	Embryos	1	6.404×10^7	6.404×10^7	19.630	<0.0001
Mate Infection	E M Weight	1	0.066	0.066	0.164	0.686
	Capsules	1	618.916	618.916	1.311	0.257
	Embryos	1	2.244×10^6	2.244×10^6	0.688	0.410
Error	E M Weight	62	24.901	0.016		
	Capsules	62	2.926×10^4	2.316×10^5		
	Embryos	62	2.023×10^8	1.311×10^9		

(Fig. 4.4A-C, Table 4.4). Slugs with productive copepods produced 73.9 % ± 23.7 lighter egg masses, 49.3 % ± 12.5 fewer capsules, and 69.1 % ± 18.4 fewer viable embryos than those with no copepod (Table 4.5). However, those slugs infected with non-productive copepods did not suffer significant reproductive losses, producing 37.6% ± 11.2 lighter egg masses, 24.0% ± 9.3 fewer egg capsules, and 34.9% ± 10.0 fewer number of embryos than uninfected slugs (Table 4.5). If I subtract the latter values of

reproductive output from the former, assuming cost to the reproductive output of the host is only used to maintain the non-productive copepod, I estimate the percentage of reproductive output usurped from the host by the parasite and re-directed to the parasite's own reproduction is 36.3% ± 26.2 lighter egg masses, 25.3% ± 15.6 fewer capsules, and 34.1% ± 20.9 fewer embryos (Table 4.5).

Table 4.4: Host-Parasite Reproduction. Results of a one-tailed, single-factor MANCOVA examining the impact of infection (i.e., no copepod, productive copepod, and non-productive copepod) on host reproductive output (i.e., Egg Mass Wet Weight (EM Weight in grams), Number of Egg Capsules (Capsules), Estimated Number of Embryos (Embryos)). Host length was run as a covariate.

Factor	RO measure	df	SS	MS	F	p
Host	E M Weight	1	0.106	0.106	8.909	0.004
Length	Capsules	1	3.367×10^{6}	3.367×10^{6}	16.779	0.0005
	Embryos	1	1.365×10^{10}	1.365×10^{10}	13.716	0.0010
Female	E M Weight	2	0.448	0.224	18.875	<0.0001
Infection	Capsules	2	3.672×10^{6}	1.836×10^{6}	9.151	0.0010
	Embryos	2	3.846×10^{10}	1.923×10^{10}	19.321	<0.0001
Error	E M Weight	19	0.226	0.012		
	Capsules	19	3.812×10^{6}	2.006×10^{5}		
	Embryos	19	1.891×10^{10}	9.954×10^{8}		

Productive copepods produced egg sacs with an average wet weight of 10.1 mg ± 0.93 and 88,359 viable embryos (± 8865) over 30 days (n=6). Average egg sac wet weight and estimated number of embryos of a single clutch (i.e., one pair of egg sacs) were 3.87 mg ± 0.26 and 33,643 ± 1241, respectively. There was no significant relationship between host mass and female copepod mass during this experiment (n=6)

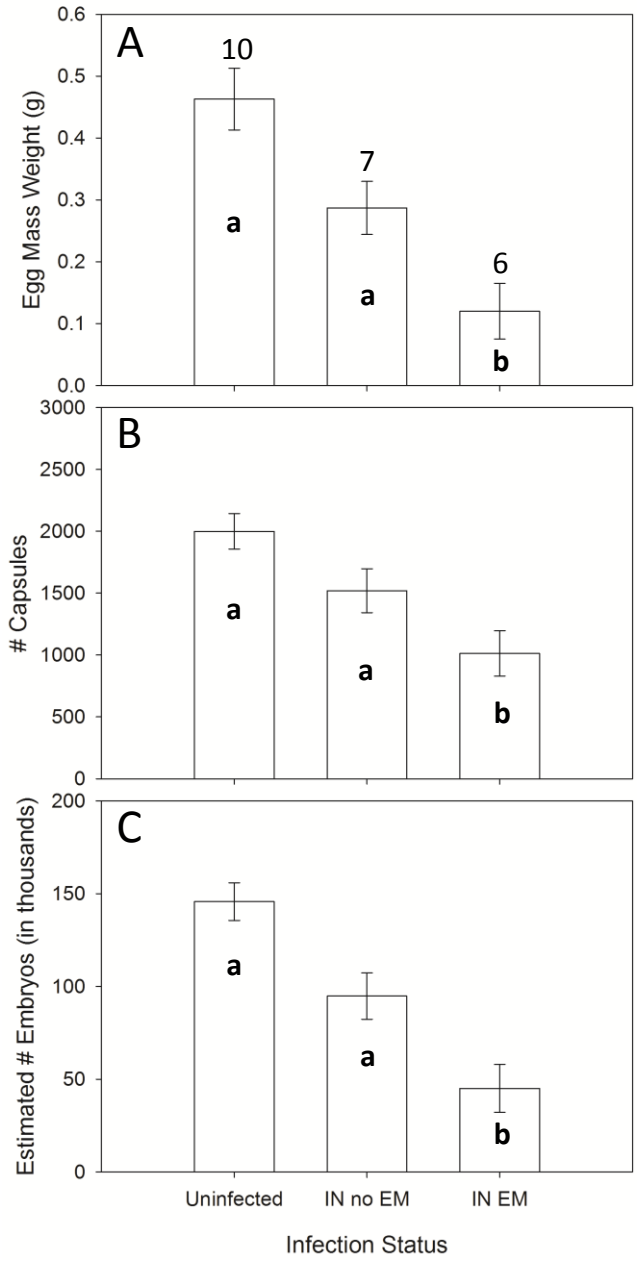


Fig. 4.4: Three measures of reproductive output (A) egg mass wet weight; (B) number of egg capsules; and (C) estimated number of embryos produced by *Janolus fuscus* with no copepod, with a non-productive female copepod (i.e., not producing egg masses, (IN no EM)) or with a productive female copepods (i.e., producing egg masses (IN EM)) in the "Host-Parasite Reproduction" experiment from August 10-September 9, 2009. Reported means adjusted for length covariate. Letters indicate significant differences. Sample size represented above each bar. Error bars represent standard error.

Table 4.5: Average reproductive cost ((Uninfected slug RO – Infected slug RO) / Uninfected slug RO) in three measures of slug reproductive output (RO) (i.e., egg mass wet weight, number of egg capsules, and estimated number of embryos) in slugs infected with productive and non-productive female copepods relative to uninfected slugs. Host RO converted to Parasite RO = the reproductive cost to host RO that is devoted only to parasite RO. The propagated error of this estimate is calculated according to Taylor (1982).

Measure of Host RO	Host RO Lost to Productive Copepod		Host RO Lost to Non-Productive Copepod		Host RO Converted to Parasite RO	Propagated Standard Error
Egg Mass Wet Weight	73.9%	-	37.6%	=	36.3%	± 26.3%
Number of Capsules	49.3%	-	24.0%	=	25.3%	± 15.6%
Number of Embryos	69.1%	-	34.9%	=	34.2%	± 20.9%

(Table 4.6). However, a previous regression analysis of *J. fuscus* and *I. belciki* mass from a greater number of dissections (n=76) suggests this is a significant positive relationship (r^2=0.218, slope=0.671, p=0.002) with adequate sample size (i.e., greater statistical power) (chapter IV). Parasite index was related negatively with host mass, though not significantly, suggesting female *I. belciki* may occupy a smaller proportion of the host's mass as the host grows (Table 4.6). Though host reproductive output varies negatively with parasite reproductive output, there were no significant linear relationships between measures of host and parasite reproductive output, egg mass wet weight and estimated number of embryos (n=6) (Fig. 4.5A, B, Table 4.6). However, with such a small sample

size (n=6) of hosts with productive copepods, statistical power was too low to detect differences in these analyses.

Table 4.6: Regression analyses of host mass and parasite mass, host mass and parasite index, host reproductive output (egg mass mass and number of embryos) and parasite reproductive output (egg sac mass and number of embryos).

I.V. [a]	D.V. [b]	Source	df	SS	MS	F	p	r^2	slope
Host Mass	Para. [c] Mass	regress.	1	0.003	0.003	0.022	0.89	0.005	0.001
		Error	4	0.464	0.116				
Host Mass	PI [d]	regress.	1	1.973	1.973	6.59	0.062	0.622	-2.208
		Error	4	1.198	0.300				
ES Mass [e]	EM Mass [f]	regress.	1	59.04	59.04	3.22	0.147	0.446	-14.226
		Error	4	73.35	18.34				
# Para. Embryos	#Host Embryos	regress.	1	738.80	738.80	2.12	0.219	0.346	-0.007
		error	4	1394.2	348.5				

[a]Independent Variable
[b]Dependent Variable
[c] Parasite
[d]Parasite Index = (parasite mass / (host mass + parasite mass)) x 100
[e]Egg sac mass / female parasite mass
[f] Egg mass mass / host mass

3.4. Host reproduction: gonadosomatic index

Gonadosomatic index (GSI) differed significantly by infection status (Fig. 4.6A, Table 4.7). Infected individuals (2.33 % ± 0.286) had significantly lower GSI than uninfected individuals (4.026 % ± 0.412) (Fig. 4.6A, Table 4.7). Gonadosomatic index

did not differ by collection date (Table 4.7, Fig. 4.6A). When double infections were included and data were pooled across collection date, GSI of uninfected individuals was significantly greater (4.02% ± 0.412) than that of slugs with both single (2.46 %± 0.372) and double (1.295 % ± 0.390) infections ($p=0.001$ and $p<0.0001$, respectively) (Fig. 4.6B) ($F_{76,2} = 34.379$, $p<0.0001$). GSI was not different between single and double infections ($p=0.136$). An a priori ANOVA revealed that uninfected slugs (average $\log_{10}$ length = -0.188 ± 0.38 g) were larger than infected slugs (average $\log_{10}$ length = -0.413 ± 0.31 g) ($F_{76,2} = 4.032$, $p=0.022$, Fig. 4.7). There was a significant negative relationship between host mass and GSI for both infected and uninfected slugs, (Fig. 4.7, Table 4.8). Therefore these analyses may underestimate differences seen in GSI of infected and uninfected slugs.

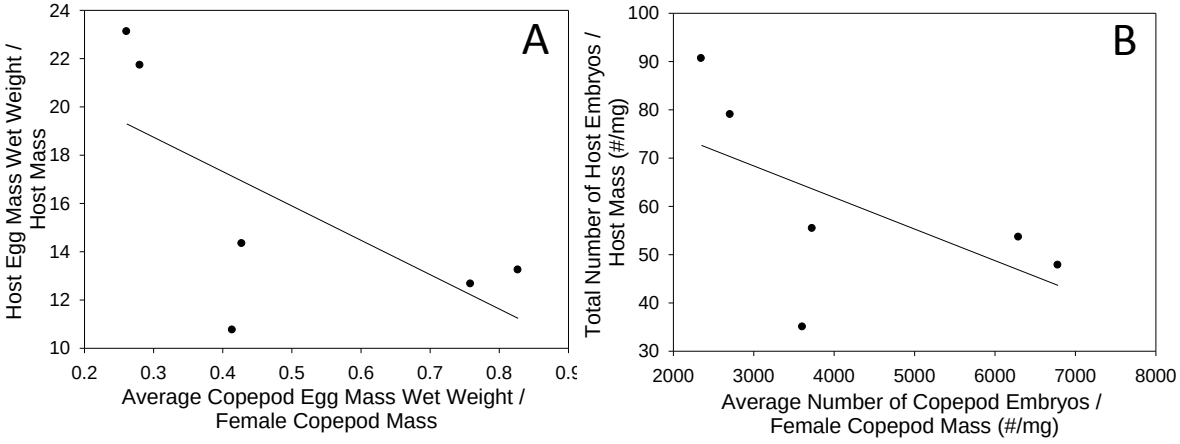


Fig. 4.5: Regression analyses of copepod and host reproductive output. (A) Average copepod egg mass wet weight / female copepod mass by total host egg mass wet weight (mg) / host mass (mg). (B) Average estimated number of copepod embryos (mg) / female copepod mass (mg) by total estimated number of host embryos (mg) / host mass (mg).

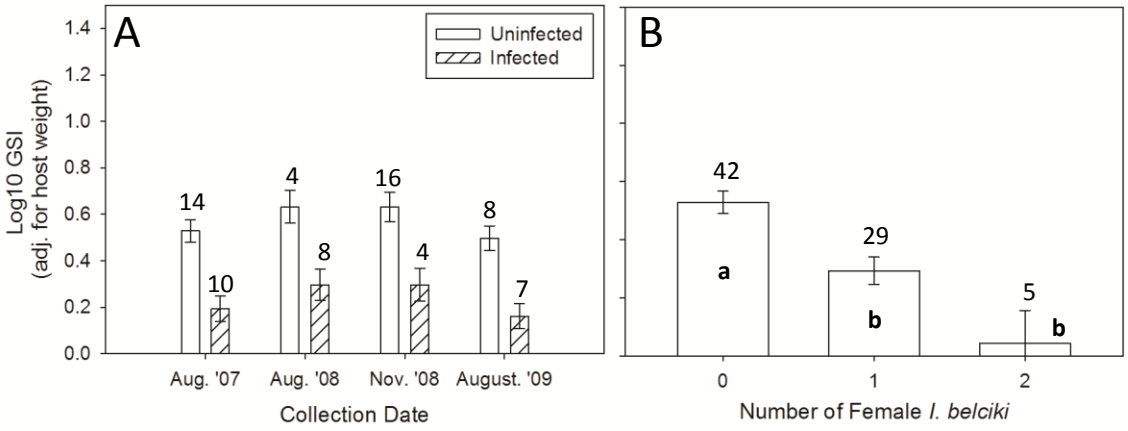


Fig. 4.6: Log_{10} gonadosomatic index (GSI) adjusted for host weight covariate of (A) uninfected (open bars) and infected (hatched bars) *Janolus fuscus* during four collection dates August 2007, August 2008, November 2008, and August 2009; and (B) uninfected *J. fuscus* and slugs infected with one or two female *I. belciki*. Letters designate significant differences in GSI from Bonferroni pair-wise comparisons with α=0.05. Numbers above bars are sample sizes. Error bars represent standard error.

Table 4.7: Results of ANOVA analysis comparing log_{10} gonadosomatic index in infected and uninfected *Janolus fuscus* during four collection dates, August 2007, August 2008, November 2008, and September 2009. There was no significant interaction of date of collection and infection status and thus the interaction was excluded from the model.

Source	df	SS	MS	F	p
Date of Collection	3	0.218	0.073	2.147	0.103
Infection Status	1	1.721	1.721	50.751	<0.0001
Log_{10} Host Weight	1	0.814	0.814	24.006	<0.0001
Error	65	2.205	0.034		

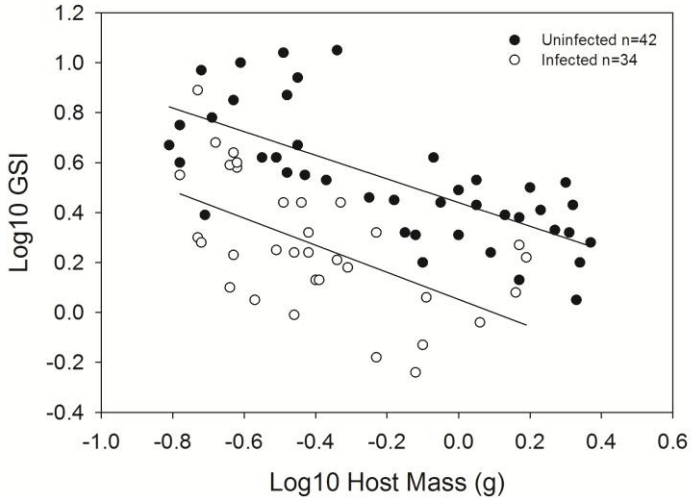


Fig. 4.7: Regression analysis of $\log_{10}$ transformed host mass and $\log_{10}$ transformed gonadosomatic index (GSI) in infected and uninfected *Janolus fuscus* from four collection dates: August 2007, August 2008, November 2008, and August 2009.

Table 4.8: Regression analyses examining $\log_{10}$ transformed slug weight by $\log_{10}$ transformed gonadosomatic index in infected and uninfected *J. fuscus* combined over four collection periods (i.e., August 2007, August 2008, November 2008, and September 2009).

Source	df	SS	MS	F	p	r^2	slope
Uninfected	1	1.314	1.314	40.536	<0.0001	0.503	-0.473
Error	40	1.296	0.032				
Infected	1	0.897	0.897	19.826	<0.0001	0.383	-0.542
Error	32	1.448	0.045				

3.5. Growth

Growth rates in *Janolus fuscus* varied from initial to maximum length across both trial periods. Differences in growth rates by length were not significant for any of the factors tested, with the exception of trial (Table 9). Slugs increased in length at a higher rate in July than in October (Fig. 4.8A, B, 4.9A, B).

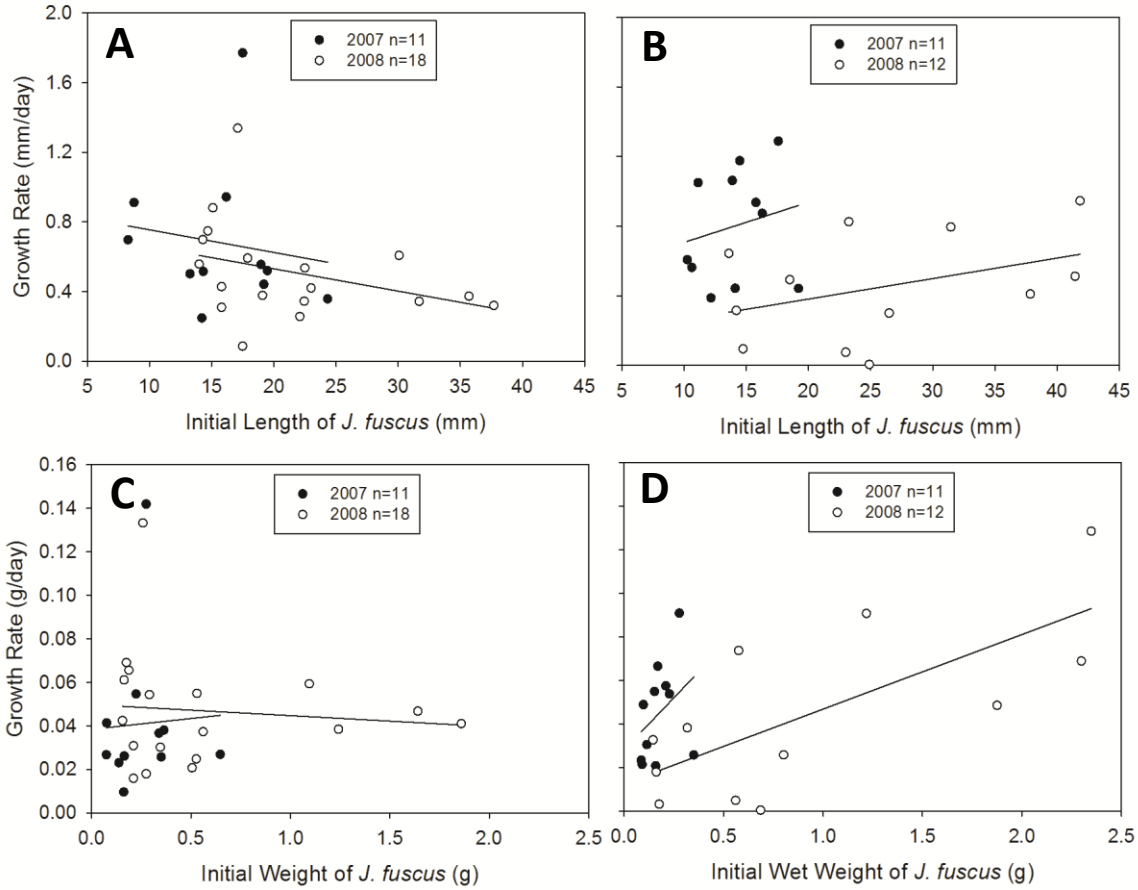


Fig. 4.8: Growth rate by initial size during two growth periods, July 14 to August 7, 2007 (25 days) and October 17 to December 2, 2008 (47 days). Growth rate (in length) by initial length for (A) uninfected and (B) infected *Janolus fuscus*. Growth rate (in weight) by initial weight for (C) uninfected and (D) infected *J. fuscus*. Closed and open circles represent 2007 and 2008 growth periods, respectively.

Growth rates determined from wet weight of *J. fuscus* varied from initial to maximum weight across both trial periods (Fig. 4.8C, D). Infection status interacted with initial slug weight because growth rate decreased with initial weight in uninfected slugs but increased with initial weight in infected slugs (Fig. 4.8C, D, Table 4.9). Though not significant, average growth rate (for both length and weight) was greater in infected than uninfected slugs in July (Fig. 4.9A, B). In contrast, average growth rate was greater on average in uninfected slugs than in infected slugs in October (Fig. 4.9A, B).

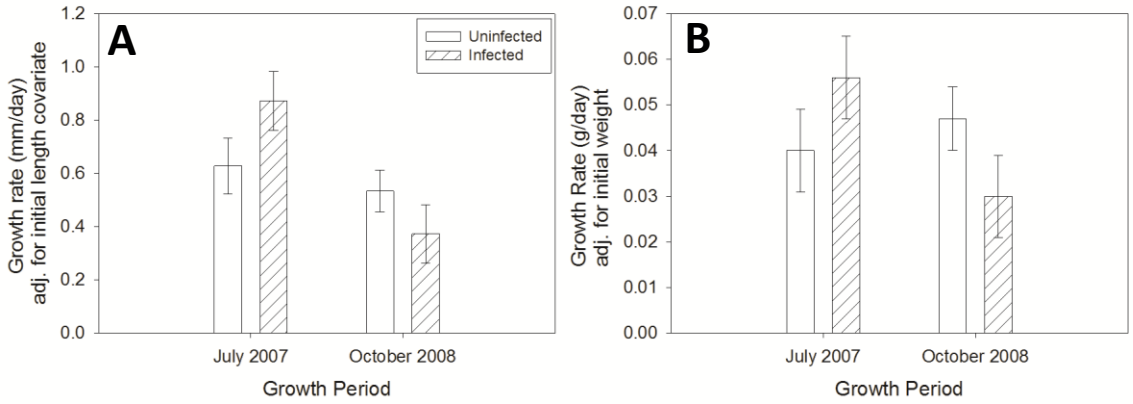


Fig. 4.9: Growth rate of infected and uninfected *Janolus fuscus* during two growth trials, July 14 to August 7, 2007 (25 days) and October 17 to December 2, 2008 (47 days). Growth rate, by growth trial and infection status, measured in (A) millimeters per day with growth rate adjusted for initial length; (B) grams per day with growth rate adjusted for initial weight.

Table 4.9: Results of two, two-factor ANCOVAs examining the effect of growth trial and infection status (fixed factors) and initial length or initial weight of *J. fuscus* (covariate) on growth rate of *J. fuscus* calculated from length and weight. Interactions with p-values above 0.1 were removed from the model.

	Growth Rate Length (mm/day)					Growth Rate Weight (g/day)				
Source	df	SS	MS	F	p	df	SS	MS	F	p
Trial	1	0.768	0.768	7.300	0.01	1	0.001	0.001	1.105	0.299
IN Status[a]	1	0.304	0.304	2.888	0.096	1	0.003	0.003	3.195	0.080
Initial Size	1	<0.001	<0.001	0.001	0.982	1	0.003	0.003	3.390	0.072
Trial x IN Status	1	0.357	0.357	3.396	0.072	1	0.003	0.003	3.338	0.074
IN Status x Initial Size	1	0.392	0.392	3.727	0.060	1	0.005	0.005	5.508	0.023
Error	46	4.838	0.105			46	0.001	0.001		

[a]Infection Status

3.6. Survivorship

During the first growth period (July 2007), 37 individuals died over the 25-day trial, 29 infected and 8 uninfected, comprising 52.7% of infected individuals and 20% of uninfected individuals (Fig. 4.10). However, there was no significant difference in survivorship (Fig. 4.10, Table 4.10). During the second trial (August 2007), 18 individuals died, 9 infected and 9 uninfected, comprising 42.9% and 27.3% of infected and uninfected individuals in the 21-day period. In contrast to the first trial, there was a significant difference in survivorship (Fig. 4.10, Table 4.10). There was a significant difference in the average length of all individuals that died between the two trials ($F_{55,2}$=31.0, p<0.0001) in which mortality was monitored, with dead individuals from the July 2007 trial (19.5 ± 0.92mm) being smaller than the August 2007 trial (27.8 ± 1.1mm).

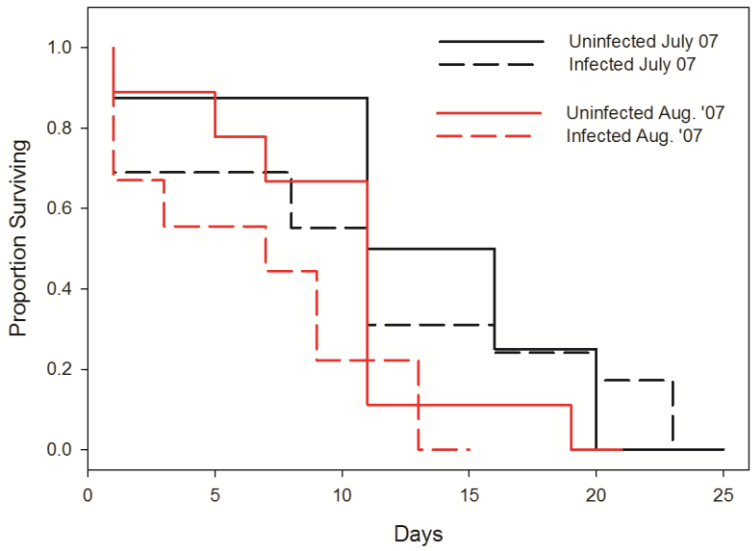


Fig. 4.10: Proportion of infected and uninfected *Janolus fuscus* surviving during two monitoring periods: July 14 to August 7, 2007 (25 days) (red lines) and August 3 to 23, 2007 (21 days) (black lines). Kaplan-Meier survival analyses suggest uninfected individuals survived significantly longer than infected counterparts during August 2007 experiment but not in July 2007. Dotted and solid lines represent infected and uninfected *J. fuscus*, respectively.

Table 4.10: Results of Kaplan-Meier survival analyses comparing survivorship in infected (IN) and uninfected (UN) *Janolus fuscus* in two trials, from July 14 to August 7, 2007 (25 days) and August 3 to 23, 2007 (21 days).

Trial	Infection Status	Average Days Survived	SE	N	X^2	$p > X^2$
July 2007	UN	18.63	1.71	8	2.013	0.1559
	IN	15.14	1.21	29		
August 2007	UN	15.44	2.02	9	5.173	0.0229
	IN	9.0	1.73	9		

4. Discussion

The endoparasitic splanchnotrophid copepod *Ismaila belciki* reduces female reproductive output in its host, the nudibranch *Janolus fuscus*. Infected female slugs are not castrated, but suffer a significant reproductive loss, as much as 73% of reproductive output compared to uninfected slugs. *Ismaila belciki* has no significant impact on male reproductive output of *J. fuscus* or on host growth, but may increase host mortality. The data presented in this manuscript not only provide evidence of the impact this parasite has on the population of *J. fuscus* in the Coos Bay region, but also provides insight to the mechanisms through which it reduces reproduction. The mechanisms by which splanchnotrophids reduce reproduction in their opisthobranch hosts are not well understood. Potential mechanisms that reduce host reproduction include direct gonad consumption (Wilson and Denison, 1980; O'Keefe et al., 2002), selective targeting of host reproduction (Reinhard, 1956; Rubiliani and Godette, 1981; de Jong-Brink et al., 1991; Hordijk, 1992; de Jong-Brink, 1995), mechanical damage or crowding of host

ovotestis (Salmen, 2008a), and nutritional drain on the host (Arme and Owen, 1967; Becker, 1980; Van Wyk, 1982; Heins and Baker, 2003, 2008; Heins et al., 2004; Schultz et al., 2006).

Male and female *Ismaila belciki* are often found attached to host gonad by their mouths, implying a high probability of consumption of this tissue. However, no obvious damage to the host ovotestis has been observed during any of the dissections conducted over the course of this study. Similarly, Salmen et al. (2008a) found a splanchnotrophid, *Ceratosomicola* species with their cephalic structures attached to host gonad but there was no apparent damage to the tissue. Many splanchnotrophids have highly reduced mouthparts and *Ismaila* species lack an anal opening (Salmen et al. 2008a; pers. obs.). Salmen et al. (2008a), therefore, inferred that splanchnotrophids are unable to consume host gonad and instead consume host haemolymph. Our observations are in general agreement, though the cephalic structures of *I. belciki* do not appear highly reduced. More studies are needed to determine the mode of nutrition in these host-parasite systems.

Infection by *Ismaila belciki* does not deter copulation or sperm transfer and does not affect mating behavior. Virgin female slugs mated with infected males were still able to produce egg masses and viable embryos, confirming infected slugs are able to produce and transfer sperm. Additionally, mating with an infected mate did not cause significant declines in female reproductive output, though uninfected females with infected mates tended toward lower reproductive output measures than uninfected females with uninfected mates. In contrast, some reports on other splanchnotrophids suggest total cessation of host copulatory behavior. Jensen (1990) noted that the sacoglossan *Elysia*

australis infected with *Splanchnotrophus elysiae* had well developed gonads but had lost the interest or ability to copulate with an uninfected conspecific.

No studies have examined whether any species of Splanchnotrophidae selectively target reproductive energy by interfering with the host's endocrine system. Crustacean and trematode parasites show sophisticated hormonal control of host physiology, enabling them to reduce or block host reproduction through modification of secondary sexual characteristics or vitellogenesis (Reinhard, 1956; Charniaux-Cotton, 1960; Rubiliani and Godette, 1981; Rubiliani, 1983, 1985; DeVries et al., 1989; de Jong-Brink et al., 1991; Hordijk, 1992; de Jong-Brink, 1995). In contrast, infected female *J. fuscus* are still able to produce viable embryos, indicating that secondary sexual organs are not modified and that vitellogenesis is not inhibited. Likewise, infected slugs do not appear to compromise functionality of the male secondary sexual organs, as infected males still copulate, produce and transfer viable sperm, fertilizing their mate's eggs. This suggests hormone-induced reduction of host reproduction is unlikely in the *I.belciki-J. fuscus* system. It seems more plausible that *I. belciki* drain energy from the host via hemolymph consumption, and the host, in turn, must balance its remaining resources to optimize growth, reproduction, and survivorship to maximize fitness.

Schrödl (1997) and Haumayr and Schrödl (2003) interpret gonad destruction (or atrophy) in *Flabellina* sp. 1 as space competition between the parasite *Ismaila damnosa* and the host rather than active tissue feeding. Like many splanchnotrophids, female *I. damnosa* are large (≤ 3 mm) relative to their host (10-18 mm) and are located posterior to the pericardium where they are likely to compete for space typically filled with host ovotestis. Large relative size and posterior position also is a likely explanation for

atrophied/damaged gonad reported in other studies of opisthobranchs with splanchotrophid infections (Bergh, 1868; Jensen, 1987; Marshall and Hayward, 2006), although rigorous tests in these pairings have not been conducted. Female *I. belciki* are also large relative to their hosts (averaging 1.7 % of the host's mass) but are found anterior to the host pericardium where the ovotestis is less developed (chapter IV). *Ismaila belciki* does not castrate its host fully, perhaps due to its reduced competition for space with host ovotestis.

Though not significant, there were negative relationships between parasite and host reproductive output, indicating that energy is likely diverted from host to parasite reproduction. Reproductive output of slugs infected with a non-productive copepod was not significantly different from uninfected slugs, suggesting that the cost to the host to maintain a parasite is minimal. In contrast, reproductive output in hosts with productive female copepods declined by 50% to 73%, on average. This suggests that energy is transferred directly from host reproductive output to parasite reproductive output, on the order of 25 to 36% of host reproductive output. Parasites typically are efficient assimilators and may allocate a much greater percentage of subsumed energetic resources to reproduction compared to somatic growth and maintenance than their hosts (Bailey, 1975; O'Brien and Wyk, 1985). For example, O'Brien and Wyk's (1985) reevaluation of Anderson's (1977) work on the energy flow to the parasitic isopod *Probopyrus pandalicola* from its shrimp host, *Palaemonetes pugio*, showed that both the isopod (4.3%) and non-parasitized hosts (4.4%) spent an equivalent proportion of the energy ingested by the shrimp on reproduction. This suggests that *P. pandalicola* allocates almost all the stolen energy (ingested by the host for host reproductive output), to its own

reproduction, minus about 0.1% for other metabolic needs compared to the 95% used by the host for other metabolic processes. Though the true energetics of the *I. belciki – J. fuscus* association are unknown, our results imply that more than a third of the host's reproductive effort may be converted directly to reproduction by this highly fecund endoparasite.

Hematophagy may lead to substantial nutrient drain and result in reduction or elimination of host reproduction (Anderson, 1977; Walker 1977; Van Wyk, 1982; Polak, 1996). Anderson (1977) and Walker (1977) estimated that the bopyrid isopod, *Probopyrus pandalicola*, ingests 10% of the host shrimp's (*Palaemonetes pugio*) energy intake and 25% of its hemolymph volume daily and eliminates host reproduction. Such high rates of hemolymph ingestion may lead to sub-optimal host nutrition resulting in complete or incomplete castration. Becker (1980) emphasized the similarity between the effects of starvation and *Schistosoma mansoni* infection on the snail *Biomphalaria glabrata*, as the fluke causes a decrease in hemolymph glucose levels, which leads to adverse effects on host oogenesis. Van Wyk (1982) suggested that nutrient drain via hemolymph consumption is the cause of partial castration of the porcellanid crab, *Pachycheles rudis* by the bopyrid isopod, *Aporobopyrus muguensis*. Like *A. muguensis*, *Ismaila belciki* does not entirely eliminate, but causes a significant reduction in, host reproduction. Additionally, infected *Janolus fuscus* experience reduced survivorship in older hosts, suggesting *I. belciki* does not selectively target host reproductive energy, but may act as a general nutritive drain on the host, most likely by consuming host hemolymph.

In true castrator systems (i.e., host reproduction completely blocked), studies have found either increased (Rothschild, 1941; Sturrock, 1966; Arnott et al., 2000; Krist, 2000; Hechinger, 2010), decreased (Kuris, 1980; Sousa, 1983; Fogelman and Grutter, 2008), or no difference in (Fernandes and Esch, 1991) host growth with infection (Gorbushin and Levakin, 1999; reviewed in Sorensen and Minchella, 2001). As *I. belciki* is not usurping and/or causing redirection of all the energy typically devoted to reproduction, I would not expect to see an increase in somatic growth in *J. fuscus*, though "gigantism" has been found in hosts of partial castrators (Pearre, 1976). Size-prevalence curves from field surveys of *J. fuscus* do not suggest gigantism (chapter IV). However, I do see some evidence of increased growth in that young, infected slugs have greater growth rates than uninfected counterparts. Increased growth of the host at a young age would increase the space available to, and decrease the damage incurred by, the parasite as it grows, a benefit to both host and parasite. Also, I found that growth rate increased in infected slugs over time, unlike uninfected slugs that experience decreased growth rates with time. Keas and Esch's (1997) found that a trematode castrator, *Halipegus occidualis*, increases growth of a freshwater snail, *Helisoma anceps*, when infection occurs before the host reaches reproductive maturity and is fed a high quality diet. Other studies suggest that enhanced growth of infected hosts relative to uninfected hosts should occur in the early phases of infection when the trematode parasite is still small relative to the host; in late phases of infection when the parasite requires more host resources, the parasite may stunt growth (Pan, 1965; Sturrock and Sturrock, 1970; Minchella et al., 1985; Gerard and Theron, 1997; Ebert, 2004). While small hosts are more likely to have early infections

than larger (older) hosts, the progression of infection is unknown in these experiments, limiting what I can infer about the disparity in growth rates between trials.

The impact of typical parasites on host mortality ranges from highly pathogenic to having no discernable effect (Roberts and Janovy, 2009). While increased survivorship has been reported in hosts infected with parasitic castrators, host survivorship varies by host-castrator system and infection does not decrease mortality as a rule (Sturrock and Sturrock, 1970; Minchella et al., 1985; Fredensborg et al., 2005; Fogelman, 2009; Lafferty and Kuris, 2009). Survivorship decreased significantly with infection in mature *Janolus fuscus* (average length = 27.8 ± 1.1 mm) but did not differ by infection status in slugs just reaching maturation (average length = 19.5 ± 0.92 mm). Mature slugs devote energy to reproduction as well as growth and maintenance. The energy drain of *Ismaila belciki* infection is more likely to surpass a threshold above which mature slugs can no longer complete all three tasks. Though I do not know the duration of infection, mature hosts also are likely to have been infected longer and with larger or more parasites (Ebert, 2004). Splanchnotrophid copepods have been shown to damage their host's internal organs (e.g., digestive gland) (Monod and Dolfus, 1932; Schrödl, 1997; Marshall and Hayward, 2006). Decreased survivorship in these cases has been attributed to this mechanical damage (Monod and Dolfus, 1932; Schrödl, 1997; Marshall and Hayward, 2006). However, I did not observe obvious destruction to internal organs in *J. fuscus* that could explain reduced survivorship. Rather, I believe that reduced survivorship is a direct impact of the nutrient drain caused by the parasite.

Ismaila belciki are highly fecund, producing an average of 33,643 embryos/egg sac every two to three weeks at the host's expense. Over a 30-day period this averages to

a total of 88,359 viable embryos. However, as *Ismaila* spp. can renew egg sacs overnight after hatching or artificial removal of eggs sacs (Schrödl, unpublished data in Salmen et al. 2008; pers. obs), estimates for copepod fecundity over the 30-day period may be slight overestimates. Though removing egg sacs before they hatch was necessary to ensure an accurate measure of copepod reproductive output, this removal could have caused the parasite to assimilate more host energy to replace eggs sacs than if sacs had not been removed. This may explain the greater proportional decrease in slug reproductive output (relative to uninfected slug reproductive output) in the "Host-Parasite Reproduction" experiment (e.g., 53% smaller egg mass wet weight) compared to the "Host Reproduction" experiment (e.g., 35% smaller egg mass wet weight). However, the reproductive output of uninfected slugs was also lower in the "Host-Parasite Reproduction" experiment compared to the "Host Reproduction" experiment suggesting some factor other than removal of copepod egg sacs limited slug reproductive output in the second experiment. Additionally, there were no significant interactions between experiment and female or mate infection status to suggest these differences in reproductive output could be explained simply by variations in the experimental conditions.

Ismaila belciki is not a parasitic castrator, though every measure of female reproductive effort (egg mass wet weight, number of egg capsules, estimated number of embryos, and gonadosomatic index) was reduced in infected *Janolus fuscus* relative to uninfected slugs. Although our statistical power was low, there was no indication that reduced reproductive output was intensity dependent and no circumstances of multiple infections resulted in total castration. In contrast with this study, a Chilean nudibranch,

Flabellina sp., infected with *I. damnosa* did not produce any egg masses over an experimental period, suggesting total castration (Schrödl, 1997). Jensen (1987) reported that the sacoglossan *Ercolania viridis*, infected with an *Ismaila* sp., were sterilized as most of their gonad tissue was missing. Likewise, the nudibranch *Phidiana lynceus* infected with *I. montrosa* had severely atrophied ovotestes (Bergh, 1868). In contrast, some studies suggest splanchnotrophids incur no damage to host gonad tissue (Jensen, 1990; Haumayr and Schrödl, 2003; Salmen et al., 2008a), but did not test for the ability to produce egg masses or viability of embryos. It is plausible that a range of castrating abilities occurs in the various species of splanchnotrophids.

Ismaila belciki share many traits with parasitic castrators (they are large relative to their host, show a positive correlation of their mass with host mass, exhibit strong site specificity and evidence of intraspecific competition within the host), but they do not completely castrate their host (Kuris, 1974; Lafferty and Kuris, 2002, 2009; chapter IV). Examples of parasites that significantly reduce reproduction without blocking it completely are rare (Pearre, 1976; Lanciani, 1982; Van Wyk, 1982; Heins and Baker, 2008). Jaenike (1996) infer from host-parasite models, that the apparent advantages of castration for the parasite are so strong that imperfect castration may be an example of suboptimal parasite virulence. Why does *I. belciki* not specifically target and completely eliminate host reproduction, minimizing its impact on host survivorship and extending the parasite's own reproductive period? Lafferty and Kuris (2009) suggest that "partial castrators" may be transitioning from an association evolving towards parasitic castration from a typical parasite adaptive strategy. *Janolus fuscus* are short-lived (~ 5 month lifespan), fast growing, voracious predators and prevalence of *I. belciki* is often high

along the Oregon coast (chapter IV). The host population available for infection each year is dependent on the previous year's reproduction, though host recruitment is open. Total castration of the host by *I. belciki* would result in a high likelihood of local extinction each year. Repeated cycles of local extinctions would place strong evolutionary pressure on the *Ismaila – Janolus* system away from complete castration. Indeed, there is strong evidence that parasitic castration is more likely to evolve in parasites that infect long-lived hosts with high reproductive investment (Hurd, 2001; Ebert, 2004, Lafferty and Kuris, 2009). Therefore, I suggest that *I. belciki* resides in the valley between the adaptive peaks of typical parasites and parasitic castrators described by Lafferty and Kuris (2009), constrained by the ecology and population dynamics of the host, *J. fuscus*.

5. Bridge IV

In chapters IV and V, I found that although *Ismaila belciki* has ecological attributes in common with parasitic castrators, it does not eliminate host reproduction but causes significant reduction in reproductive output of *Janolus fuscus*. In chapter VI, I address another facet in the life history of *I. belciki*, its development. In the following chapter nauplius larvae of *I. belciki* were reared in the laboratory and incubated with *J. fuscus* in an attempt to infect potential hosts. Dissections were also conducted to look for internal copepodid stages. Larval stages were described with the aid of light, scanning electron, and confocal microscopes.

CHAPTER VI

LARVAL STAGES IN THE LIFE CYCLE OF *ISMAILA BELCIKI* (COPEPODA: CYCLOPOIDA; SPLANCHNOTROPHIDAE), AN ENDOPARASITIC COPEPOD OF THE NORTHEAST PACIFIC NUDIBRANCH, *JANOLUS FUSCUS*

Introduction

The Copepoda includes free-living species and species that are parasitic on various animals. The life cycle and larval stages of free-living species typically include six naupliar stages followed by five copepodid stages before the final molt to a juvenile (Izawa, 1987; Gotto, 1979). However, in those orders of the Copepoda that have parasitic representatives, Siphonostomatoida and Cyclopoida, the number of larval stages may vary from zero to six naupliar stages and one to five copepodid stages (Costanzo, 1959; Dudley, 1964; Gotto, 1979; Do et al., 1984; Izawa, 1987). Over one thousand species of poecilostome copepods (Cyclopoida) are known to infect marine fish and invertebrates, but the larval stages of most species are unknown (Izawa, 1987). Much of the work on the life cycles of poecilostome copepods addresses species infecting fish hosts, with relatively few studies focusing on species infecting invertebrates (Pesta, 1907; Paterson, 1958; Costanzo, 1959; Bocquet et al., 1963; Dudley, 1964, 1966; Gotto, 1979; Nakamura et al., 1979; Do et al. 1984; Humes, 1986; Izawa, 1986b; Lamb et al., 1998; Dojiri et al., 2008). Copepods of the family Splanchnotrophidae infect marine

invertebrates and this is one of many poecilostome families in which the life cycle is unknown and the larval stages are virtually unstudied (Belcik, 1981; Ho, 1987b).

Copepods from the family Splanchnotrophidae are highly modified endoparasites of marine opisthobranch gastropods. These parasites that inhabit the mantle cavity and/or cerata of opisthobranch gastropods and are found worldwide. *Ismaila*, with eleven described host-specific species, is the most speciose of the five described splanchnotrophid genera,. While many studies have addressed the morphology and systematics of splanchnotrophids (Bergh, 1868; Belcik, 1965, 1981; Ho, 1981, 1987a; Jensen, 1987; Huys 2001; Haumayr and Schrödl, 2003), only two studies have described any larval stages from this group (Belcik, 1981; Ho, 1987b). Belcik (1981; and Dudley, in litt., 1965 therein) described the first nauplius of *Ismaila belciki* from Oregon and suggested there are at least two planktotrophic nauplius stages in the life cycle. Ho (1987b) described three copepodid stages (copepodid II, III, and both sexes of copepodid IV) of a congener *Ismaila occulta* from a single nudibranch host, *Dendronotus iris*, collected in Long Beach, California. Though he did not find the first and fifth copepodid stage, he surmised that five copepodid stages occur and that copepodid I is the infective stage (Ho, 1987b).

Up to 81% of the population of the arminacean nudibranch *Janolus fuscus* in Coos Bay, Oregon can be infected with *Ismaila belciki* (chapter IV). The adult morphology of male and female *I. belciki* has been described in detail by Belcik (1965, 1981 as *I. monstrosa* before re-examination by Ho, 1987a). Female *I. belciki* are large (up to 5.5 mm) relative to their hosts (average 1.7% of host mass) (Belcik, 1965; chapter IV). Males are smaller (up to 1.9 mm) (average of 0.12% of host mass) and typically

associated with a female (Belcik, 1965, 1981; chapter IV). Female copepods are typically oriented perpendicular to the axis of the host and reside in the host hemocoel just anterior to the host pericardium, with her posterior protruding through the host's dorsal integument to produce two egg masses (chapter IV). Though Belcik (1981) described the first naupliar stage, which emerges from these egg masses, the rest of the larval stages are unknown.

In this study, nauplii of *Ismaila belciki* were reared in the lab and incubated with potential hosts, *Janolus fuscus*, in an attempt to determine the infective stage of this copepod. To piece together development within the host, dissections of host specimens collected from the field were dissected in search of internal copepodids, copepodid molts and juvenile stages. Copepodid stages are compared to those described by Ho (1987b) for the congener *I. occulta*. This study adds several life-history stages to the known life-cycles of splanchnotrophid copepods.

Materials and Methods

Janolus fuscus were collected from 2005 through 2009 at a site near Fossil Pt. (43°21'32.4N, 124°18'45.36W) in Coos Bay, Oregon, and from North Cove (43°18'30.6N, 124°23'58.92W) on the nearby outer coast at Cape Arago. All laboratory work was conducted at the Oregon Institute of Marine Biology, less than 16 km from the collection sites. Nudibranchs were held in individual plastic containers (10.5x10.5x12.5 cm) submerged in flowing seawater tables, where they were fed arborescent bryozoans *Bugula pacifica*, *Tricellaria circumternata*, and *Scrupocellaria diegensis* to satiation. Nauplii of *Ismaila belciki* were collected as they hatched from the female's external egg masses about two weeks after the egg masses appeared on the dorsal surface of the host.

Nauplii were cultured in 0.45 μm filtered seawater at a concentration of 1larva/ml in 1- or 2- L glass jars. Culture jars were partially submerged in flow-through seawater tanks at 11-13°C and stirred by paddles attached to a stir rack, unless otherwise indicated. Larvae were fed 1:1 *Thalassiosira weissflogii* (diatom) and *Isochrysis galbana* (flagellate) (~1.5 x 10^4/ml) every other day (Strathmann, 1987). Light, scanning electron, and confocal microscopes were used to examine nauplii, copepodids and molts of *I. belciki* to assess potential morphological changes between stages. Specimens were fixed for SEM using 2% osmium tetroxide followed by filtered seawater and freshwater rinses, dehydrated through a graded alcohol series, and critical point dried before viewing with a Tescan Vega II SEM. For confocal microscopy, specimens were stained over night in 1.5 mg/ml of the florescent marker Congo Red in 100% EtOH and glycerine (as described in Michels and Buntzow (2010)) and viewed on an Olympus Fluoview FV1000 Confocal Microscope System with an He/Ne 543 nm excitation laser.

In July 2008, I used the vital stain Neutral Red (0.1g/L FSW) as described by Anstensrud (1989) to stain two-day-old stage I nauplii of *Ismaila belciki*. Nauplii took up the stain and there were no obvious differences in mortality between stained and unstained cultures from the same parental batches. 100 two-day old nauplius I larvae were incubated with one of 10 uninfected *Janolus fuscus* in 1 L cultures for 15 days under culture conditions described above. Additionally, single *I. belciki* nauplii were kept in unstirred 10-ml culture wells also held in flow-through seawater tanks at 11-13°C. Nudibranchs were removed every other day and examined under an Olympus 7x dissecting scope for Neutral Red-stained copepod stages within the host integument. Nudibranchs were fed arborescent bryozoans, *Bugula pacifica* and *Tricellaria*

circumternata overnight, rinsed in 0.45 μm filtered seawater, and returned to the incubation culture with copepod nauplii. During the absence of nudibranchs cultures of larval copepods were examined for naupliar molts and later stage nauplii. Representative nauplii and molts were examined and photographed on an Olympus BX50 compound microscope at 40x magnification. Cultures were also cleaned, filtered seawater replaced, and algae added during this period. Following the incubation period, *J. fuscus* were anesthetized in 7.5% MgCl and preserved in 10% formalin in seawater.

In September 2010, a second infection trial was conducted over 14 days. Nauplii were collected immediately after hatching as described above but not stained with neutral red. In 150 nauplii were immediately added to each of three150-ml unstirred culture dishes with a potential host and fed *Isochrysis galbana* only at a concentration of 1.5 x 10^4 cells/ml. Two stirred 1-L cultures at a concentration of 1 larva/ml were maintained without nudibranchs. Cultures were examined and ten or more representative larvae were removed two, three, five, eight and 11 days after hatching from both stationary and stirred cultures. Nudibranchs were examined, fed and rinsed as described above. Two nudibranch hosts were preserved (as above) on day five and the third nudibranch on day 14.

Dissections following incubation periods were conducted on a Leica Fluo III epiflorescent dissecting scope (8x magnification). Copepods autofloresced when specimens were excited using a mercury lamp and emitted light filtered through a GFP Plus filter. Larval stages were stained with Congo Red and viewed on a confocal microscope as described above.

Supplemental dissections of 76 infected *Janolus fuscus* collected from field sites from 2005 through 2009 were conducted under a Unitron LWZ dissecting microscope at 4.5x. To better view copepod stages found during dissections, specimens were preserved for scanning electron and confocal microscopy as described above.

Identification of copepodid stages of *Ismaila belciki* relied heavily on the work of Ho (1987b) which gives extensive detail of the morphology of these stages in a congener, *I. occulta*. The overall size and shape, number and relative size of somites (segments), and protopod shape and relative size of endo and exopods of the legs were used to assign stages to copepodid II-IV. The the first antennae of many samples appeared fused with the second antennae and fine structure could not be used as clues in stage identification.

Results

Egg sacs were opaque white, cream, or pink just after they were laid but became more transparent with development (Fig. 5.1A). Unhatched embryos of *Ismaila belciki* were 68 ± 1 μm (standard error) in length. Naupliar eyes were visible within embryos before hatching (Fig. 5.1B).

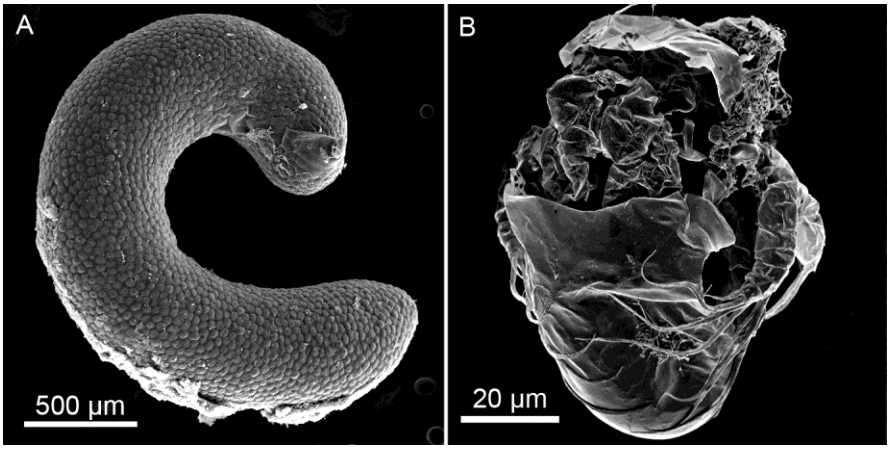


Fig. 5.1: Scanning electron micrographs of A, an egg sac of *Ismaila belciki* with many embryos and B, a nauplius I hatching out of the egg capsule.

Infection attempts

When fed, naupliar stages consumed *Isochrysis galbana* and *Thalassiosira weissflogii* as both algae were visible within the larval gut. In both the July 2008 and September 2010 incubation cultures and non-incubated stirred cultures, first molts were found on the third day after hatching. In July 2008, individual nauplii kept in isolated wells of plate cultures did not molt and thus, a molting event could not be tied to individual larvae. No further naupliar stages (past nauplius two) or molts were found in cultures, and cultures died by day 13 in the July 2008 incubation experiment. One *Janolus fuscus* incubated with nauplii of *Ismaila belciki* in July 2008 contained a copepodid stage III (see below). In the September 2010 trial, a third naupliar stage (second molt) was found in a stationary culture and in each of two non-incubated 1-L cultures on day eight. Dissections revealed no copepod stages within the two *J. fuscus* preserved on day five or in the single host preserved on day 14.

Dissections

Of the 76 infected *Janolus fuscus* dissected, only 12 copepodid stages and six molts of *I. belciki* were found within hosts, primarily in the host's cerata. This is probably an undersampling of the true number of copepodid stages found within these host specimens. The large size and similar color of the host's gonad and viscera made finding small copepodid stages difficult. Copepod exoskeletons autofloresced when excited with a mercury lamp under the epiflorescent dissecting microscope, but so did many parts of the nudibranch, including the gonad, digestive diverticula and buccal mass. I attempted to use dyes that preferentially stained chitin as found in copepod exoskeletons

(e.g., Calcaflor White and Congo Red) in the hopes of making copepod stages more apparent within the host tissue. Unfortunately, these dyes also stained nudibranch tissue.

External stages

Nauplius I

The first planktotrophic naupliar stage of *Ismaila belciki* ranged in size (carapace length x width) from 87.5 x 47.0 to 92.4 x 51.8 µm (Fig. 5.2A-D). The labrum was prominent with five spinules arranged in a row along the posterior margin (Fig. 5.2C, D). The labium was raised with a marginal row of fine spinules (nine+) on the margin and either side of a posterior protrusion (Fig. 5.2C, D). The first antenna was uniramous and three-segmented with three and two setae on the second and third segments, respectively (Fig. 5.2A-D). The second antenna was biramous with a two-segmented protopod (with a coxa and basis), one-segmented endopod and five-segmented exopod (Fig. 5.2A, B). The coxa had one prominent feeding spine and the basis had one long and two short spines (Fig. 5.2A-D). The exopod had six setae, one on each of four proximal segments and two on the terminal segment. The endopod was one-segmented with four setae, two distal and two medial. The mandible was biramous with a two-segmented protopod. The coxa bore one spine with two spinules and the basis had two spines, one with spinules. The exopod was four-segmented with one seta on each segment. The endopod was two-segmented, the first segment with two spines and the second segment with four spines (Fig. 5.2C, D). The body was ornamented on the ventral surface with three flap-like processes, one medial and two more posterior with small spinules on the posterior margins (Fig. 5.2D). Furcal armature consisted of a median process between the caudal

rami with 10+ small spinules (Fig. 5.2C). The caudal ramus supported a singular plumose seta (Fig. 5.2A-D).

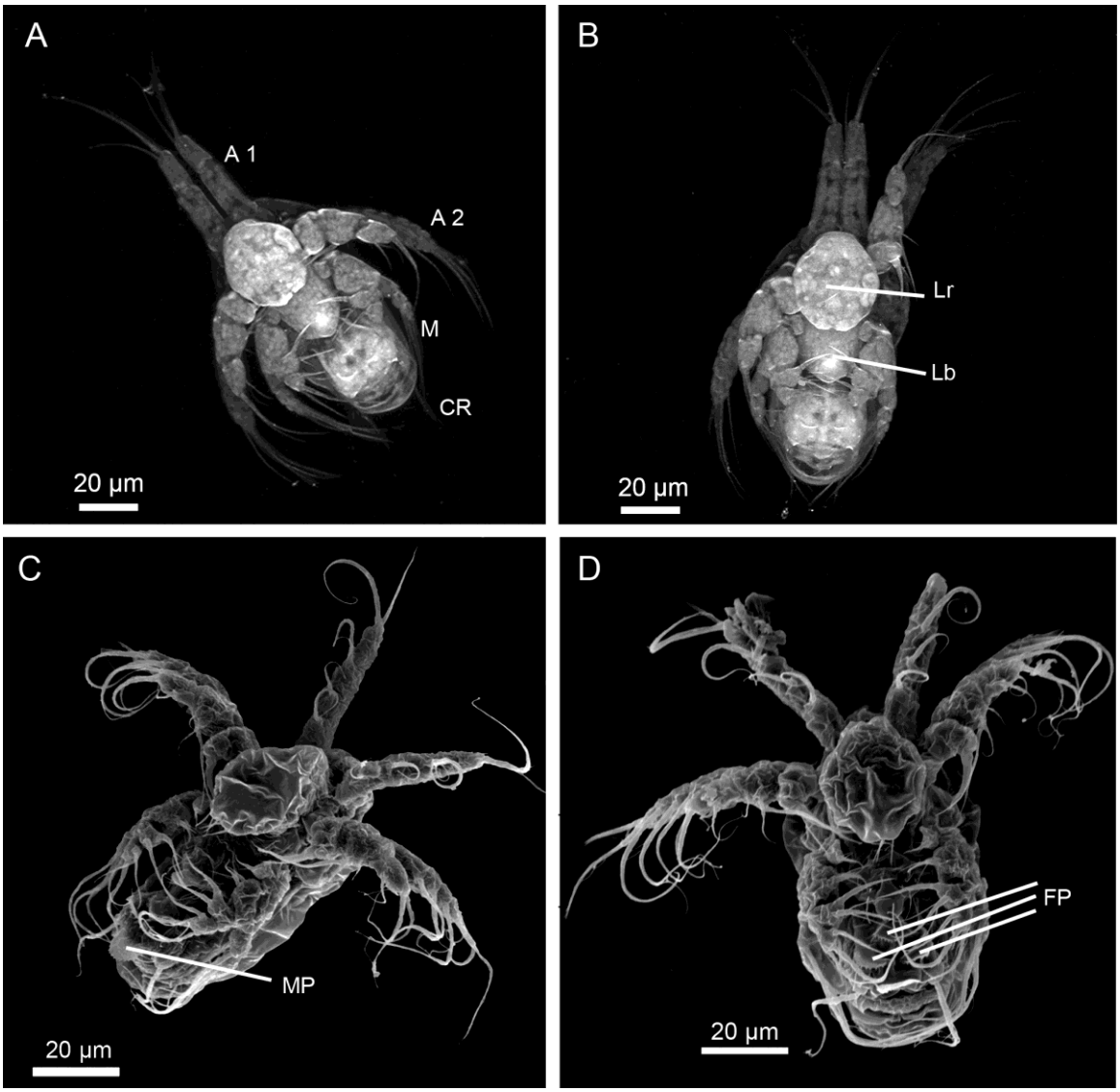


Fig. 5.2: Nauplius I of *Ismaila belciki* A, and B, confocal micrographs of two nauplii, stage I; C and D, scanning electron micrographs of two nauplii, stage I. A1, first antenna; A2, second antenna; CR, caudal ramus, FP, flap processes, Lr, labrum, Lb, labium, M, mandible, MP, median process of caudal ramus.

Nauplius II

Nauplius II stages ranged in size (carapace length x width) from 94.4 x 49.6 µm to 102.9 x 49.4 µm (Fig. 5.3A, B). The first antenna had an additional seta on the last

segment (now three total) (Fig. 5.3A, B). The exopod of the second antenna supported a total of seven setae, the two most proximal very minute. Additional setae added to the inner distal end of segment one of the endopod of the second antenna for a total of five setae (Fig. 5.3A). An additional spine was added to the first segment of the mandible endopod (three total) (Fig. 5.3B). A rudiment of the first maxilla was present on the post-mandibular ventral surface (Fig. 5.3A).

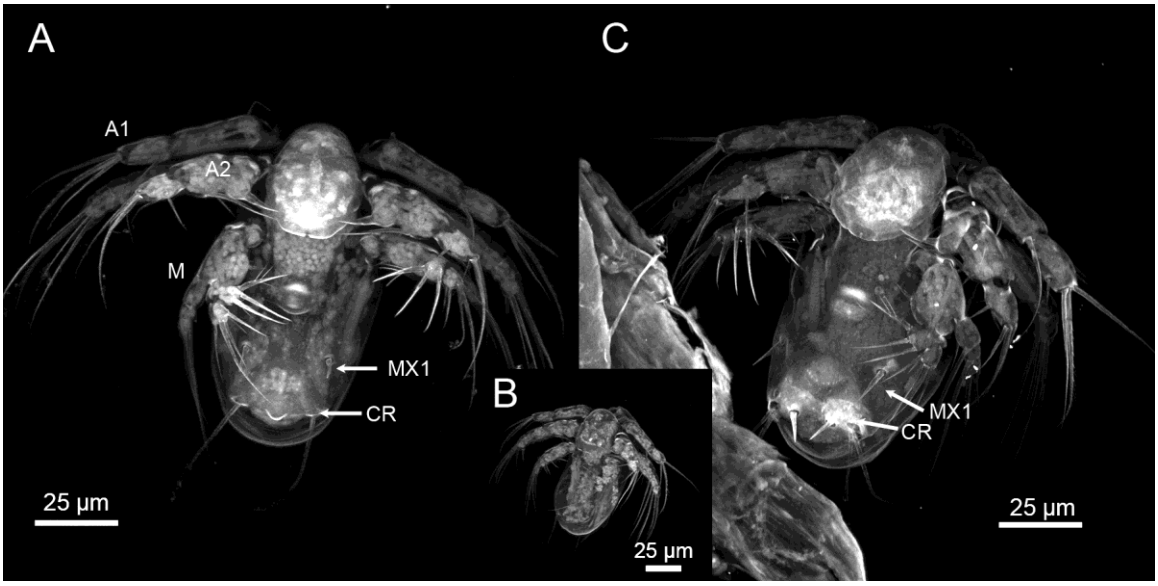


Fig. 5.3: Confocal micrographs of A, nauplius II of showing most ventral serial sections, note first maxilla; B, nauplius II showing overall shape from all serial sections; and C, nauplius III of *Ismaila belciki* with all serial sections, note one spine and two setae of caudal ramus. A1, first antenna; A2, second antenna; CR, caudal ramus; M, mandible; MX 1, first maxilla.

Nauplius III

Nauplius III stages ranged in size (carapace length x width) from 107.0 x 49.0 µm to 112.0 x 55.9 µm. The first antenna possessed three additional setae on the last segment for a total of six (Fig. 5.3C). The exopod of the second antenna had two additional setae (nine total). The endopod had at least one additional seta (six+ total). The mandible

basis had three spines (two minute). The first segment of the mandible endopod had an additional spine (total four) (Fig. 5.3C). The caudal ramus supported an additional seta and one spine (Fig. 5.3C).

Internal stages

Copepodid II

Only a single specimen of a copepodid II (250.0 x 99.3 μm) (total length x width) was found in a ceras of a young host (2.4 mm). This individual moved in the host hemocoel from a ceras to the lower host body and back while being observed. The copepodid had a straight cephalothorax, five somites and a prominent round protrusion in the rostral region characteristic of the copepodid II described in *Ismaila occulta* (Ho, 1987) (Fig. 5.4A, B). A detailed description of cephalic and thoracic appendages could not be made as the specimen was destroyed in the critical point drying stage of the SEM preservation process.

Copepodid III

Two copepodid III specimens were 424.5 x 163.8 μm and 385.2 x 146.3 μm (total length x width) (Fig. 5.4C-F). The cephalothorax of copepodid III was elongate and straight and followed by somites two through six (Fig. 5.4C, D, E, F). Segmentation was not obvious in either specimen as the exoskeleton was not visible (Fig. 5.4C, D, F). More subtle contours and constrictions visible in the thorax as well as serial Z-projections through the specimen (seen with a confocal microscope) revealed points of segmentation. The round protrusion of the rostral area found in the copepodid II stage was absent (Fig. 5.4A, D, E). An endopod was absent in the first leg but present in the second leg (Fig.

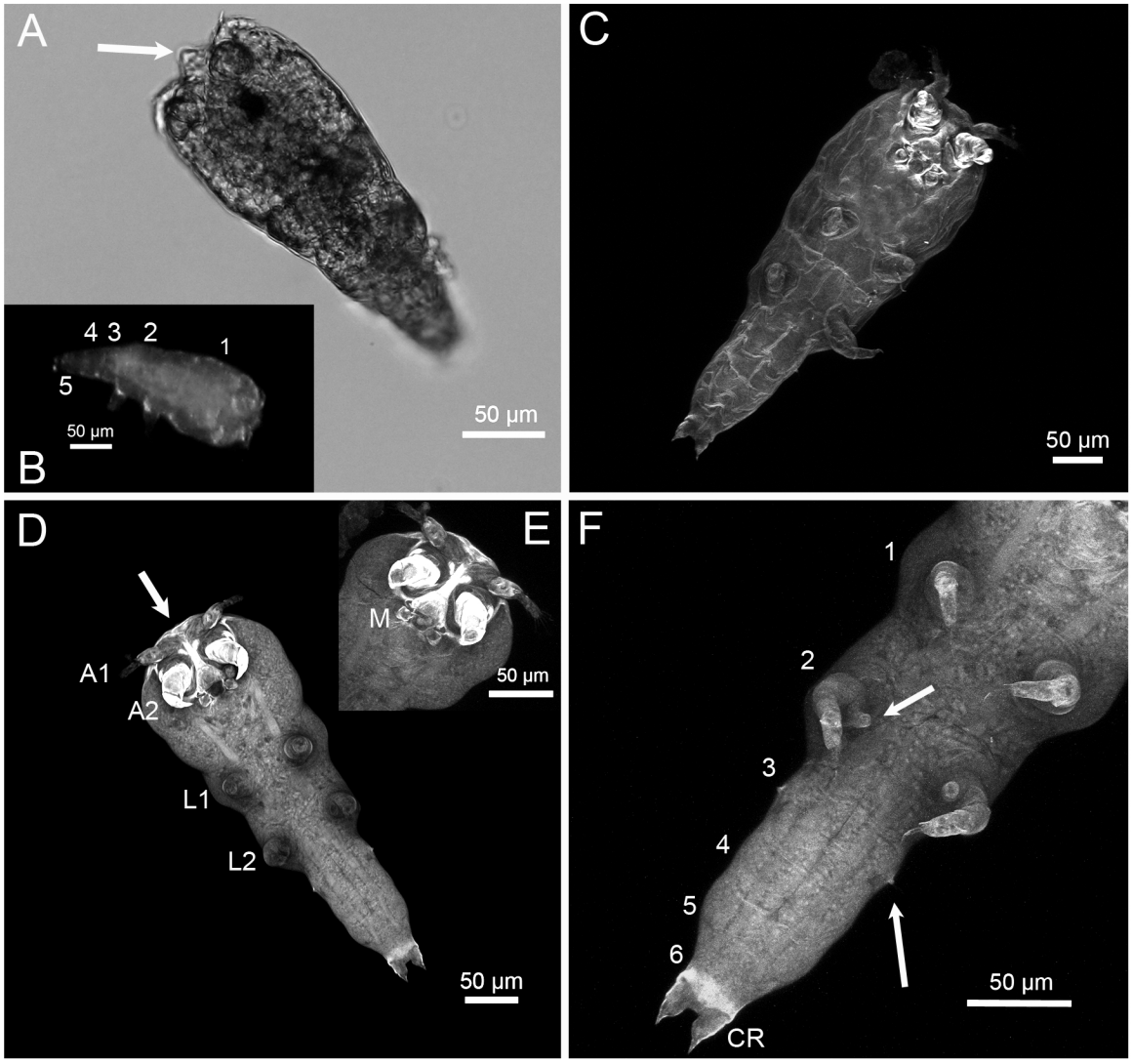


Fig. 5.4: Copepodid II and III of *Ismaila belciki* found inside *Janolus fuscus* A and B, light micrographs a copepodid II. Arrow indicates round rostral protrusion. C, confocal micrograph of copepodid III (424.5 x 163.8 µm) D) copepodid III (385.2 X 146.3 µm). Arrow indicates absence of round protrusion at this stage. E, cephalosome of same copepodid III; F, the same copepodid III showing uniramous first leg and biramous second leg with small endopod. Arrows indicate endopod of second leg and leg three on the third somite. Numbers indicate number of somites. A1, first antenna; A2, second antenna; CR, caudal ramus; M, mouthparts (mandible and first and second maxilla); L1, leg one; L2 = leg two.

5.4F). The first leg and exopod of the second leg were tipped with a hook. The third leg was a small knob tipped with a short seta (7.3 μm) located on the third somite (Fig. 5.4C, D, F). The mouthparts of copepodid III and later copepodid stages took up the Congo Red stain more than other body parts, suggesting cephalic structures are more heavily fortified with chitin (Fig. 5.4C-F).

Copepodid IV female

Three samples of copepodid IV females ranged in size from 438.0 x 184.2 μm to 545.6 x 247.75 μm (total length x width). The cephalothorax of copepodid IV females showed transformations indicative of the adult stage (Fig. 5.5). The cephalosomal portion of the cephalothorax exhibited lateral pouching and pocketing and was distinct from the pedigerous portion (Fig. 5.5). The second somite was more distinct from the cephalosome and held the second, biramous legs (Fig. 5.5A, C). The base or protopod of the first leg was swollen (Fig. 5.5), a feature not seen in the male copepodid V or juveniles (see Fig. 5.6, 5.7) but present in post-copepodid IV females (see Fig. 5.8A, 5.9). Additionally the second leg had a long endopod, roughly half the length of the exopod (Fig. 5.5). The third and fourth somites were roughly equal in size (Fig. 5.5B, D). The fifth and six somites combined to form the genital somite (Fig. 5.5A, C). The abdomen had two somites of roughly equal size that were distinct from the thorax (Fig. 5.5B, D). There appeared to be a leg 6, represented by a flap at the posterior of the genital somite (Fig. 5.5C).

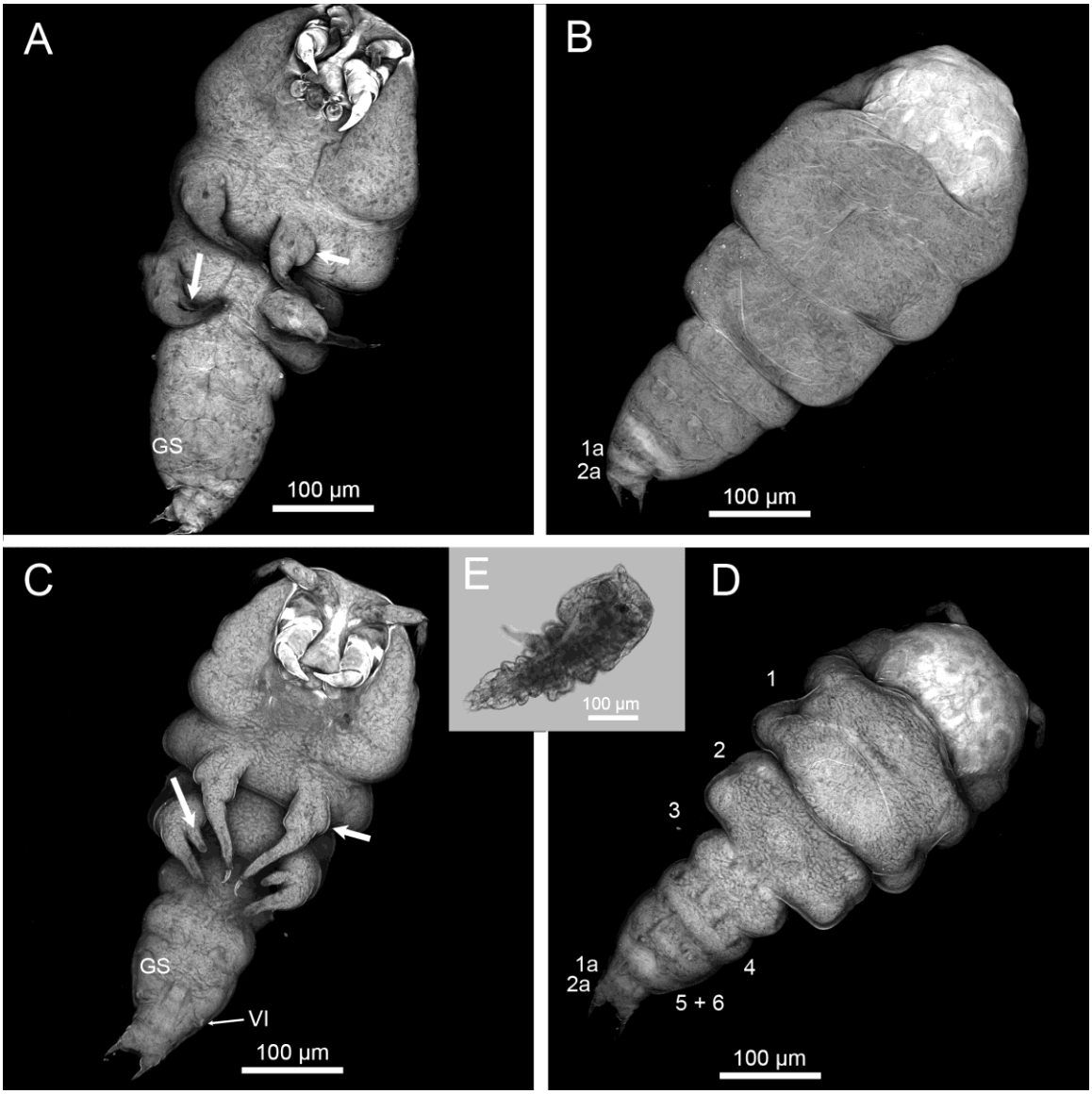


Fig. 5.5: Confocal and light micrographs of female copepodid IV of *Ismaila belciki* A, ventral and B, dorsal view of same female copepodid IV (545.6 x 247.75 µm); C, ventral and D, dorsal view of second female copepodid IV (508.8 x 228.0 µm); E, light micrographs of a third female copepodid IV. Numbers indicate number of somites. 1a and 2a, first and second abdominal segments; GS, genital somite (5 + 6 = fusion of thoracic somite five and six). Arrows indicate the swollen protopod of first leg and endopod of second leg.

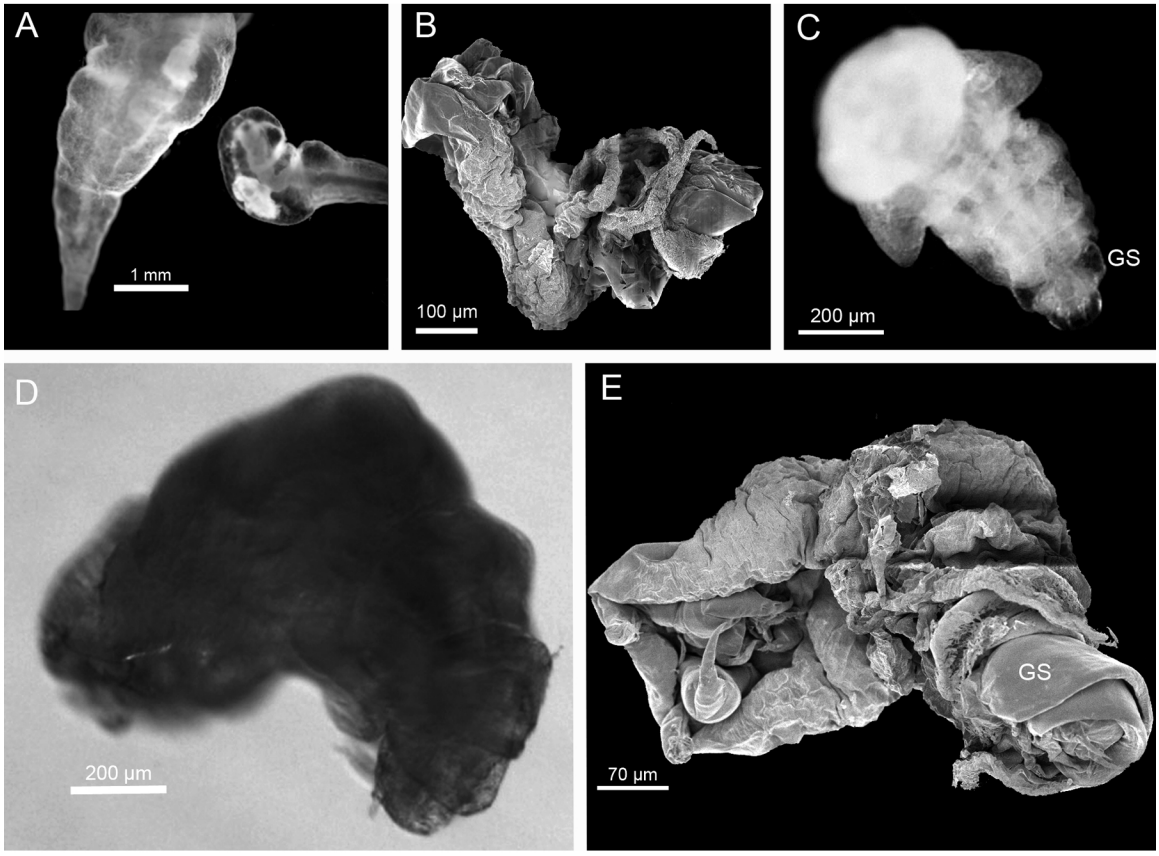


Fig. 5.6: Light and scanning electron micrographs of two potential copepodid IV males of *Ismaila belciki*. A, Two copepodid stages inside of the cerata of *Janolus fuscus*; B, scanning electron; and C, light micrograph of same copepodid IV of *I. belciki* (815 µm) (dissected from ceras on right); D, light and E, scanning electron micrographs of same second copepodid IV male (dissected from ceras on left) GS, genital somite.

Copepodid IV male

Two copepodids found in the cerata of *Janolus fuscus* were not photographed in clear detail on a compound microscope (Fig. 5.6A, C, D) and shrunk severely in the SEM preservation process (Fig. 5.6B, E). From preliminary observations on the compound scope, these two specimens were called copepodid stage IV males as both exhibited over 6 segments with distinct genital somites and were larger (845 µm and 815 µm, in length) and more ventrally bent than copepodid IV females, as described by Ho for *Ismaila*

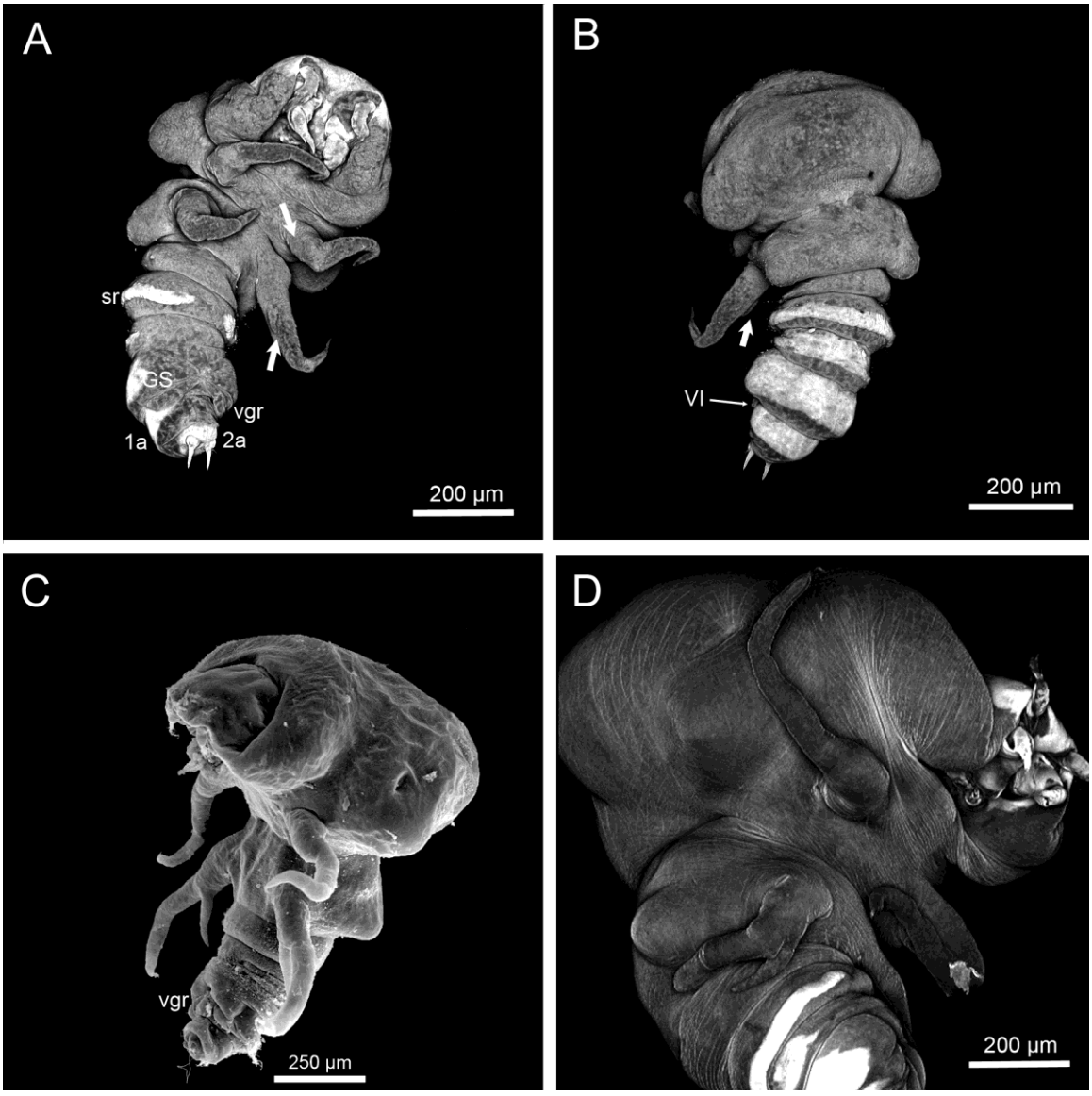


Fig. 5.7: Confocal micrographs of male copepodid V, juvenile male and adult male *Ismaila belciki* A, Ventral and B, dorsal view of a male copepodid V (833.8 x 467.0 µm) (total length x width); C, Scanning electron micrographs of juvenile male *I. belciki* before expansion of the dorsal gonadal lobes; D, Confocal micrograph adult male *I. belciki* with great expansion of the dorsal gonadal lobes. Arrows indicate the slender protopod of first leg and small endopod of second leg, GS, genital somite; 1a and 2a, first and second abdominal somites; VI, leg six represented as a flap on the posterior of the genital somite; sr, sclerotized ring; vgr, ventral genital ridge.

occulta (1987) (Fig. 5.6B-E). They were not as advanced as the copepodid V male, as neither had developed a ventral genital ridge (Fig. 5.7). However, a close examination of first and second leg protopods and the relative size of the endo and exopods of the second legs was impossible in these preserved specimens (Fig. 5.6B, E). Based on their size, it is also possible that these individuals could be post-copepodid IV females.

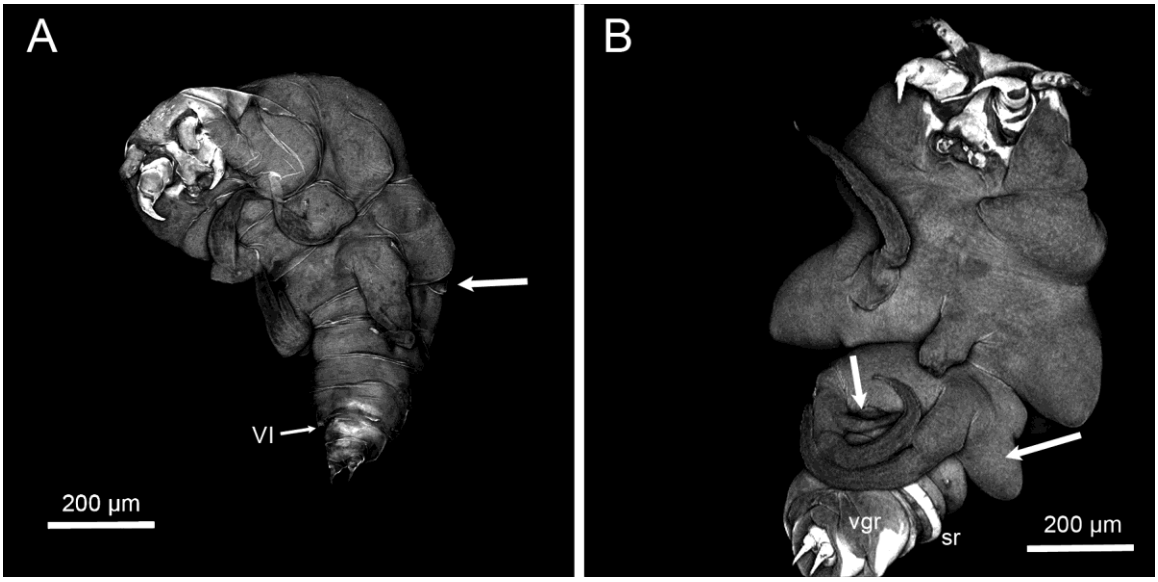


Fig. 5.8: Confocal micrographs of copepodid V female *Ismaila belciki* A, 729.0 x 428.9 µm (total length x width), the arrow indicates developing somite between somite two and three; VI, leg six with spine; B, potential copepodid V female 1036.1 x 622.2 µm. Arrows indicate endopod and outside lateral lobe of tripartite second leg. sr, sclerotized ring; vgr, ventral genital ridge.

Copepodid V and juvenile male

The cephalosomal portion of the cephalothorax in the male V copepodid stage (833.8 x 467.0 µm) (total length x width) was displaced ventrally and was distinct from the pedigerous portion (Fig. 5.7A, B). The pedigerous portion of the cephalosome expands in the juvenile into the large dorsal gonadal lobes of the adult male (Fig. 5.7C, D). The protopod of the first leg was uniramous and slender compared to that of

copepodid IV female and post-copepodid IV female *Ismaila belciki* (Fig. 5.5, 5.8A, but see Fig. 5.8B). The second leg was also slender with a relatively smaller endopod (compared to exopod) than seen in female copepodids and adult males (Fig. 5.7A, B). This endopod is longer in the juvenile and adult stage (Fig. 5.7C, D). The fourth somite beared an incomplete sclerotized ring also found in the adult (Fig. 5.7). The fifth and sixth somites combined to form the genital somite as seen in the female and proposed male copepodid IV (Fig. 5.5, 5.6, 5.7). Additionally, the ventral genital ridges were developing as this stage with the posterior ridge carrying the leg 6 flap (Fig. 5.7). There were two abdominal somites as seen in the copepodid IV male and females, the first larger than the second (Fig. 5.7). The sclerotized ring on the fourth somite and dorsal side of the posterior somites and caudal ramus stained brightly with Congo Red, suggesting that these areas were more heavily fortified with chitin (Fig. 5.7A, B, D). This was also seen in juvenile and adult male and female specimens (see below).

Copepodid V and juvenile female

Two specimens exhibited traits associated with copepodid IV females and juvenile females (Fig. 5.8A, B). Specimen A (729.0 x 428.9 μm) (total length x width) was larger than the copepodid IV females (438 to 546 μm in length) and possessed a cephalosome more ventrally displaced than the later. Swollen first and second leg protopods and a long second leg endopod were indicative of both the copepod IV and juvenile females (Fig. 5.8A, 5.9A). Below the dorsal second somite, there was an emerging segment in the exoskeleton that may lead to the dorso-lateral lobes characteristic of female juveniles and adults (Fig. 5.8A, 5.9A, B).

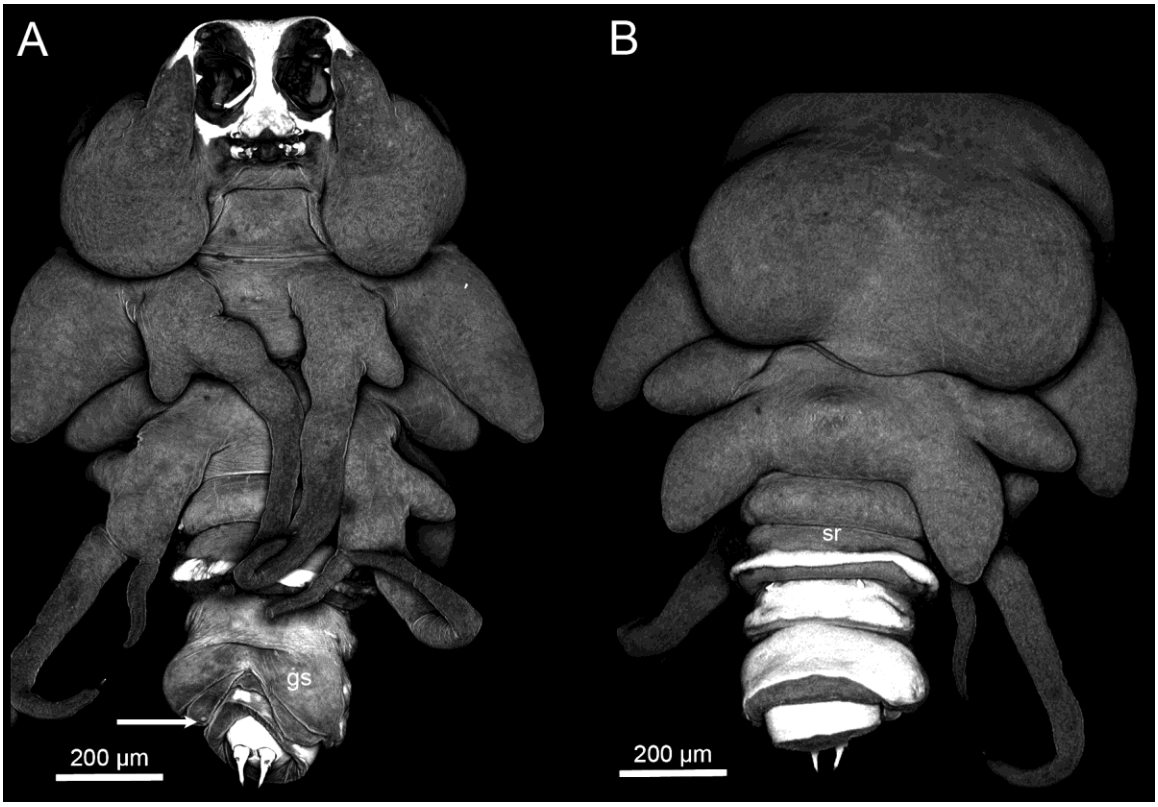


Fig. 5.9: Confocal micrographs of the A, ventral and B, dorsal view of a juvenile female *Ismaila belciki*. Arrow points to the sixth leg spine, gs, genital somite; sr, sclerotized ring. Serial sections of the first and second antennae were omitted as they were so brightly stained with Congo Red and made that area of the image oversaturated.

Specimen B was large (1036.1 x 622.2 µm) (total length x width) and close to the size of the juvenile female specimen (1040 x 1001 µm) (total length x width) (Fig. 5.8B, 5.9A, B). This specimen had distinct lateral pouches that protruded from the pedigerous region of the cephalothorax, a feature found in juvenile stages (Fig. 5.8B, 5.9A). Oddly, the protopod of the first leg was very narrow, a feature associated with male copepodids and adults (Fig. 5.7, 5.8B). Also, one of the first legs was damaged and missing. The second leg, however, was very similar to the tripartite juvenile female second leg with a long exopod and endopod and outside lateral lobe (Fig. 5.8B, 5.9A). The fourth somite had a developing sclerotized ring and the dorsal side of posterior segments were heavily

stained as found in the juvenile and adult of both sexes (Fig. 5.7A, B, D, 5.8B, 5.9). Additionally, two large flaps were developing on the genital somite (Fig. 5.8B). Aside from the narrow first leg, these features suggest this may be a copepodid V female.

The juvenile *Ismaila belciki* female specimen still retained the large second antennae and mouth structures later reduced relative to the rest of the cephalosome in the adult female (base only visible in Fig. 5.9A, 5.10). The juvenile female possessed three lateral lobe precursors of the adult's three lateral processes, though the single dorsal process seen in the adult was still absent at this early stage (Fig. 5.9A, B and 5.10). First legs were biramous, though the external ramous was only a nub, not the extended ramous found in the adults (Fig. 5.9A, 5.10). Likewise, the outer nub of the tripartite second leg was more extended in adult females (Fig. 5.9A, 5.10). The fourth somite possessed a nearly complete sclerotized ring (Fig. 5.9). The genital somite had two large flaps with the most posterior still possessing a spined vestige of leg 6 (Fig. 5.9A). The dorsal side of posterior somites stained brightly with Congo-Red, similar to mouth structures, caudal spines, and the sclerotized ring that may contain more chitin than other parts of the copepod (Fig. 5.9).

Copepodid molts

A few copepodid molts were found in the cerata of *Janolus fuscus*. Though they could not be identified to the copepodid stage, two early copepodid stage molts (169.6 μm x 173.6 μm) (total length x width), missing its posterior and 259 x 279.1 μm) had more prominent second antenna claws and cephalic structures compared to a later copepodid molt (458.6 x 200.3 μm) (total length x width) (Fig. 5.11A-D).

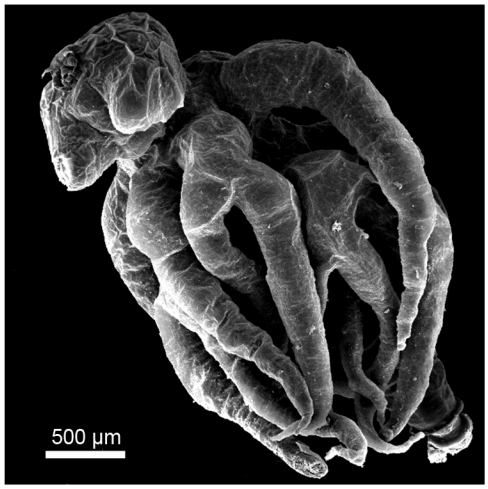


Fig. 5.10: Scanning electron micrograph of adult female *Ismaila belciki*.

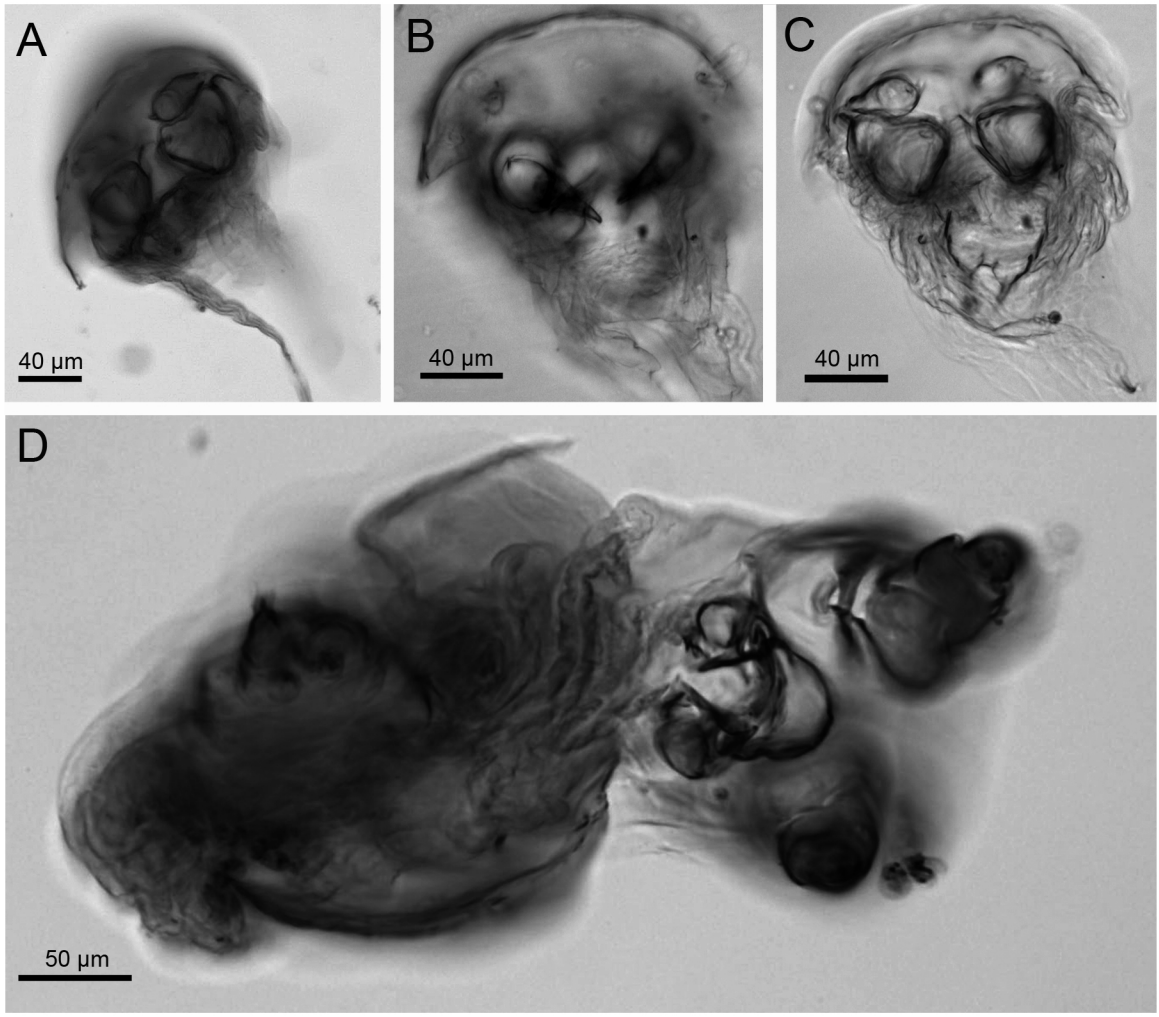


Fig. 5.11: Light micrographs of copepodid molts of *Ismaila belciki* found in the cerata of *Janolus fuscus* A-C, early copepodid molt highlighting large second antennae and four legs with hooked tips; D, later copepodid stage molt.

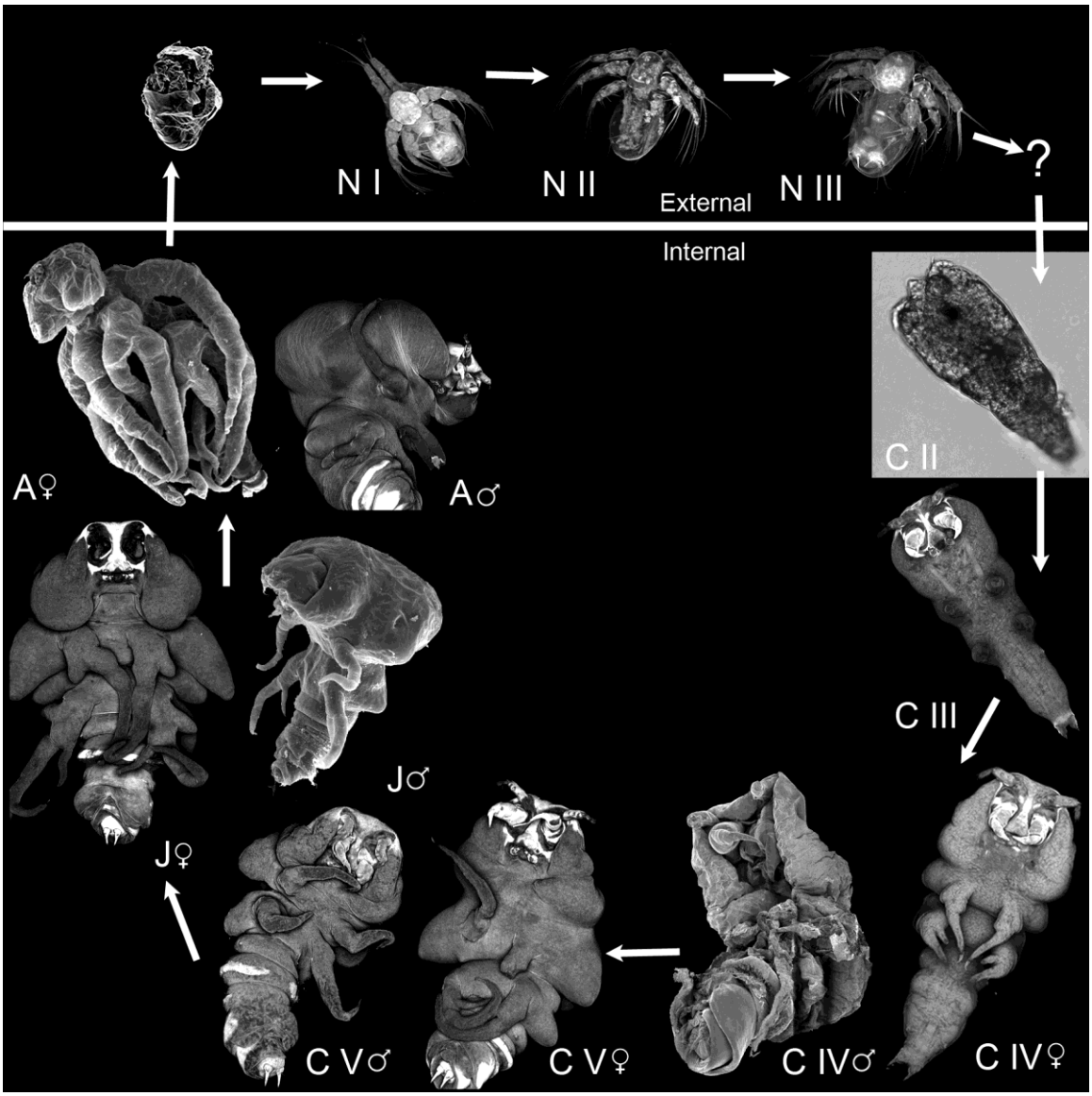


Fig. 5.12: The life cycle of *Ismaila belciki*, including at least three nauplius and likely five copepodid stages. A, adult male and female; C II, copepodid two; C III, copepodid three; C IV, copepodid four male and female; C V, copepodid five male and female; J, juvenile male and female; N I, nauplius one; N II, nauplius two; N III, nauplius three. Male and female are designated by male and female symbols from the copepodid IV to adult stages. Arrows designates direction of development and the line separates stages found outside the host (external) from those inside the host (internal).

Discussion

As described by Belcik (1981) and Dudley (in litt, 1965, in Belcik 1965), *Ismaila belciki* hatch as a planktotrophic nauplius I stage with a patent gut and three appendages. The eggs and first nauplius stage is similar to that described by Belcik's (1981) with a few deviations. Embryos and recently hatched *I. beliki* in the current study varied in size from 65-78 μm (in diameter) and 87.5-92.5 μm (carapace length), respectively. Only those embryos and larvae shrunken by the SEM preservation process (via alcohol dehydration and critical point drying) in our study were in the size range reported by Belcik (1981) (53 μm and 55-60 μm, for embryos and nauplii respectively). A similar shrinking effect may have occurred with Belcik's (1981) samples as they were preserved in Lavdowsky's Solution (AFA) or 70% ethyl alcohol. While there were slight differences in the number of setae of the nauplius I, this may be error in either study. However, Belcik (1981) reports that *I. belciki* nauplius I has a uniramous mandible with two terminal setae. In the current study, nauplii had biramous mandibles with numerous setae as typically found in poecilostome Cyclopoid nauplii (Izawa, 1987).

Nauplius II and III of *Ismaila belciki* occurred from days three to five and eight to 11 post-hatching, respectively, but no further naupliar stages, growth or changes in morphology were evident for the remaining three to six days with cultures slowly degrading. The addition of the first maxilla in the second nauplius and the additional seta and spine on the caudal ramus in the third nauplius stage (as well as other subtle morphological changes) were similar to those described in the first three naupliar stages of other poecilostome Cyclopoida (reviewed in Izawa, 1987). A typical copepod has six naupliar and five copepodid stages (Izawa, 1987; Ruppert et al., 2004; Boxshall, 2005).

However, in parasitic copepods the life cycle is often abbreviated, with fewer than six naupliar stages (Costanzo, 1959; Dudley, 1964, 1966; Gotto, 1979; Do et al., 1984; Izawa, 1986b, 1987; Lamb et al., 1998). The poecilostome Cyclopoida include species with one to six naupliar stages (Do et al., 1984; Izawa, 1987). However, those species with abbreviated naupliar stages are typically lecithotrophic (i.e., non-feeding) nauplii that hatch, from large eggs (>120 μm) with substantial yolk reserves, into large larvae (>120 μm) (Izawa, 1973, 1975, 1986, 1987; Nakamura et al., 1979). Lecithotrophic development in the poecilostome copepods is often associated with a morphological simplification of appendages used in feeding and very little growth during naupliar stages (Dudley, 1964; Izawa, 1973, 1975, 1987; Do et al., 1984). For example, Izawa (1973, 1975) found two species of poecilostome Cyclopoida, *Colobomatus pupa* (Philichthyidae) and *Sarcotaces pacificus* (Sarcotacidae) infecting a frogfish and goatfish, respectively, did not grow at all over five naupliar stages. In contrast, planktotrophic (i.e., feeding) nauplii of poecilostome Cyclopoida typically hatch from small eggs (<100 μm), have well-developed feeding structures, and grow substantially through six naupliar stages (Costanzo, 1969; Izawa, 1986, 1987). For example, *Lichomolgus canui* (Lichomolgidae) found in an ascidian, hatch from 50 μm eggs and growth from an 85 μm first nauplius to a 175 μm sixth nauplius stage (Costanzo, 1969). *Ismaila bleciki* hatch from small eggs (68 μm) into small (87.5 – 92.5 μm) (carapace length), planktotrophic naupliar larvae, with well-developed feeding structures (e.g., presence of feeding spines on coxa and basis of second antenna and mandible). Additionally, larvae can be seen with the microscopic alga, *Isochrysis galbana*, in their gut after feeding. Despite these features, only three naupliar stages were found for *I. belciki*, while such small egg and

first nauplius size suggest six naupliar stages likely exist. Most poecilostome copepods with planktotrophic species progress through six nauplius and the first copepodid (infective) stage in 7- 15 days (reviewed in Izawa, 1987). It appears that *I. belciki* may have a longer larval period than most poecilostome copepods (reviewed in Izawa, 1987). However, a single copepodid III stage found in a *J. fuscus* incubated with *I. belciki* nauplii for 15 days (17 days after hatching) suggests *I. belciki* infect earlier than 17 days after hatching. If this is true, *I. belciki* may not have nauplius four though six stages or may undergo successive molts past the third nauplius stage but grow little or not at all, displaying discrete morphological changes missed by the author.

Most studies of the larval development of poecilostomatoid copepods have found or implied that these parasites infect their hosts at the first copepodid stage (Pesta, 1907; Dudley, 1964; Costanzo, 1969; Izawa, 1973, 1975, 1986, 1987; Do et al., 1984; Humes, 1986; Ho, 1987b; Boxshall, 2005), though rare accounts of nauplius, metanauplius, and second copepodid infective stages are known (Dudley, 1966; Gotto, 1979; Dojiri et al., 2008; Roberts and Janovy, 2009). The first copepodid stage of species from families of poecilostome Cyclopoida are alike in having two pairs of biramous, unimerite swimming legs that are well-equipped with spines and plumose setae (Pesta, 1907; Costanzo, 1969; Izawa, 1973, 1975, 1986; Do et al., 1986). This includes species that are highly modified as adults such as *Sarcotaces pacificus* that lives in the dermal gall of frogfish and those of *Lichomolgus canui*, which is an unmodified copepod living loosely in the pharynx of ascidians (Costanzo, 1969; Izawa, 1973). Such an early copepodid with swimming legs was not found by Ho (1987b) in his examination of the copepodid stages of *Ismaila occulta*. For this reason the earliest copepodid stage found in his study was designated the

copepodid II stage. He suggested a copepodid I infects the host and rapidly molts to the copepodid stage II. Likewise, a copepodid I stage was not found in culture or within dissected hosts of *I. belciki* in the current study. Further studies are needed to locate the infective stage both outside and inside the host.

A single copepodid III was found in one *Janolus fuscus* incubated with *Ismaila belciki* from the July 2008 trial. This suggests that infection and growth to a copepodid III stage may occur within 15 days of incubation and 17 days after hatching. While uninfected nudibranchs are thoroughly examined before incubation periods and small potential hosts (< 20 mm) chosen for their relative transparency, a previous infection with an infective stage or copepodid II may have gone unnoticed in those areas of *J. fuscus* that are not entirely transparent. However, if infection occurred, the larval period of *I. belciki* is around two weeks and the growth inside the host is rapid. This suggests I missed the infective stage and time in at least one culture in the July 2008 trial. The growth rate of *I. belciki* inside the host is unknown. However, a few rare samples (n=3) of infected small new recruits of *J. fuscus* (2 - 3.4 mm) were collected from a survey site near Fossil Pt. in November and December 2009. One copepodid II and two copepodid III of *I. belciki* were found in these small individuals. A newly metamorphosed *J. fuscus* can grow to 2.5 mm in 23 days (chapter III). While *I. belciki* may be capable of infecting newly metamorphosed hosts (280 μm), the size of the host relative to that of a potential infective larva (estimated as >112 μm) would be very small. Therefore the first few days after metamorphosis seem an unlikely time of infection. This suggests that *I. belciki* may infect and grow to a copepodid III in under 20 days, though the larval duration is unknown.

The site of infection on the host is unknown. Copepodids and their molts are typically found in the cerata just outside the digestive diverticulum, though copepodids occasionally occur in other sites in the host hemocoel. Adult male and female *Ismaila belciki* are never found in the cerata of *Janolus fuscus* but reside in the hemocoel of the main body. This suggests that *I. belciki* may infect through the cerata. However, Ho (1987b) found copepodids and adults in the digestive diverticulum of a large *Dendronotus iris* host. This suggests *Ismaila* species could infect via host consumption, passing through the digestive system up into the digestive diverticulum. It is possible that small early infective stages could go unnoticed among the small bryozoan pieces in the digestive diverticulum, explaining the difficulty in finding this stage. Though I found no *I. belciki* in the digestive diverticulum, it is possible they pass out of the diverticulum quickly at an early stage and reside just outside in the cerata where they are more visible. However, the digestive diverticulum of *J. fuscus* is not large enough to hold (without obvious distortion) late copepodid stages or adults.

Male and female copepodids II through IV of *Ismaila belciki* found in *Janolus fuscus* were identified using Ho's (1987b) detailed study of 312 copepodids found in a single specimen of *Dendronotus iris*. In the current study, many fewer copepodids were found. Additionally, some samples preserved poorly for SEM, making morphological description difficult. Therefore, representative stages should be viewed with caution, recognizing the great variation that likely exists within stages. The single copepodid II specimen was distinctive in possessing five somites and a rostal protrusion absent in the copepodid III. Copepodid III had an additional somite and had a uniramous first and biramous second leg, as described by Ho (1987b). In some samples (of both old and new

specimens), the exoskeleton seemed thin, wrinkled, or absent. This may have been due to preservation in 10% formalin, or the onset or occurrence of a recent molting event.

At the copepodid IV stage, male *Ismaila belciki* are larger than females and display slight differences in morphology, as described in *I. occulta* (Ho, 1987b). Both female and presumed males exhibit a cephalosome with distinct cephalosomal and pedigerous regions of the cephalothorax, though males appeared more ventrally bent than females. The fifth and six somites are fused to form the genital somite visible on the ventral surface of both copepodid IV sexes. Copepodid IV females had swollen protopods and long second leg endopods. These could not be compared to male copepodid IV legs, but copepodid V males had narrow protopods and small endopods, suggesting earlier morphological differences. Ho (1987b) found male copepodid IV *I. occulta* to have narrower protopods and a relatively smaller endopod on the second leg than female counterparts. Interestingly, the flap representing leg six in male *I. occulta* of copepodid IV also appears in the female copepodid IV of *I. belciki* though this structure lacked a spine as described by Ho (1987b) until the female copepodid V stage and persisted in the juveniles. The sixth leg could not be confirmed or denied in the male copepodid IV samples, though it is present with a single spine in the male copepodid V.

Ismaila belciki have a copepodid V stage. Ho (1987b) did not find a copepodid V stage of male or female *I. occulta*, nor did he describe juvenile stages of this species. He did, however, suggest that a copepodid V exists for *I. occulta*, as the ventral genital ridge found in adult males was not yet developed in the male copepodid IV stages. In *I. belciki* male copepodid V have developed the ventral genital ridge. By this stage females are larger than males, a feature evident in the juveniles and adults. Male and female

copepodid V have developing sclerotized rings on the fourth somite that become complete and incomplete, in female and male adults, respectively. Additionally the dorsal surface of the genital and first abdominal somite stain with Congo Red more than other parts of the parasite, suggesting these areas (along with cephalic structures) are more chitinous. The function of this sclerotized ring in the adult female is to serve as a stopper against the body wall of the host to control the level of protrusion of her posterior out of the host's integument (Ho, 1981). The sclerotized ring is also found in the male and may serve a similar function as he can protrude his posterior out of the host integument when *in copula* and held in the "embrace" of the female (pers. obs.). Greater chitinous fortification of the dorsal surface of the genital and abdominal somites may serve a protective function for mating male and female *I. belciki* with posteriors exposed to the outside.

Copepodid V males and females possess features that will extend and change as adults. In the male copepodid V, the cephalosome region will shrink and be pushed forward with the expansion of the gonadal lobes of the pedigerous portion of the cephalothorax with maturation. Additionally the second leg protopod will extend, more so in the adult male *I. belciki* than *I. occulta* (Ho, 1981). In general, the first and second leg endopods were relatively larger in most copepodid stages of *I. belciki* compared to *I. occulta*, in accordance with the size of the adult endopods (Ho, 1981, 1987b). Interestingly, the copepodid V female had a very narrow first leg, a feature common in male stages. The explanation for this leg is unknown but could be related to damage associated with the missing leg. The juvenile female stage had nubs on the first leg exopod and lateral lobes that will extend into long biramous first legs and lateral

processes, respectively, in the adult. Additionally the distinctive dorsal process absent in the juvenile will develop in the adult. The prominent cephalic structures of the juvenile female with shrink in relation to the rest of the cephalosome that will develop broad genital lobes as an adult.

These data suggest that *Ismaila belciki* have a least three free-living planktotrophic naupliar stages. Though the infective stage is unknown, early copepodid stages found in very young hosts from the field and a copepodid III found in a host after 15 days of incubation with *I. belciki* nauplii suggest *I. belciki* may infect and grow rapidly to a copepodid III within 15-20 days. As suggested for *I. occulta*, *I. belciki* likely have five copepodid stages, as typically found in copepod development, though the copepodid I has not been observed (Fig. 5.12).

CHAPTER VII

GENERAL CONCLUSIONS

The population dynamics and reproduction of nudibranch molluscs can be influenced by a number of factors such as prey abundance and composition, abiotic conditions, developmental constraints, and parasitic infection. In this dissertation I have addressed each of these factors. I examined changes in the demography of *Janolus fuscus* from two field sites in relation to prey abundance and storm effects. These data were combined with a description of the development, growth and lifespan of *J. fuscus* to characterize this nudibranch's life cycle. Additionally I examined the ecology of an endoparasitic copepod, *Ismaila belciki*, and its impact on reproduction, growth, and survival of its host, *J. fuscus*. Last, I described larval stages of *I. belciki* and attempted to piece together the life cycle of this parasite.

Chapter II of this dissertation demonstrated that differences in the density of *Janolus fuscus* between sites and among seasons is likely a product of the combination of biotic and abiotic factors. Density of *J. fuscus* was consistently higher at the protected site (Fossil Pt.) than the exposed site (North Cove). Bryozoan abundance was also greater at the protected site, perhaps explaining the difference in nudibranch density. Additionally, the density of *J. fuscus* differed by season, with peak densities in spring at Fossil Pt. and summer at North Cove, corresponding to peak abundance of the dominant bryozoan prey species at each site (*Bugula pacifica* and *Tricellaria circumternata* at

Fossil Pt. and North Cove, respectively). New recruits were found throughout the year, though only on *B. pacifica*. The greater abundance of *B. pacifica* at the protected site may also contribute to earlier recruitment and greater adult densities at this site. Adult slugs were absent in both populations during winter months. Prey abundance and larval recruitment were not limiting factors and unlikely explanations for adult absence in the winter. However, density of *J. fuscus* was negatively correlated with winter storms. Winter storms may explain the earlier disappearance of *J. fuscus* at the exposed site on the outer coast compared to the more protected site inside Coos Bay. This study suggests that *J. fuscus* is a subannual species, exhibiting continuous recruitment, rapid growth of the population, and overlapping generations that disappear in the winter.

Embryological development of *Janolus fuscus* follows the patterns characteristic of opisthobranchs and other invertebrates that undergo holoblastic, spiral cleavage (e.g., nemerteans, annelids, and other molluscs) (Wilson, 1892; Casteel, 1904; Thompson, 1958). Embryos passed through the trochophore-like stage in the capsule and hatch as veliger larvae. The growth pattern for *J. fuscus* larvae was similar to other planktotrophic nudibranch larvae (Perron and Turner, 1977; Chia and Koss, 1978; Hubbard, 1988) with rapid shell growth early in the larval period and cessation of shell growth just prior to metamorphic competence. *Janolus fuscus* settled and metamorphosed on its bryozoan prey *Bugula pacifica*, supporting findings from chapter II in which new recruits were found on *B. pacifica* collected in the field. Induction of metamorphosis by adult prey has been documented for many opisthobranch species (Thompson, 1958; Harris, 1975; Hadfield, 1977; Kempf and Willows, 1977; Harrigan and Alkon, 1978; Hadfield and Pennington, 1990; Lambert et al., 1994; Avila 1998; Trowbridge and Todd, 2001).

Juvenile growth was rapid in the lab with 2.5 μm slugs reaching maturity (19 mm) in 25 days and growing into maximum-sized adults before dying after a five-month lifespan. This supports the conclusion from chapter II that *J. fuscus* is a subannual species that has overlapping generations, continual recruitment, and rapid growth before dying within a single year.

In chapter IV, I found that prevalence of *Ismaila belciki* varied by site and season, increasing with the density of *Janolus fuscus*. Intensities of infection generally mirrored peaks in parasite prevalence and host density. *Ismaila belciki* appears to be a rare intermediate between a typical parasite and a parasitic castrator. This copepod shares many characteristics with castrating parasites, as demonstrated in chapter IV. Female *I. belciki* are large relative to their host and parasite mass is positively correlated with host mass. Female copepods exhibit high site specificity, preferring a position in the host hemocoel just anterior to the host pericardium. There is some evidence of intraspecific competition among female *I. belciki*, which may lead to the observed underdispersed distribution of female *I. belciki* and weakly overdispersed distribution of male and female copepods in the host population. Additionally, infected *J. fuscus* are larger than uninfected counterparts. However, a comparison of growth and survivorship in infected and uninfected slugs in chapter V does not suggest the parasite causes increased growth ("gigantism") or survival in their hosts, as found in some castrating parasites. Chapter V clearly shows that *I. belciki* does not completely castrate its host, but causes a significant decline in host reproductive output. Studies suggest parasitic castration is more likely to evolve in parasites with long-lived hosts with high investment in reproduction (Hurd, 2001, Ebert, 2004, Lafferty and Kuris, 2009). While, *J. fuscus* do invest heavily in

reproduction, chapter III shows that *J. fuscus* only live five months, a period that may be too short for *I. belciki* to reap the potential benefits (e.g. prolonged life) of total castration. Thus, the ecology and population dynamics of *J. fuscus* may effectively constrain this copepod parasite to a life history somewhere between that of a typical parasite and a parasitic castrator.

Ismaila belciki has a least three planktotrophic naupliar stages and four copepodid stages within the host as found in chapter VI. As *I. belciki* nauplii are small, planktotrophic (feeding) larvae, I would expect them to have six naupliar stages, as found in both free-living and parasitic copepods with small feeding nauplii. While the infective stage is still elusive, it is likely the copepodid I, as typically found in poecilostome Cyclopoida copepods. However, a copepodid I with distinctive swimming legs (as described in other studies) was not observed outside or inside the host. However, early copepodid stages (II and III) found in very young hosts from the field and a copepodid III found in a host after 15 days of incubation with *I. belciki* nauplii suggest *I. belciki* may infect and grow rapidly to a copepodid III within 15-20 days. Though the infection site is unknown, the presence of copepodid stages and molts in the cerata of the host suggest *I. belciki* may penetrate the host at this site.

In this dissertation I have explored aspects of the interconnected life histories of a nudibranch host *Janolus fuscus* and its parasitic copepod, *Ismaila belciki*, addressing the development and life cycle of each, changes in the host and parasite population, and the impact of infection on host condition.

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CHAPTER I

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CHAPTER VI

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