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Disease Ecology of the Caribbean Spiny Lobster Panulirus argus: The Effect of a Nemertean Egg Predator, Carcinonemertes conanobrieni, on the Host's Reproductive Performance and Active Parental Care

Natalie C. Stephens
Clemson University, nsteph3@g.clemson.edu

DISEASE ECOLOGY OF THE CARIBBEAN SPINY LOBSTER *PANULIRUS ARGUS*: THE EFFECT OF A NEMERTEAN EGG PREDATOR, *CARCINONEMERTES CONANOBRIENI*, ON THE HOSTS' REPRODUCTIVE PERFORMANCE AND ACTIVE PARENTAL CARE

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Dr. J. Antonio Baeza, Committee Chair
Dr. Michael J. Childress
Dr. Matthew W. Turnbull

Abstract

Anthropogenic stressors and climate change are contributing to the rapid deterioration of marine ecosystems and the important ecological services they provide to coastal communities; concomitantly, this allows infectious agents that cause disease to proliferate in marine environments. At the forefront of marine disease ecology are the impacts of pathogens and parasites on commercially and recreationally exploited decapod crustaceans, including the Caribbean spiny lobster *Panulirus argus*. While there are few pathogens and parasites known to infect *P. argus*, emerging threats such as the nemertean micro-egg predator *Carcinonemertes conanobrieni* pose new, unexposed problems across its Greater Caribbean distribution. Beyond its current distribution in host populations and its sole infestation site of egg-bearing female brood clutches, there is limited knowledge that exists for this host-egg predator system. Therefore, I investigated the effect of *C. conanobrieni* on egg-bearing female *P. argus* reproduction and active parental care behaviors. In my second chapter, spiny lobsters were used to formally test if *C. conanobrieni* has an effect on reproductive performance (fecundity, reproductive output, embryo mortality). In my third chapter, *P. argus* with *C. conanobrieni* were used in experimental assays to determine if lobsters use active parental care as behavioral mitigation to limit the negative effects of infestation on host broods. To achieve my objectives, I collected female lobsters across the Florida Keys reef tract for two summer field seasons (2022 and 2023). Across both years and all coral reef sites, my collected females were infested with one or more stages of *C. conanobrieni* (100% prevalence). Declines in female lobster reproductive performance were attributable to *C. conanobrieni* based on my findings. I found that the life history of this egg predator aligns closely with the cycle of *P. argus* embryo development. During my investigation into the effect of *C. conanobrieni* on *P. argus* active

parental care, there was limited evidence to suggest that this nemertean had a significant effect on these behaviors. Additionally, I found no evidence that *P. argus* are actively sensing *C. conanobrieni* when this nemertean is infesting their brood masses. As a whole, I have defined the costs associated with *C. conanobrieni* and explored putative behavioral adaptations the host may use in the face of brood infestation.

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Chapter 1:

Introduction to study system

Background

Human populations are heavily dependent on marine ecosystems and the services they provide towards meeting nutritional needs, economic purposes including fisheries revenue, coastal protection from storms and erosion, transportation, carbon sequestration, among many others (Barbier 2017; Selig et al. 2019). The combined consequences of climate change and anthropogenic disturbance, including habitat alteration and overfishing, on marine ecosystems has caused significant deterioration of ocean productivity, major disruptions to food web dynamics, increased losses in biodiversity, and other negative changes to these important ecosystems and their services (Hoegh-Guldberg and Bruno 2010). A lesser studied, but equally important, effect that these stressors may lead to is the greater proliferation of infectious agents and the diseases they may cause in marine organisms (McCallum et al. 2003; Tracy et al. 2019; Hoegh-Guldberg and Bruno 2010). Climate change, through the warming of global temperatures, facilitates increased pathogen transmission and outbreaks in previously healthy host communities, leading to accelerated ecosystem failures across marine environments (Harvell et al. 2004; Hayes and Goreau 1998). A lack of baseline knowledge of disease in various marine communities has created challenges in the field of marine epidemiology, but recent studies have focused on the current rate of disease transmission to interpret the responsible factors for greater host susceptibility to infection (Ward and Lafferty 2004). Investigating the impact of increased disease outbreaks on a global scale has led researchers to identify marine ‘hotspots’, areas

warming at unprecedented rates, making them targets for understanding adaptation options for ecosystems and fisheries (Hobday and Pecl 2014).

The Caribbean basin over the past few decades has been denoted a ‘disease hot spot’ due to the growing frequency of diseases reported in organisms across its coral reef ecosystems, coupled with its warming temperatures, invasive species introductions, changing sea levels, nutrient runoff, pollution, and more (Ellison and Farnsworth 1996; Lafferty et al. 2004; Weil and Cróquer 2009; Harvell et al. 1999; Harvell et al. 2004; Williams and Bunkley-Williams 2000). The Wider Caribbean basin, which comprises the Gulf of Mexico, Florida, Bahamas, and Bermuda, has seen an increased incidence of pathogens sweeping through its coral reef ecosystems since the 1980s through the emergence of red band disease (RBD), yellow blotch (YBS), white pox disease (WPX), and many more targeting numerous coral and octocoral species (Weil 2004). Increased intensity and frequency of diseases has left ecologists questioning future coral reef survival predictions, especially across geographic scales, but corals are not the only organisms in this basin being impacted (Weil et al. 2006). Widespread disease in this area has additionally affected sea fans (Nagelkerken et al. 1997), reef sponges (Wulff 2007), and mass mortalities among marine invertebrates, such as sea urchins, and fishes with protistan parasites in the Caribbean have become well documented (Williams and Bunkley-Williams 2000; Landsberg and Blakesley 1995). One important marine invertebrate that is susceptible to this increase in diseases spanning the Caribbean region is the Caribbean spiny lobster, *Panulirus argus* (Latreille 1804).

The Caribbean spiny lobster, *Panulirus argus,* belongs to the family Palinuridae, and spiny lobsters within this family are commercially and recreationally exploited across major fisheries in tropical, subtropical, and temperate oceans (Booth and Phillips 1994). *Panulirus*

argus constitutes one of the largest fisheries across the Caribbean basin and regionally has a fishery value estimated to have reached US$456 million in recent years (Atherley et al. 2020b, 2021; Ehrhardt 2005). Aside from their lucrative economic value, spiny lobsters play a significant part in marine ecosystem functioning due to their role as benthic consumers (Segura-García et al. 2016) and as a top predator across their entire Caribbean distribution, which includes populations in North Carolina, Central America, the Bermudas, the southern Yucatan, and the Antilles (Cruz et al. 2015). *Panulirus argus'* life cycle is complex and spans many different marine environments. Specifically, they undergo an 11-stage oceanic planktonic phyllosoma for about 6-8 months until metamorphosis occurs over the continental shelf break (Cruz et al. 2001). The late phyllosoma molts into a puerulus larvae that migrates to coastal inshore nurseries and settles into red algae by detecting environmental cues, such as light (Butler et al. 2011a). Here, they grow into benthic, gregarious juveniles that inhabit coral crevices, sponges, and rocks with other juvenile lobsters (Childress and Herrnkind 1997). The dietary preferences of juvenile *P. argus* in their rocky, inshore habitats comprises mostly crustaceans, hermit and brachyuran crabs, and gastropods (Briones-Fourzán et al. 2003). Older juveniles will migrate from their nearshore hard bottom habitats to larger fishing grounds offshore (Cruz et al. 2001), where they continue to grow into adults. Previous studies have demonstrated that while reef herbivores make up the majority of adult *P. argus* diets, foraging of forams, fish, mollusks, and even some macroalgae and coralline algae occurs (Segura-García et al. 2016).

In addition to the complex life cycle of *P. argus* that spans multiple environments, adult spiny lobsters have a unique courtship display. Male spiny lobsters search for receptive females to mate with, make contact, and deposit a black spermatophore, or external sperm packet, onto the female's sternum (Lipicius et al. 1983; Butler et al. 2011b). Anywhere from 1-28 days after

the initial courting event, female *P. argus* use their pereopods to scratch at the stored sperm until it successfully fertilizes the extruded embryos (Butler et al. 2011b). Then, the embryos become attached to the lengthened setal hairs on female pleopods where they develop underneath the female's abdomen prior to hatching in 3-4 weeks (Cruz and Bertelsen 2009).

The distribution of the various life stages of *P. argus*, spanning both inshore and offshore environments and hundreds of miles, increases their exposure to new pathogens. In general, *Panulirus* spp. have very few incidences of pathogens and parasites despite their ecosystem importance and expansive fishery size (Shields 2011). White spot syndrome virus (WSSV) has previously been studied in penaeid shrimp, but laboratory studies have revealed its high potential to infect wild *P. argus* populations (Ross et al. 2019). *Panulirus argus* virus 1 (PaV1) is a species-specific virus that was discovered in the Florida Keys *P. argus* juvenile population over a decade ago (Shields and Behringer 2004). Its identification marked the first pathogenic virus known to infect any lobster and by targeting hemocytes, this virus compromises lobster metabolism and energy, which could ultimately lead to extreme lethargy and death (Shields and Behringer 2004). The digenean trematode *Cymatocarpus solearis* uses *P. argus* as a second intermediate host and the effects of infection, namely higher serotonin and lower glucose levels, may contribute to changes in host behavior (Franco-Bodek et al. 2023;Briones-Fourzán et al. 2016). More recently, the Caribbean spiny lobster has been found to host a newly identified species of nemertean worm, formally described as *Carcinonemertes conanobrieni*, that infests female embryo masses (Simpson et al. 2017). The discovery of *C. conanobrieni* on egg-bearing *P. argus* in the middle Florida Keys was the first reported occurrence of an embryo predator in this species (Baeza et al. 2016). The prevalence of *C. conanobrieni* has since been detected

among populations of *P. argus* in Colombia (Gonzalez-Cueto and Quiroga 2018; Berben et al. 2023) and Saint Kitts & Nevis (Atherley et al. 2020a).

Carcinonemertes conanobrieni belongs to Phylum Nemertea, family Carcinonemertidae and presently, 17 species of *Carcinonemertes* worms are described formally (Leiva et al. 2021). Morphological characters that separate *C. conanobrieni* from other species in the family Carcinonemertidae include having a wide stylet basis, a mucus sheath covered in external hooks that surrounds the worms' body, and little sexual dimorphism between females and males throughout their life cycles (Simpson et al. 2017). Beyond their morphology, these nemerteans exhibit a suite of different behaviors when attached to the host, including becoming encysted on individual embryos in the hosts brood mass, free-roaming among the hosts eggs, or encapsulated within mucus sheaths that are attached to the host's pleopods (Simpson et al. 2017). The unique morphology and behaviors of *C. conanobrieni* in female brood masses may contribute to its success on this host, which includes disrupting reproduction.

Nemertean egg predators (family Carcinonemertidae) such as those in the genus *Carcinonemertes* have been linked to reduced reproductive performance in decapod crustaceans when they infest the host's brood mass and directly feed on embryos (Shields and Wood 1993; Shields et al. 1990; Wickham 1986; Kuris et al. 1991; Berben et al. 2023; Torchin et al. 1996). For example, *C. errans* was suggested to be the primary driver of embryo mortality in *Cancer magister*, capable of causing over 50% mortality in host brood masses (Wickham 1979). Across its Alaskan distributions the red king crab, *Paralithodes camtschatica*, experienced seasonal outbreaks of *C. regicides*, which resulted in widespread embryo mortality ranging from 30-70% or greater (Kuris et al. 1991). While these studies, among many others, demonstrate the negative effect of these nemerteans on decapods, other *Carcinonemertes* sp. have not had a severe impact

on their host. Egg-bearing blue swimmer crabs *Portunus pelagicus* experienced no significant declines in embryo mortality when infested by *C. mitsukurii* (prevalence = 42.6%; Shields and Wood 1993). Most recently, Schneider et al. (2023) demonstrated that low prevalence (66.6%) and intensity (53.9 worms per infested host) of *C. carcinophila* on brooding blue crabs, *Callinectes sapidus*, resulted in limited, if any, detectable effects on host fecundity and reproductive output. Previous studies of *C. conanobrieni* infesting *P. argus* limitedly illustrate that this nemertean causes significant changes in host reproductive performance (Berben et al. 2023), which this study seeked to confirm and elaborate on.

The negative effects of *Carcinonemertes* sp. could also extend to affecting host behaviors. The invasion of parasites infecting susceptible hosts in their natural environments has direct implications on natural selection shaping and modifying host behaviors that limit infection and subsequently allow for survival and reproduction in their presence (Hart 1990). To restrict initial contact with pathogens, hosts may use behavioral avoidance to avoid infected conspecifics and/or environments with increased risk of infection (Behringer et al. 2018; Hart 1990). However, when parasite establishment becomes inevitable hosts have adapted their behaviors to limit parasite spread (Boots et al. 2009). Mitigation behaviors are expected to help mediate the negative effects of parasite infection and, thereafter, be maintained by selection for removing parasites when detected (Poulin 1995; Bauer 2013; Moore 2013; Vale et al. 2018; Gibson and Amoroso 2022). Other behaviors that are essential to host survival and successful reproduction, including foraging and mating, facilitate the spread of parasite loads across susceptible hosts, which highlights the need for traits that control or limit disease spread, such as active parental care behaviors (Ezenwa et al. 2016; Hart 1990).

The origins of parental care in invertebrates have been explained through the lens of various ecological pressures, including conditions imposed by the physical environment (Trumbo 2012), and through the need for adequate food resources to supplement nutritional needs (Tallamy and Wood 1986; Smiseth et al. 2012; Royle et al. 2012). In aquatic ecosystems, brooding embryos externally, as seen for many marine animals including cnidarians, echinoderms, and crustaceans (Adiyodi and Adiyodi 1989) not only offers embryos protection from their environment, but also may increase their overall fitness and survival (Smiseth et al. 2012; Royle et al. 2012). Beyond essential body grooming in response to fouling, crustaceans that incubate embryos underneath their abdomen may use the third maxilliped, specialized chelae, or pereopods to engage in specialized embryo cleaning, which is predicted to aid hosts in gathering information directly from the brood mass (Bauer 2013; Baeza and Fernández 2002, Fernàndez et al. 2020; Baeza et al. 2016). This becomes especially critical when there is an accumulation of foreign objects or parasites from their environment, with exposure to the latter leading to direct, negative effects for the host and its brood (Trumbo 2012). In line with reports from other decapod crustaceans, including the peppermint shrimp *Lysmata boggessi* (Baeza et al. 2019) and the brachyuran crab *Cancer setosus* (Baeza and Fernàndez 2002), active parental care behaviors of brooding female *P. argus* have previously been associated with oxygen provisioning throughout embryo development and investigating the space within their embryo masses that they carry on their abdomen (Baeza et al. 2016). Engaging in active parental care, including grooming using the 4th and 5th pereopods, may act favorably for female *P. argus* if these behaviors aid to mitigate for increasing *C. conanobrieni* loads or act as a compensatory tool to the negative effects they cause (Poulin 1995; Hart 1990), which is the sole focus of my third chapter.

The first objective of my thesis was to describe the effect of the nemertean worm *C. conanobrieni* on egg-bearing *P. argus* reproductive performance. While investigating *C. conanobrieni* prevalence across this host's Florida Keys distribution, I simultaneously quantified the reproductive performance of my sample *P. argus* population, including female fecundity and reproductive output. The estimates of *P. argus* fecundity reported herein were in line with those reported for similar sized spiny lobsters. *Carcinonemertes conanobrieni* infestation contributed to lowered fecundity and reproductive output, due to the feeding of this egg predator leaving behind a significant number of consumed and dead embryos compared to live embryos. Based on behavioral observations, I proposed a life cycle of *C. conanobrieni* when present in female brood masses, however I suggest that future studies focus solely on describing this in closer detail throughout the entire duration of lobster embryo incubation.

My thesis' second objective assessed the effect of *C. conanobrieni* on the series of active parental care behaviors performed by egg-bearing *P. argus*. In agreement with Baeza et al. (2016), I observed this species perform these behaviors towards their brood mass in a specific sequence. I predicted that these behaviors may be secondarily adapted for egg predator mitigation, but my results suggest that their primary function is most likely to meet embryo development requirements.

Goals

The broad project goal for this master's thesis was to investigate the effect of *Carcinonemertes conanobrieni* on female *Panulirus argus* reproductive performance and behavior. To address these goals, I collected egg-bearing *P. argus*, assessed and reported the prevalence of *C. conanobrieni* on spiny lobsters from three geographic regions along the Florida Keys reef tract,

and performed laboratory and behavioral assays to investigate the various roles of the egg predator on its host.

The second chapter of my master's thesis investigated whether *C. conanobrieni* had an effect on the reproductive performance of its female spiny lobster host. I focused solely on fecundity, reproductive output, and embryo mortality as the measures of female reproductive performance. I expected that, if present, *C. conanobrieni* would negatively affect female *P. argus* reproduction through declines in fecundity and reproductive output, coupled with increased embryo mortality.

The goal of the third chapter was to determine if this egg predator has an effect on *P. argus* active parental care behaviors. I investigated if these hosts have an explicit mode of behavioral mitigation, namely grooming, in light of *C. conanobrieni* infestations across different stages of embryo development, and secondarily tested whether spiny lobsters sense and react to the presence of the nemertean in their brood mass. I expected that females with *C. conanobrieni* would groom their embryos more frequently to attempt at limiting the negative effects associated with infestation, including increased embryo mortality. Additionally, I predicted that female *P. argus* are directly sensing nemerteans in their brood mass via chemical cues, which will be met by an increase in active parental care, explicitly grooming bouts, to remove them.

Chapter II:

The effect of the nemertean egg predator *Carcinonemertes conanobrieni* on female Caribbean spiny lobster *Panulirus argus* reproductive performance in the Florida Keys National Marine Sanctuary, USA

Abstract

There are few pathogens reported for the commercially important and widespread Caribbean spiny lobster *Panulirus argus*; however, emerging pathogens are always a threat. While overfishing contributes to its declining fisheries status, a nemertean egg predator, *Carcinonemertes conanobrieni*, recently discovered infesting brooding female *P. argus* poses a rising threat. The goal of my study was to assess the prevalence and effect of *C. conanobrieni* on the reproductive performance of female *P. argus*. Ovigerous lobsters were collected using SCUBA from across the Florida Keys reef tract (USA) and examined for infestation by *C. conanobrieni*. Reproductive performance was determined as the reproductive output (%) and fecundity (nº eggs female^{-1}), of egg-bearing lobsters. I used redundancy analysis to determine which host traits could be used to explain differences in the abundance of *C. conanobrieni* life stages found within a female's brood mass and to determine the effect of *C. conanobrieni* on lobster reproductive performance. I observed 100% prevalence of *C. conanobrieni* among brooding *P. argus* across the Florida Keys reef tract over two consecutive years (2022, 2023), the highest prevalence to date in this host. Mean intensity of infestation was 20.06 nemerteans per 1,000 embryos. The reproductive performance of females carrying late-stage embryos was lower, and coupled with higher embryo mortality (%), compared to females with early-stage embryos. Lobsters carrying early-stage embryos also appeared to be infested primarily with juvenile *C.*

conanobrieni, while those carrying late-stage embryos had *C. conanobrieni* adults and egg masses present. Declines in overall *P. argus* reproductive performance could be attributable to *C. conanobrieni* based on my findings. The life history of this egg predator aligns closely with the cycle of *P. argus* embryo development. *Carcinonemertes conanobrieni* outbreaks might impact fisheries targeting *P. argus* across the Caribbean distribution, thus heightened surveillance for this nemertean is important.

Keywords: Egg predator; Fecundity; Reproductive output; Embryo mortality

Introduction

Climate change and anthropogenic stressors, such as overfishing and habitat destruction, are contributing to the rapid deterioration of marine ecosystems and the services they contribute to coastal populations; this concomitantly allows the proliferation of diseases in marine environments (Lafferty et al. 2004; Hughes et al. 2003). The relationship between increased disease occurrence and climate change can be linked to warming global temperatures facilitating increased pathogen transmission and host susceptibility, leading to an uptick in ecosystem failures across marine environments (Harvell et al. 2004). Ecologists have given priority to disease 'hot spots' including the Greater Caribbean basin due to mass mortalities of marine organisms, including corals, sea squirts, and sea urchins, among other invertebrates, that are occurring on large-scales and often caused by unknown pathogens (Hughes et al. 1985; Aronson and Precht 2001; Harvell et al. 2004). At the forefront of marine disease ecology is the combined effects of pathogens and parasites on ecologically and economically important marine organisms, including decapod crustaceans. Commercially and recreationally exploited populations of crabs, shrimps, and lobsters are extremely susceptible to disease outbreaks (Shields 2012), both directly

if disease causes mortality and indirectly if the parasite or pathogen impedes host growth. One important decapod crustacean that is susceptible to this increase in diseases spanning the Caribbean region is the Caribbean spiny lobster, *Panulirus argus* (Latreille 1804).

The abundance of *P. argus* is declining across the Western Atlantic Ocean, Caribbean Sea, and Gulf of Mexico, putting it at risk of a fisheries collapse (Butler et al. 2009). Landings of *P. argus* in Cuba and the Gulf of Mexico have equated to US$70 million and $37 million, respectively, and have reached over 20.3 tons in Colombia (FAO 2019; De la Hoz and Manjarrés-Martínez 2016). Overfishing might contribute to its declining fisheries status in addition to environmental stressors such as increasing ocean temperatures, ocean acidification, and sea level rise (Ross and Behringer 2019). Moreover, both of these stressors may exacerbate the incidence of diseases infecting *P. argus*. There are few pathogens reported for this species but that does not mean it is not at risk from an emerging pathogen. One such example is *Panulirus argus* virus 1 (PaV1), a species-specific virus discovered among the juvenile *P. argus* population in 2000 that attacks lobster hemocytes, ultimately leading to mortality from metabolic exhaustion (Shields and Behringer 2004). While some infections can lead to serious disease for *P. argus*, including pathogens like *Vibrio* sp., others may indirectly harm the host through impacts on host reproduction. This includes nemertean worms in the genus *Carcinonemertes*, which have been tied to the collapse of commercially important decapod crustacean fisheries (Shields 2011).

Nemertean egg predators (family Carcinonemertidae) such as those in the genus *Carcinonemertes* and *Ovicides* have been linked to reduced reproductive performance because they infest the host's brood mass and directly feed on embryos (Shields and Wood 1993;

Wickham 1986; Kuris et al. 1991, Berben et al. 2023). For instance, *Carcinonemertes errans* was the main driver of host embryo mortality in the Dungeness crab *Cancer magister* in the late 1970s (Wickham 1979). Outbreaks of *C. errans* ultimately contributed to the collapse of this fishery in Central California for several years (Wickham 1979, 1980). In 2017, *Carcinonemertes conanobrieni* was the first nemertean reported infesting the embryo masses of *P. argus* in Florida (Simpson et al. 2017). This egg predator has since been found across portions of *P. argus'* Caribbean distribution, including Saint Kitts and Nevis and Colombia (Baeza et al. 2016; Gonzalez-Cueto & Quiroga 2018; Simpson 2018; Atherley et al. 2020a).

Distinguishing characteristics that separate *C. conanobrieni* from other species in its family include a wide stylet basis, external hooks covering the exterior of its mucus sheath, and minimal sexual dimorphism (Simpson et al. 2017). Their feeding and persistence on female embryo masses at high densities during embryo incubation could threaten female *P. argus* reproductive performance. Given the effects of worms in the family Carcinonemertidae and the increased reporting of *C. conanobrieni* among ovigerous female *P. argus* from several locations in their Caribbean range, investigating the relationship between *C. conanobrieni* and *P. argus* reproduction across brood masses of different stages, sizes, and locations is critical to fully capturing its potential effect.

This study focused on determining the prevalence of *C. conanobrieni* across the Florida Keys and testing if *C. conanobrieni* has an effect on the reproductive performance of host Caribbean spiny lobsters. Brooding females were collected across multiple coral reefs in *P. argus'* middle, upper, and lower Florida Keys distribution for comparison of egg predator prevalence. I evaluated the prevalence and mean intensity of *C. conanobrieni* across female *P.*

argus carrying early and late-stage embryos along the Florida Keys National Marine Sanctuary. To understand the effect of this nemertean worm on host reproduction, I estimated two measures of reproductive performance: reproductive output and fecundity to then conduct redundancy discriminatory analyses (RDA) to determine what specific traits from the host explained the presence of different *C. conanobrieni* life stages present on a female's brood mass and to detect the effect, if any, of *C. conanobrieni* on lobster reproductive performance. The prevalence of PaV1 was also assessed. This study will allow for comparisons over multiple years across different localities in the Greater Caribbean, including studies conducted within the Florida Keys. *Carcinonemertes conanobrieni* outbreaks might impact fisheries targeting *P. argus* across the Caribbean, thus surveillance of heavily fished populations is important.

Material and Methods

Sample Panulirus argus *population and prevalence of* Carcinonemertes conanobrieni *across the Florida Keys*

Collections of female Caribbean spiny lobsters for this study were made under the Special Activity License (SAL-21-1674B-SR) granted by the Florida Fish and Wildlife Conservation Commission across two years: 2022 and 2023. In 2022, egg-bearing *P. argus* were collected from June - July 2022 at three coral reef sites located in the middle Florida Keys reef tract off Long Key, Florida: Tennessee Reef (approximately 5km off Long Key, Florida; N 24°44.726', W 080°46.915') and surrounding patch reefs (15-30 feet depth), Critter Ridge Reef (approximately 5km off Duck Key, Florida; N 24° 43.95', W 80° 54.726')), and Long Key Ledge (N 24° 44.2667', W 80° 51.1333') (Figure 1). To account for potential geographic differences in

the prevalence of *C. conanobrieni*, from June - July 2023, I collected egg-bearing *P. argus* from three geographic regions along the Florida Keys reef tract, the Upper, Middle, and Lower Florida Keys (Fig. 1). In the Middle Florida Keys, female *P. argus* were all collected from Tennessee Reef (N 24°44.726', W 080°46.915'). In the Upper Florida Keys, females were collected from Molasses Reef outside of the sanctuary preservation area (SPA) (N 25°00.528', W 080°22.590') and Conch Reef outside of the SPA (N 24°56.541', W 080°28.525'). In the Lower Florida Keys, females were collected from American Shoals (N 24° 31.400', W 081° 31.266'), Big Pine Shoals (N 24°34.222', W 081°19.300'), and Pelican Shoals (N 24°29.986', W081°37.883').

Brooding *P. argus* were collected via SCUBA with the aid of a tickle stick and hand net. In 2022, upon collection in the field, a zip-tie with a unique number was securely attached to the base of the female's antennae to enable us to track the identity of each lobster from the field through all laboratory analyses. The numbered zip tie allowed for a direct comparison of laboratory analyses with environmental characteristics and *in situ* host behavior. The collected female's behavior, categorized as solitary (found in her den with no other *P. argus*) or aggregated (found in her den with another *P. argus* present), was recorded. An image of the collected females den with as much visible substrate as possible was captured using a Hero GoPro 6. The tag number of the individual and the individual's relative location on the reef (edge of the reef or not) were also recorded. The program Coral Point Count with Excel Extensions (CPCe - Kohler and Gill 2006) was used to determine the dominant substrate of each individual female *P. argus'* den. Using CPCe, 25 randomly generated points were overlaid on the GoPro image. The substrate at each point was inspected and classified into categories: Sand, Rubble, Rocky Reef Substrate (RRS), or a combination of these substrates. The most abundant substrate within the image's randomly placed points was used to categorize the dominant substrate of the

lobsters den and used in my redundancy analyses. All habitat analyses was performed by an undergraduate student.

Females were transported in coolers to the Keys Marine Laboratory (Layton, Florida USA) for further processing. Previous studies have shown that transfer of *C. conanobrieni* from one female to another during transportation is very unlikely due to their inability to swim and maneuver between egg masses (NCS, AB, and JAB; Berben et al. 2023). Once back at the Keys Marine Laboratory, carapace length (CL) was recorded using a dial caliper (precision = 0.1 mm) and carapace features (e.g. barnacles on the mouthparts, missing appendages, scratched spermatophore) were noted. Using a 30G 1ml/cc fine-tip needle syringe, approximately 1 mL of hemolymph was extracted from a walking leg and was added to an automatic temperature-compensated digital refractometer (Fisherbrand™ Handheld Analog Clinical Refractometer) to collect specific protein (sp), refractive index scale (nd), and specific gravity (UG) and convert it to blood serum protein (BSP), a measure of lobster nutritional condition (Behringer and Butler 2006; Butler et al. 2022). Any females not identified as intermolt were excluded in BSP analyses since molt stage is well known to affect BSP (Smith & Dall 1982, Depledge & Bjerregaard 1989). I haphazardly selected two pleopods, with eggs attached to them, on the left side of the lobster and two pleopods on the right side of the lobster and snipped them at the base using blunt edge scissors. In 2023, female *P. argus* were processed onboard the research vessel. The pleopods were placed in a 15 ml falcon tube filled with seawater labeled with the side of the abdomen collected from (left or right), date, and location of collected. After snipping the pleopods, a notch was then cut into one of the uropods as an indication to us to avoid recapture of that individual and the lobster was released at the same site of capture. Pleopods were transported on ice back to the Keys Marine Laboratory and processed immediately.

For each pleopod, I recorded the embryo stage of development and molt status of the individual. Embryo stage of development of each female's brood mass was determined using a Leica S8AP0 stereomicroscope or Wild M5-97874 dissecting scope. Embryo stage of development was classified for each female's embryo mass under the stereomicroscope in accordance with Baeza et al. (2016): Early stages were subdivided into stage I embryos with uniformly distributed yolks, and stage II embryos were classified as those that exhibited cell differentiation and had yolk clusters present. Late-stage embryos were subdivided into stage III embryos with well-developed eyes and starting chromatophore features appearing, and stage IV embryos had well-developed eyes, chromatophores, and thoracic appendages present. For simplicity during data collection and analyses, I defined all females carrying stage I and II embryos as early-stage and all females carrying stage III and IV into late-stage. Molt status of each female (pre-molt, intermolt, or post-molt) was assessed by snipping a pleopod and observing for defining features under a Leica S8AP0 stereomicroscope (Lyle and MacDonald 1983).

After assessing embryo stage and molt status, using fine-tip forceps, I scanned the entirety of the pleopods, one by one, for evidence of *C. conanobrieni* infestation. Here, prevalence is defined as the presence or absence of the nemertean egg predator *C. conanobrieni* among female *P. argus* embryos and was calculated as the number of hosts infested with one or more *C. conanobrieni* worms divided by all hosts collected. I scanned each pleopod for any evidence of *C. conanobrieni* infestation and expected to see either live worms, dead worms, or consumed embryos. Once all pleopods were scanned for evidence of *C. conanobrieni*, they were completely submerged in falcon tubes with 4% formalin labeled with the individual's ID, date collected, and site of collection.

The effect of **Carcinonemertes conanobrieni** *on female* **Panulirus argus** *reproduction*

To investigate the effect of *C. conanobrieni* on female *P. argus* reproductive performance, four subsamples of ~1,000 embryos per female were observed under a Leica S8AP0 stereomicroscope or Wild M5-97874 dissecting scope prior to being weighed and dried in the oven. Within each embryo subsample, counts were made of the a) number of live embryos, b) number of dead embryos (those that appear neon orange or otherwise cloudy in color), and c) number of consumed embryos (those that the yolk has been completely removed, leaving an oval-shaped, empty capsule) (Baeza et al. 2016). Embryos considered dead could be due to the effect of the egg predator, lack of fertilization, genetic abnormalities, fouling, or other causes (Baeza et al. 2016; Kuris 1990). If any empty embryo cases were present, they were expected to be due to the worm consuming the contents of the embryo dry using its eversible proboscis (Simpson et al. 2017). Additionally, three life stages of the egg predator have previously been found in the lobster's embryo mass (Simpson et al. 2017), therefore I counted a) the number of *C. conanobrieni* juveniles (usually encysted on lobster embryos), b) the number of *C. conanobrieni* adults (either encysted, free-roaming, or within a mucus sheath), and c) the number of *C. conanobrieni* egg masses (spherical or spiral egg cases wrapped around the lobster embryos) separately. Additionally, under the microscope, I observed adult *C. conanobrieni* in mucus sheaths lining the edges of the pleopod or adults roaming the pleopod surface. Adult *C. conanobrieni* infesting the pleopods has been noted before in this species before (Simpson et al. 2017), but I quantified this finding by counting the number of adults present on the pleopods that were used in my subsamples to estimate mean intensity of the egg predator on the outer edges of pleopods.

Egg predator intensity per host and mean intensity, as defined in Bush et al. (1997) were calculated for each captured female across my four embryo subsamples per lobster. Intensity of *C. conanobrieni* per female *P. argus* was defined as the total number of *C. conanobrieni* found across a single female's four embryo subsamples. Mean intensity was estimated by summing the total number of *C. conanobrieni* found across all infected female *P. argus* divided by the total number of *C. conanobrieni* infested females. Lastly, embryo mortality within the four embryo subsamples was calculated as the average of the total dead and consumed embryos, when combined, and expressed as a percentage (%).

Reproductive performance of female **Panulirus argus**

Beyond prevalence and mean intensity of *C. conanobrieni*, this study was interested in investigating the reproductive performance of egg-bearing *P. argus* to further understand the impact of *C. conanobrieni* on the host. Lobsters collected during summer 2022 were used to assess this impact. As noted above, in 2022, all lobsters were transported back to the laboratory for processing and analysis. Before euthanization (within 24 hours of capture), the pleopods (n = 8) of each captured female were gently snipped off their abdomen using blunt-edge scissors and immersed in individual Petri dishes filled with seawater. Following Atherley et al. (2020b), I completed a multi-step approach to humanely euthanize lobsters by injecting females in the walking leg with 1mL of potassium chloride followed by placing them in coolers filled with ice. The weight of the hepatopancreas was obtained by removing the entirety of the organ, weighing it to the nearest 0.01 mg (OHAUS Discovery, DV215CD, USA), and drying it individually from the rest of the body to calculate lobster nutritional condition. Hepatopancreas dry weight index,

DWI, is the measure for lobster nutritional condition and is calculated as the dry weight of the hepatopancreas divided by the animal wet weight times 100 (Gutzler and Butler 2017).

I was interested in determining whether the prevalence and infestation intensity of *C. conanobrieni* had a negative effect on host reproductive performance by estimating fecundity and reproductive output. To measure fecundity, fine-tip forceps were used to haphazardly pluck four, approximately 1,000 embryos from the egg masses of each of my collected females. These counted subsamples were the same ones used for estimating lobster embryo stage and *C. conanobrieni* prevalence/intensity (see section above). The remaining embryos were carefully stripped from their respective pleopods using fine-tip forceps and placed in a pre-weighed aluminum foil boat. Once all counts were performed, the four embryo subsamples per female were placed in individually labeled aluminum foil boats, weighed using an analytical balance to the nearest 0.01 mg (OHAUS Discovery, DV215CD, USA), and dried, alongside the rest of the female's stripped embryos, in the oven for at least 72 hours at approximately 70°C. Once completely dried, the samples were re-weighed using an analytical balance to the nearest 0.01 mg. The equation used to estimate lobster fecundity was adapted from Baeza et al. (2016).

Reproductive output, an estimation of the female's total investment of resources towards reproduction (Baeza et al. 2016), was estimated by drying the entire female body, including the pleopods once stripped of embryos. Reproductive output was calculated as the ratio of the dry weight of the entire embryo mass, adding in the four subsamples, to the dry weight of the female body. The allometric model $y=a*x^b$ (Hartnoll 1978) was used to test whether reproductive output increases on a linear scale (isometrically) with female body size (Baeza et al. 2016). A *t*-test was performed to test if the slope (*b*) between the dry weight of the entire embryo mass and the dry

weight of the female body mass, after being log-transformed, deviates from 1, the expected slope of unity (Baeza et al. 2016). The slope, *b*, of this relationship can represent a rate of increase ($b>1$) or rate of decrease ($b<1$) in reproductive allocation with each unit increase in lobster body dry mass (Baeza et al. 2016). Prior to performing the *t*-test, I checked for assumptions of normality and homogeneity of variances and found them to be satisfactory.

Two separate analyses of covariance (ANCOVA) were carried out to investigate differences in individual-level female reproductive parameters: fecundity and reproductive output. My first ANCOVA was used to test the effect of embryo stage of development (early versus late) and carapace length (covariate; CL, measured in mm) on female fecundity. A second ANCOVA was used to test the effect of embryo stage of development and female carapace length (covariate) on lobster reproductive output. Prior to performing both ANCOVAs, I tested that the data met statistical assumptions of normality of residuals and homogeneity of variances by looking at residual plots, performing a Shapiro-Wilk normality test on the model residuals, and testing for homogeneity of variance using a Levene's test.

Lastly, I investigated brood loss across embryo development and carapace length of my collected egg-bearing females by using the formula adopted by Oh and Hartnoll (1999b): $100\ [1 - \exp(a_l - a_e)]$ where a_l is the intercept for late-stage embryos and a_e is the intercept for early-stage embryos. All statistical analyses were conducted in RStudio v. 2022.2.0.443 (R Development Core Team 2021).

Considering that all lobsters collected for this study were infested with *C. conanobrieni*, this study relied on redundancy analyses, a multivariate constrained ordination technique, to understand a) what host traits explained the abundance of different *C. conanobrieni* life stages:

juvenile, adult, egg masses, and b) the effect of *C. conanobrieni* on lobster reproductive performance. Redundancy analysis (RDA) involves two data matrices: a response variable matrix, Y, that contains dependent variables, and an explanatory variable matrix, X, that contains predictor variables measured in the same samples (Legendre et al. 2011). The variance of the response variables explained by the explanatory variables is the 'redundancy' and the fraction of total variance calculated through this relationship is key to understanding how much variance in Y is due to differences in X between samples (Paliy and Shankar 2016). RDA assumes that the relationships between variables are linear, like principal components analysis (PCA), and the output is a 2-D 'triplot' with axes representing the constrained RDA dimensions (Paliy and Shankar 2016). The eigenvalues, their contribution to variance, and accumulated constrained eigenvalues represent the axes of rescaled variables from the environmental matrix and include each axis' cumulative proportion of explained variance in the final product (Legendre and Legendre 2012). The significance of the redundancy analysis was evaluated using Monte Carlo tests with 1,000 permutations (Legendre et al. 2011). Statistically significant differences were determined at $\alpha \leq 0.05$. The adjusted R^2 was computed for both RDAs. All redundancy analyses were conducted in RStudio using the package vegan (Oksanen et al. 2019; Oksanen 2015).

For my first redundancy analysis, I was interested in investigating the relationship between female *P. argus* traits and the subsequent abundance of *C. conanobrieni* found in the hosts' brood mass. For host features (X-matrix), the analysis included carapace length (measured in mm), lobster embryo stage of development (Early or Late), female molt status (intermolt, pre-molt, or post-molt), dry female body weight (dFBM), dry embryo mass weight (dEM), lobster blood serum protein (sp), lobster refractive index (nD), and lobster nutritional condition (DWI). Additional host factors included habitat characteristics such as the status of the female when

collected, specifically whether found solitary or aggregated with another individual (Solitary or Aggregated), and the substrate that the female was found on upon collection (sand, rubble, rocky reef substrate, or a combination). Since embryo stage of development, female molt status, and habitat characteristics were all categorical variables, I coded them as 'dummy' variables taking the value of 1 (presence of respective category) or 0 (absence of respective category). The response variables (y-matrix) consisted of n° *C. conanobrieni* egg masses, n° *C. conanobrieni* juveniles, and n° *C. conanobrieni* adults summed across the four subsampled pleopods. The abundances of *C. conanobrieni* for each lobster were transformed using the Hellinger transformation, which takes the square root of the variables (Borcard et al. 2011). The response variables (y-matrix) were standardized, centered around a mean of 0, and scaled to have a standard deviation of 1. Multicollinearity was examined using variance inflation factor (VIF) scores. All VIF scores were <10, therefore did not justify the removal of any variables from my analysis.

The second redundancy analysis investigated the effect of *C. conanobrieni* on spiny lobster reproductive performance. The predictor matrix included the number of *C. conanobrieni* juveniles, adults, and egg masses summed across all four subsampled pleopods per female. The predictor variables (x-matrix) were square root transformed prior to performing the analysis. The response variables (y-matrix) were the counts of total dead and total consumed lobster embryos across all my subsampled pleopods per female (n=4), lobster fecundity, and lobster reproductive output. I performed a z-score transformation on the response matrix prior to performing the analysis. When assessing multicollinearity, all VIF scores were <10, therefore did not justify the removal of any variables from my analysis.

Assessing for the prevalence of **Panulirus argus** *virus 1 (PaV1)*

In addition to *C. conanobrieni* prevalence and female *P. argus* reproductive performance, I was interested in investigating whether the prevalence of other diseases in spiny lobsters, specifically *Panulirus argus* virus 1 (PaV1) might be indirectly influencing the prevalence and intensity of *C. conanobrieni* infestation in egg-bearing *P. argus*. To assess for PaV1 infection in my collected *P. argus*, 1mL of hemolymph was collected from the walking legs using a 30G 1ml/cc fine-tip needle syringe. I recorded visible clinical signs of PaV1, notably milky hemolymph that does not coagulate and any unusual red discoloration of the exocuticle (Huchin-Mian et al. 2013). After this assessment, the hemolymph was carefully dispensed into a 1.5 uL microcentrifuge tube filled with 0.5 mL of 95% ethanol. Samples were stored under refrigeration and transported on ice to Clemson University for further analysis. Once brought back to Clemson, samples were frozen at -20 degrees Celsius. Prior to extractions, samples were thawed at room temperature for 15-20 minutes. A hemolymph pellet was formed using a centrifuge at 14.950 rpm for 1 minute which allowed for separation of the hemolymph and ethanol. Approximately 25 mg of the resulting hemolymph pellet was transferred into a pre-weighed 1.5 uL microcentrifuge tube, weighed to the nearest 0.01 mg (OHAUS Discovery, DV215CD, USA). Genomic DNA (gDNA) was extracted using the E.Z.N.A Blood DNA Mini Kit (Omega Bio-Tek) according to the manufacturer's protocol. I used the primers 45aF (Primer sequence in 5'to 3' order 5': TTC CAG CCC AGG TAC TAC - 3') and 543aR (5' - AAC AGA TTT TCC AGC AGC GT - 3') (from Montgomery-Fullerton et al., 2007 with modifications from Moss et al., 2012), to diagnose PaV1 infection using PCR amplification. Post-extraction, I used a spectrophotometer (Thermo Fisher Scientific, NanoDrop One, USA) to quantify the DNA concentration and purity of all of my samples. The PCR reaction contained 0.5 uM 45aF (forward primer), 0.5 uM 543aR (reverse

primer), 1.0 unit of GoTaq® Green Master Mix (Promega), and 1.0 unit of Nuclease-free water (Promega) along with 2.5 uL of DNA template for a total volume of 25 uL. The thermal cycling conditions were an initial denaturation at 94°C for five minutes, followed by 40 cycles of 94°C, 63°C for 45 seconds, and 72°C for one minute. The final elongation occurred at 72°C for 10 minutes. PaV1 PCR product was loaded and run in a 1% agarose gel with 3 uL of Midori Green (NIPPON Genetics EUROPE) and a 3 uL 1 kb DNA step ladder (Promega) and electrophoresed at 145 volts for 45-60 minutes. It is expected that after running the 1% agarose gel I would visualize the 499 bp PaV1 amplicon as a clear and distinct band on the gel under UV light, indicating PaV1 infection. For my positive control, I extracted DNA from a previously identified positive PaV1 lobster from hemolymph using the same protocols as above and my negative control was run using only Nuclease-free water (no DNA).

Laboratory observations on the behavior of Carcinonemertes conanobrieni

I was also interested in further exploring the behaviors of these worms by isolating them from the brood mass. After counting and classifying individual *C. conanobrieni* during their three different life stages (juvenile, adult, egg mass) found among my four ~1,000 *P. argus* embryo subsamples per female, I isolated individuals in the laboratory to visualize and describe their behaviors. Using fine-tip forceps, individual *C. conanobrieni* were gently removed from the subsampled embryos and immediately placed in plastic Petri dishes and glass bowls, of various sizes (90 x 15 mm; 35 mm), filled to the top rim with seawater. The worms were sexed following Simpson et al. (2017) and their life stage (juvenile, adult, or embryo) was recorded. Worms were separated into groups as follows: male-female pairs, same sex pairs (female-female, male-male), or solitary. Worms were fed *ad libitum* clumps of fresh *P. argus, Mithrax* sp., or *Menippe*

mercenaria embryos. Embryos were counted and placed into petri dishes with worms and replaced every 2-3 days to avoid biofouling (e.g., bacterial/mold growth). The Petri dishes were kept indoors at room temperature (~72 C) on a lab benchtop. Worms were checked daily, two to three times per day, to observe and record any *C. conanobrieni* behavior in the Petri dishes under a Leica S8AP0 stereomicroscope or Wild M5-97874 dissecting scope. I specifically focused on *C. conanobrieni* mating, feeding, roaming, and mucus sheath-forming behaviors. During behavioral check-in periods (morning (between 8:00 AM - 9:00 AM) and evening (between 6:00 PM - 7:00 PM), I observed the behaviors of *C. conanobrieni* worms in the Petri dish, recorded where in the Petri dish they were (side, bottom, top), where in the water column they were located, and how many embryos they had consumed during a 15-20 minute observation in the selected time slot. Complete water changes were performed at each behavior check using small transfer pipettes. When a *C. conanobrieni* female laid an egg mass, it would be removed from the petri dish with the worm and placed in a separate dish to determine if any larvae would hatch in the laboratory.

Results

Prevalence of Carcinonemertes conanobrieni *across the Florida Keys*

I sampled egg-bearing female *P. argus* across the Florida Keys reef tract in the summer of 2022 and 2023 to determine *C. conanobrieni* prevalence. In 2022, I collected 98 females from the Middle Florida Keys with carapace lengths (CL) ranging from 60.6 - 87.7 mm and a mean (± SD) of 74.3 ± 5.03 mm. Of these, 45 females carried early-stage embryos and 53 females carried late-stage embryos. The brood masses of all were infested with *C. conanobrieni*, making prevalence 100% in 2022.

In summer 2023, I assessed egg-bearing lobsters for *C. conanobrieni* infestation at three locations along the Florida Keys reef tract (Upper, Middle, Lower Keys). From the Middle Florida Keys, I collected 60 females with carapace lengths ranging from 63.0 - 92.0 mm CL and a mean (± SD) of 73.82 ± 5.74. Of these, 17 females were carrying early-stage embryos and 40 were carrying late-stage embryos. From the Upper Keys, I collected 28 egg-bearing lobsters with carapace lengths ranging from 60.0 - 90.0 mm CL and a mean (± SD) of 78.27 ± 6.18. Of these, 10 females were carrying early-stage embryos and 18 were carrying late-stage embryos. From the Lower Keys, I collected 38 egg-bearing *P. argus* with carapace lengths ranging from 59.60 - 86.0 mm CL and a mean (± SD) of 75.24 ± 5.47. Of these, 17 carried early-stage embryos and 21 carried late-stage embryos. All females, across all localities, were found to be infested with *C. conanobrieni* in their brood mass (100% prevalence).

Female Panulirus argus *sample population and egg predator prevalence*

Of the 98 egg-bearing females collected in 2022, 36 were classified as pre-molt, 47 as intermolt, and 15 as post-molt. Eighty-two females (84%) had partially or completely scratched spermatophores and 23 females (23%) had one or more appendages missing. Nutritional condition, assessed using the DWI, ranged from 0.42 - 3.38 with a mean (± SD) of 0.92 ± 0.34. For my measure of nutritional condition from hemolymph, blood serum protein was calculated only for intermolt females and ranged between 7.21 to 15.99 with a mean (± SD) of 11.20 ± 1.85.

Across all of the subsampled lobsters, I found a total of 1,966 *C. conanobrieni* which included three nemertean life stages (juvenile, adult, and egg masses). Female *P. argus* collected for this study were all found with one or more *C. conanobrieni* juveniles, adults, or egg masses

in their brood masses. Egg predator prevalence was 100% for all female lobsters collected in the Middle Florida Keys. Mean intensity of *C. conanobrieni*, including all life stages present (juvenile, adult, egg masses) was 20.06 egg predators per 1,000 embryos (Table 1). Additionally, 246 ± 3.27 *C. conanobrieni* worms were found either in mucus sheaths along the pleopod edges or roaming the pleopod's surface.

Assessing for the prevalence of **Panulirus argus** *virus 1 (PaV1)*

I performed PCR to assess for the presence/absence of PaV1 viral DNA in the hemolymph of the collected females used for my reproductive performance study (2022). Five females presented with suspected clinical signs of PaV1, specifically cloudy or milky hemolymph or unusual carapace discoloration. However, all samples tested negative for PaV1 in the PCR assay.

Reproductive performance of **Panulirus argus**

Fecundity for female spiny lobsters carrying early-stage embryos ranged from 70,411.57 to 851,281.11 embryos with a mean (± SD) of 457,012.7 ± 160,965.9 embryos. For females carrying late-stage embryos, fecundity ranged from 25,266.62 to 695,638.75 embryos with a mean (± SD) of 362,131.6 ± 165,886.5 embryos. The estimated number of eggs carried by the smallest egg-bearing female *P. argus* (60.6 mm CL) was 76,068 while the largest female (87.7 mm CL) had a clutch size of 682,865 embryos. Female carapace length had a significant effect on fecundity (ANCOVA; $F = 34.424$, df = 1, 94; $p<0.001$). Additionally, embryo stage of development had a significant effect on fecundity ($F = 20.887$, df = 1,94: $p<0.001$) (Table 3). The interaction between embryo stage of development and carapace length was not statistically significant ($F = 0.299$, df = 1,94; $p = 0.5858$). Overall, fecundity increased with female body size

and was greater for females carrying early-stage embryos compared to females carrying late-stage embryos at any given female body size (Figure 2A, Table 3).

Reproductive output estimates for females carrying early-stage embryos ranged from 1.8% to 15.1% with a mean (± SD) of 9.9% ± 2.2%. Reproductive output (RO) for females carrying late-stage embryos ranged from 0.9% to 13.6% with a mean (± SD) of 7.8 % ± 2.6%. Female body mass (log-transformed) had a significant effect on female embryo mass (log-transformed) (ANCOVA; $F = 21.4659$ $df = 1, 94$; $p < 0.001$). Additionally, embryo stage of development had a significant effect on female embryo mass ($F = 12.1297$, $df = 1,94$; $p = 0.000755$). The interaction between embryo stage of development and female body mass was not statistically significant ($F = 1.8368$, $df = 1, 94$; $p = 0.178573$) (Table 3). After log-transforming the values of female body mass (mg) and female embryo mass (mg), I calculated the slope of the regression as $b = 0.97$ ($SEb = 0.28$); meaning that there is no change in resource allocation to reproduction with every unit increase in female body dry mass (Figure 2B). The slope of the line, after female body dry weight and embryo dry weight were log-log transformed, did not deviate from 1 therefore, reproductive output is independent of female body size (Table 3).

Embryo mortality, the average of dead and consumed embryos, per 1,000 embryos subsampled, ranged between 0% and 15% with a mean (± SD) of 2.55% ± 3.27 for egg-bearing lobsters carrying early-stage embryos. For females carrying late-stage embryos, embryo mortality was 25.27% ± 38.15 with a range of 0% to 100%. Brood mortality from early to late-stage embryos in my collected females was estimated as a loss of 94,880.4 eggs or 20.1% of the total number of eggs.

The effect of **Carcinonemertes conanobrieni** *on female* **Panulirus argus**

The variation in specific host traits collected from my sample female *P. argus* population, including female nutritional condition (DWI and blood parameters), female body and embryo mass condition (weight, carapace length, molt status), embryo stage, and habitat characteristics such as the substrate surrounding the female's den and status of the female at collection (solitary or aggregated with other lobsters), explained 39.8% of the variation in *C. conanobrieni* abundance (juvenile, adult, egg masses) on the host. Eigenvalues for the first and second canonical axes were 0.31 and 0.06, respectively. The RDA model was significant ($p = 0.001$). Only the first canonical axis, RDA1, was significant in my model ($p = 0.001$) and explained 31.12% of the variance in the data. The second canonical axis, RDA2, only explained 5.64% (Figure 3a). The adjusted R^2 of the model was 0.28. Lobster nutritional condition (DWI), dry embryo mass weight (dEM), dry female body weight (dFBM), and embryo stage were all statistically significant host variables (p-values = 0.006, 0.001, 0.001, and 0.001, respectively) explaining infestation intensity of *C. conanobrieni* in the embryo masses of female *P. argus*. There was a negative relationship between *C. conanobrieni* juvenile infestation intensity and *P. argus* nutritional condition (DWI). My first RDA analysis also showed that as female embryo mass weight increased, the infestation intensity of *C. conanobrieni* adults declined in the brood mass (Figure 3b). Furthermore, as female *P. argus* body mass increased a sharp decline in the abundance of *C. conanobrieni* juveniles was observed.

The second redundancy analysis model investigating the effect of *C. conanobrieni* on lobster reproductive performance had a total explained variance of 45.20%. Eigenvalues for the first and second ordination axes were approximately 0.4362 and 0.00886, respectively. The

second RDA model was significant (p = 0.001). The adjusted R^2 of the model was 0.43. All three life stages of *C. conanobrieni* quantified i.e. juveniles (p = 0.010), adults (p = 0.001), and egg masses (p = 0.001) significantly affected *P. argus* reproductive performance as seen in my RDA. Specifically, a decline in the number of live *P. argus* embryos in a given female's clutch was associated with increases in *C. conanobrieni* abundance. *Carcinonemertes conanobrieni* adults and their egg masses explained the largest increase in the number of dead and consumed *P. argus* embryos (Figure 3c). The presence and increases in abundance of *C. conanobrieni* juveniles in female embryo masses showed slight decreases in fecundity and reproductive output (RO), however the most notable declines in fecundity and RO occurred in females with heavy *C. conanobrieni* egg mass and adult loads. My second redundancy analysis supported my prediction that all life stages of *C. conanobrieni* contribute to significant losses in host fecundity and reproductive output, coupled with increased embryo mortality (Figure 3d).

Laboratory observations of* Carcinonemertes conanobrieni *behavior

Among my collected embryo subsamples, I observed *C. conanobrieni* larvae, juveniles, adults, and their egg masses. I observed hatched larvae from worm egg masses engaging in erratic, fast-paced swimming among clumps of late-stage embryos that appeared close to hatching. Juvenile *C. conanobrieni* were found either encapsulated in an ovoid cyst structure on individual *P. argus* embryos or I witnessed an empty juvenile mucus sheath attached to a consumed lobster embryo with the juvenile nemertean inside the empty egg capsule. When exploring the behaviors of these worms by isolating them from *P. argus* brood masses in the laboratory, females of *C. conanobrieni* were observed to lay their egg masses in the Petri dishes on the walls or the bottom of the dish. Nemertean egg mass organization ranged from spherical to spiral strings encased in mucus. Worms either laid a single egg mass or more than one egg mass over the span of a few

days. Females that were solitary in Petri dishes since collection from the host laid their own embryos. On some occasions, females of *C. conanobrieni* were observed to lay multiple egg masses over the span of a few days to then die, usually entrapping themselves within their own mucus sheaths, and did not respond to prodding with forceps.

Additionally, I observed mating in *C. conanobrieni* when maintained in heterosexual pairs; the male and the female would wrap their posterior ends around each other and surround their bodies, entirely, within their mucus sheaths. Approximately 2-4 days after mating, strands of egg masses were observed at the bottom of the Petri dish (see above). However, no nemertean larvae hatched in any of the Petri dishes. I did not observe any direct *C. conanobrieni* feeding despite several attempts to feed *C. conanobrieni* with *P. argus* embryos and supplementing them with other decapod embryos, such as *Mithrax* spp., failed. I also observed worms on the pleopods of female *P. argus*, either in mucus sheaths along the edges of the pleopods or roaming the surface of the pleopod.

Discussion

Prevalence of Carcinonemertes conanobrieni *on egg-bearing female Panulirus argus*

Prevalence of *Carcinonemertes conanobrieni* along the Florida Keys reef tract has risen markedly from the baseline levels reported on female *P. argus* during its initial discovery in 2015 (7.4% - Baeza et al. 2016), to 100% in 2022 and 2023 (this study), making mine the highest reported across *P. argus'* Caribbean distribution. *Carcinonemertes conanobrieni* has been detected in other Caribbean localities including Colombia (87.78% - Berben et al. 2023) and Saint Kitts and Nevis (87%- Atherley et al. 2020a) (Table 4). Prevalence estimates for *C. conanobrieni* have so far been limited to female *P. argus* brood masses (Baeza et al. 2016;

Simpson 2018; Gonzalez-Cueto and Quiroga 2018; Atherley et al. 2020a; Berben et al. 2023; my study). However, Atherley et al. (2020a) also detected branchial nemerteans in non-ovigerous *P. argus* but studies performed in the Florida Keys, including mine, have found no evidence of *C. conanobrieni* in the gills of female *P. argus* (Baeza et al. 2016; Simpson 2018). Atherley et al. (2020a) also included female *P. argus* whose brood masses contained an undescribed *Carcinonemertes* sp., which I failed to detect among any of my sampled females. Future studies need to corroborate the prevalence of *C. conanobrieni* in the gills and other host microhabitats beyond *P. argus* reproductive peaks.

Mean intensity estimates frequently accompany prevalence and may provide insight into *C. conanobrieni* dispersion on female *P. argus'* brood mass. Infestation intensity by *Carcinonemertes* spp. can vary from extremely high to very low, even with the high prevalence of *C. conanobrieni* observed in my female *P. argus* sample population (Shields and Kuris 1988). At its discovery, *C. conanobrieni* mean intensity was 0.24 ± 0.19 worms per 100 embryos in the Florida Keys population (Baeza et al. 2016). In female *P. argus* with *C. conanobrieni* in Saint Kitts and Nevis, mean intensity was 5.7 worms lobster^{-1} ± 10.56 or 0.0064 worms 1,000 lobster eggs^{-1} ± 0.014 (Atherley et al. 2020a). My study's mean intensity estimates, including three life stages of the egg predator, is the highest reported for *P. argus*. Mean intensity estimates are critical to measure over time, especially given this study's 100% prevalence, because they can indicate outbreak versus non-outbreak periods of *Carcinonemertes* spp. on their decapod hosts. For instance, over numerous years, *C. epialti* infestations on the shore crab *Hemigrapsus oregonensis* cycled between a mean intensity of 26 worms and a prevalence of 47% (Kuris 1978) to an outbreak year with a mean intensity of 296 worms per crab and a prevalence of 97% ten years later (Shields and Kuris 1988). While ecologists have yet to identify outbreak versus non-

outbreak years of *C. conanobrieni*, continued research investigating this host-egg predator relationship will allow us to monitor for *C. conanobrieni* outbreaks in the future.

Reproductive performance of *Panulirus argus*

I investigated female *P. argus* reproductive performance to explore the effect *C. conanobrieni* has on this host. Fecundity estimates for female *P. argus* reported herein are within the expected range of those previously reported for this species, in this size range, when compared to previous studies in the Florida Keys (Baeza et al. 2016; Simpson 2018), Cuba (Cruz 1980), Colombia (Berben et al. 2023), and Mexico (Ramírez-Estévez and Briones Fourzán 1996; Fonseca-Larios and Briones-Fourzán 1998) (Table 2). My smallest egg-bearing lobster had a carapace length of 60.6 mm, while my largest measured at approximately 87.7 mm CL, however, females have been reported smaller at 51.3 mm CL (Baeza et al. 2016) or as large as 145 mm CL (Ramírez-Estévez and Briones Fourzán 1996). Previous studies on female *P. argus* fecundity have shown that larger females (105 - 120 mm CL) contribute more eggs to the population and usually spawn more than once per year (Fonseca-Larios and Briones-Fourzán 1998). Significant overfishing of the largest *P. argus* in the Florida Keys explains the lack of large females found on these reefs and missing from my study (Bertelsen and Matthews 2001).

My estimates of fecundity in *P. argus* are also in line with those reported for similar-sized spiny lobsters, however, some *Panulirus* spp. are more or less fecund than *P. argus*. For instance, in the ornate spiny lobster, *P. ornatus*, females tend to be larger in size (CL range: 104.4 - 145.1 mm) and fecundity estimates range between 518,181 - 1,979,522 eggs per female (Vijayakumaran et al. 2012). In turn, in the relatively small, congeneric *P. penicillatus* (CL range: 46.6 - 92.2 mm), fecundity ranges between 31,162 and 296,115 eggs per female (Juinio

1987). I reviewed the literature for other spiny lobster species in the genus *Panulirus* to estimate a fecundity-to-carapace length relationship for all *Panulirus* spp. based on mean values for fecundity and carapace length. The equation Fecundity = -522,961.94 + 9726.53CL (Adjusted R^2 = 0.59) demonstrates that female body size is a major determinant of fecundity and overall reproductive potential in the genus (MacDonald 1988). Predicting fecundity based on spiny lobster carapace length can also be useful for stock assessments and is shown to be the most cost-effective way to estimate fecundity at larger scales (Green et al. 2009). Moreover, fecundity-size-based relationships could supplement data-deficient fisheries, especially since it has been identified that 84% of countries with commercial and/or artisanal fisheries do not use reproductive parameters to predict recruitment processes (Cruz and Bertelsen 2009). Continued reporting and use of fecundity from this and other studies is paramount due to the continued commercial and recreational exploitation of *P. argus*, contributing to its overfished status across the Greater Caribbean region.

Stock and recruitment estimates should be supplemented by reproductive performance measures other than fecundity, such as reproductive output (Chubb 2000). Surprisingly, estimates of reproductive output for ~~s~~piny lobster species is nonexistent other than for *P. argus* (Lunden 2018; Baeza et al. 2016; Berben et al. 2023). Reproductive output has greatly ranged for female *P. argus* in the Florida Keys (49.21% ± 8.17 lobster body dry weight - Baeza et al. 2016) compared to female *P. argus* in Colombia (9.05% ± 1.95 (early stage) versus 6.97% ± 2.03 (late stage embryos) - Berben et al. 2023). Given these estimates, in addition to this study, I conclude that reproductive output is limited to approximately 7-11% of female *P. argus* body dry weight. Allometric constraints on brood size in *P. argus* and other brooding decapods has been suggested to be driven by energetic or physical/mechanical limitations (Hines 1982; Hines 1992; Ediritanti

et al. 2016; Oh and Hartnoll 1999b; Kuris 1990; Fernández et al. 2020). Female *P. argus* are limited by space available in the cephalothorax for yolk accumulation, similarly to brachyuran crabs whose reproductive output (~10%) is limited by the compaction of their ovaries in the cephalothorax (Hines 1982; Hines 1992). Unlike the constraints of a well-developed and hard exoskeleton exhibited by brachyuran crabs and spiny lobsters, including *P. argus* (Thessalou-Legaki and Kiortsis 1997; Anger and Moreira 1998) the soft exoskeleton of caridean shrimps, that allows for an extended abdomen and ovary, may explain their greater reproductive output (15-22% - Corey and Reid 1991; de Moraes et al. 2017; Anger and Moreira 1998).

Reproductive output has previously been identified as positively allometric with body weight in *P. argus* (Baeza et al. 2016; Berben et al. 2023), however, in this study, I report an isometric relationship between female body mass and embryo mass ($b = 0.97 \pm 0.28$); reproductive output scaled proportionally with female body weight in my population. I acknowledge that there is a lack of studies focusing on the reproductive output of brooding invertebrates, including other species of spiny lobsters with a body size similar to that of *P. argus*. In agreement with previous studies focused on reproductive performance in this species, I encourage other studies to incorporate estimates of reproductive output to get a better understanding of how much female spiny lobsters invest in reproduction compared to growth and other processes given their body sizes (Baeza et al. 2016).

Over the duration of female reproduction, the range of brood loss seen across a few *Panulirus* spp. (n = 3) has been previously estimated at 10-28% (Groeneveld et al. 2005), with female *P. argus* from this study (20.1%) fitting in on the high end of brood loss in spiny lobsters. Other studies comparing brood loss across multiple taxa of decapod crustaceans indicate that

brood loss can range widely from 4-71% (Kuris 1990). Causes of increased embryo loss in brooding decapod crustaceans include increasing egg volume throughout development (Ediritanti et al. 2016), incomplete fertilization or improper egg attachment to the pleopods (Oh and Hartnoll 1999a), active parental care behaviors such as heightened embryo ventilation frequency (Kuris 1990; Fernández et al. 2020) or increasing age and size making larger and older females more susceptible to significant brood loss (Kuris 1990). Even so, for some crustaceans, brood loss is generally only noticeable at the extremes, earliest and latest stages of embryo development (Oh and Hartnoll 1999a; Kuris 1990).

Despite mechanical and energetic causes of brood loss, my study alludes away from those and shows that mortality by brood predators can also explain embryo loss in *P. argus* (Shields 2011). Specifically, brood loss has occurred frequently for brooding decapod crustaceans infested by *Carcinonemertes* spp. (Shields 2011). Indeed, there is strong evidence suggesting that egg predators, such as nemerteans in the genus *Carcinonemertes*, contribute to significant embryo mortality throughout all stages of host embryo development due to their feeding (Shields and Kuris 1988; Kuris 1990; Kuris et al. 1991). Prevalence and infestation intensity of these worms in the brood mass closely align with the reproductive cycle of their host (Shields 1993), thereby providing a mechanism for these worms to contribute to significant brood mortality and further disrupt host reproductive performance.

Carcinonemertes conanobrieni **impacts the reproductive performance of** ***Panulirus argus***

Carcinonemertes conanobrieni can be directly tied to significant declines in female *P. argus* reproductive performance. Robust evidence for this conclusion includes the effect of *C. conanobrieni* explained by my redundancy analyses. Infestation by *C. conanobrieni* in *P. argus*

embryo masses was best explained by the stage of lobster embryo development, the molt status of the female, and the size/weight of the female and her embryo mass. The results from my second RDA signified that *C. conanobrieni* adults and their egg masses contributed to the most significant declines in *P. argus'* reproductive performance. Female *P. argus* bearing late-stage embryos had more *C. conanobrieni* adults and their egg masses within their own embryo mass which ultimately corresponded with them having lowered fecundity and reproductive output, coupled with increased embryo mortality, compared to females bearing early-stage embryos.

Baseline levels of dead and consumed *P. argus* embryos when *C. conanobrieni* was first discovered in the summer of 2015 averaged 7.2% and 12.7%, respectively. In the subsequent summer sampling period at the same locality, embryo mortality for females carrying early-stage embryos (stage I and II) was 1.03% compared to females with late-stage embryos (stage III and IV) exhibiting 6.74% embryo mortality (Simpson 2018). Estimates of embryo mortality in egg-bearing *P. argus* in the middle Florida Keys have greatly increased since last surveyed, with embryo mortality exceeding 50 -75% in some of my subsamples. With such wide-ranging values, previous studies have used embryo mortality as a way to classify nemertean infestation on the host as an outbreak versus non-outbreak year (Shields and Kuris 1988; Roe 1979). In the case of *H. oregonensis* infested with *C. epialti,* over 50% of egg-bearing females experienced 75-100% brood mortality, leading researchers to presume a *C. epialti* outbreak period (Shields and Kuris 1988). This is in stark contrast with a non-outbreak *C. epialti* period where mean embryo mortality for infested females was 5.6% (Shields and Kuris 1988). For the Dungeness crab *Cancer magister,* over 50% embryo mortality due to *C. errans* was suggested to significantly contribute to the collapse of this commercially important decapod fishery (Wickham 1979, Wickham 1980). The continuation of heightened embryo mortality by *C.*

conanobrieni on female *P. argus* could detrimentally affect this heavily fished decapod and cause a fisheries collapse like the one seen for *C. magister.* This data could then be used to inform fisheries management, as fishing pressure has been suggested to facilitate nemertean outbreaks (Shields and Kuris 1988).

Beyond the effect of *C. conanobrieni* on female *P. argus* reproductive performance, I hypothesized that the prevalence of other diseases, such as PaV1, might indirectly influence the prevalence and intensity of *C. conanobrieni* on this host. However, I found that none of the collected females were infected with the virus. A drawback to these results includes limited DNA integrity testing on the extracted samples and, therefore, future work should consider validating this to be able to make strong inferences on the relationship between PaV1 prevalence and *C. conanobrieni* infestation.

The life cycle of *Carcinonemertes conanobrieni* on egg-bearing female *Panulirus argus*

My observations in the laboratory, alongside previous studies, allowed me to propose a model for the life cycle of *C. conanobrieni* that starts with a hoplonemertean planuliform larvae hatching from egg strands deposited among *P. argus* embryos (Simpson et al. 2017). *Carcinonemertes conanobrieni* larvae are ovoid or spherical in shape and usually have ciliary tufts, presumably to aid with efficient swimming (Simpson et al. 2017). During their larval stage, *C. conanobrieni* are likely to disperse in the water column. While their larval duration is unknown, other cold-water congeneric species (e.g., *C. errans*- Wickham 1980) are suggested to undergo a larval period of ~ 8 months, however further research into the larval period of *C. conanobrieni* and other tropical congeneric species is needed. How long *C. conanobrieni* larvae can travel and survive before finding a host remains to be addressed, but settlement on the host may initiate from detecting physical (e.g., temperature - Pawlik 1992) or chemical (e.g., biogenic

chemicals produced by other organisms - Keough and Raimondi 1994) cues, including those emanating from conspecifics, known to mediate gregarious settlement in other nemerteans (Dunn and Young 2014). Despite past research showing *Carcinonemertes* sp. utilizing these larval cues, the settling cues used by most nemerteans, including *C. conanobrieni,* to locate their host are still largely unresolved.

Preceding studies in congeneric species have shown that larvae settle on their host's exoskeleton, metamorphosize into juveniles that ensheath on the host by secreting mucus, and remain on the host exoskeleton, without feeding, for months (Wickham and Kuris 1985; Kuris 1978). My study, and others investigating this species (Simpson et al. 2017; Berben et al. 2023), have not detected larval or juvenile *C. conanobrieni* on the exoskeleton of female *P. argus* (Simpson et al. 2017, Atherley et al. 2020a, Berben et al. 2023). I hypothesize that *C. conanobrieni* settlement occurs only among the host's egg mass when larvae sense chemical cues associated with female *P. argus* oviposition. In the embryo mass, *C. conanobrieni* larvae metamorphose into juveniles that encyst on individual *P. argus* embryos (Simpson et al. 2017). In its congeneric, *C. errans,* nemerteans frequently migrated into the brood mass 1-2 days following host oviposition (Wickham 1980).

After settlement and metamorphosis, juvenile *C. conanobrieni* can emerge from their cyst and begin actively feeding on host embryos. Feeding *C. conanobrieni* juveniles subsequently grow into adults that can reach 12.71 mm and 16.73 mm in total body length for males and females, respectively, but unlike some members of the genus *Carcinonemertes*, sexual dimorphism is minimal in *C. conanobrieni* (Simpson et al. 2017). While I did not directly observe *C. conanobrieni* feeding, previous studies have confirmed that this species exhibits the

same suctorial feeding as other *Carcinonemertes* worms, which explains my observations of dead and consumed *P. argus* embryos alongside healthy embryos (Simpson et al. 2017; Kuris 1993; Roe 1984). Specifically during feeding, *C. conanobrieni* punctures the lobster embryo with its stylet, and using its everted proboscis and muscular contractions, the nemertean extracts the yolk from the egg until the embryo is fully or partially consumed (Simpson et al. 2017; Simpson 2018). The aforementioned observations confirm that *C. conanobrieni* is a voracious egg predator, capable of living and growing on *P. argus* through the consumption of embryos.

Once *C. conanobrieni* adults reach sexual maturity, distinguishable by the presence of gametes visible across the body wall (Roe 1984), male and female worms begin mating within the embryo mass of female *P. argus*. Mating in this species, as seen by Simpson (2018) and observed in my study, occurs when male and female *C. conanobrieni* intertwine themselves and produce a shared mucus sheath that males eventually leave while females remain inside to finish depositing an egg mass before host eclosion, as seen in *C. epialti* and *C. errans* (Kuris 1978, Wickham 1980). Similar to Simpson et al. (2017), I observed spherical and spiral egg mass strings covered in mucus and wrapped around clumps of late-stage *P. argus* embryos. *Carcinonemertes* egg masses typically hatch in synchrony with their host's embryo mass (Kuris 1978), which aligns with my behavioral observations of hatched *C. conanobrieni* larvae swimming among late-stage spiny lobster embryos in the laboratory. It is unknown whether *C. conanobrieni* worms slough off and die when done laying their egg masses or ensheath on the host exoskeleton or gills post-reproduction and regress, as seen in species such as *C. epialti* (Kuris 1978) and *C. errans* (Wickham 1980). Future studies should continue to rear and observe *C. conanobrieni* in a laboratory setting to fill in the gaps of their life cycle missed by this study.

Conclusions

This study has tied the high prevalence and infestation intensity of *C. conanobrieni* with its negative effect on egg-bearing *P. argus* reproductive performance. The presence of *C. conanobrieni* on female *P. argus* in the upper, middle, and lower Florida Keys provides further evidence supporting the hypothesis that this egg predator may be present across this host's entire Caribbean distribution (Gonzalez-Cueto and Quiroga 2018; Atherley et al. 2020a). I have demonstrated that this nemertean contributes to significant declines in reproductive performance, coupled with increased embryo mortality, throughout female spiny lobster embryo development. Specifically, I saw a significant decline in female *P. argus* fecundity and reproductive output in later stages of embryo development (III and IV) which coincided with heavier *C. conanobrieni* adult densities in the host's brood mass. This was in stark contrast with seeing more *C. conanobrieni* juveniles in female spiny lobsters brooding early-stage embryos. Furthermore, I compared the fecundity and reproductive output of female *P. argus* from my study to other spiny lobsters and estimated a fecundity-carapace length equation that quantifies fecundity across multiple *Panulirus* sp. This quantification will supplement spiny lobster fisheries that lack sufficient reproductive performance data. Lastly, my behavioral observations allowed me to propose a model for the life cycle of *C. conanobrieni* which yields further insight into the interconnectedness of spiny lobster and egg predator ecology and reproduction. Continued monitoring of this Florida Keys female *P. argus* population beyond its reproductive peaks could aid disease ecologists in identifying the patterns of *C. conanobrieni* outbreaks experienced by this host. In addition, winter sampling of *P. argus* could help close the gaps in the life cycle of *C. conanobrieni* that ecologists are uncertain about and provide clues to where the egg predator may settle, including if it uses microhabitats like the gills or exoskeleton, during the cessation of

female reproduction. Future studies should investigate co-occurring crustaceans, such as the spotted spiny lobster *P. guttatus*, for the presence of *C. conanobrieni* as they may act as reservoirs for these egg predators before making their way to egg-bearing *P. argus*. This data will supplement future studies further exploring the association between female *P. argus* and egg predator prevalence.

Abbreviations

CL	Carapace length
SPA	Sanctuary preservation area
RO	Reproductive output
ANCOVA	Analysis of covariance
RRS	Rocky Reef Substrate
VIF	Variance inflation factor
RDA	Redundancy Analysis
DWI	Lobster nutritional condition
dEM	Dry embryo mass weight
dFBM	Dry female body mass weight
sp	Lobster blood serum protein
nD	Lobster refractive index
Min	Minimum
Max	Maximum
SD	Standard deviation

Table 1. Breakdown of total *Carcinonemertes conanobrieni* from all female *Panulirus argus* (N=98) across all four lobster embryo subsamples. Statistics include the sum, mean, standard deviation (SD), and range of *C. conanobrieni* by each life stage present in the host's embryo mass.

Egg Predator Life Stages				
Statistics		**Juvenile**	**Adult**	**Egg Mass**
	Sum	926	502	538
	Mean	9.45	5.12	5.49
	SD	11.65	7.31	13.10
	Range	0 (min), 57 (max)	0 (min), 55 (max)	0 (min), 68 (max)

Table 2. Fecundity estimates (range) and fecundity-carapace length equations for spiny lobster (*Panulirus*) species gathered from the literature and this study.

Species Name	Locality	Carapace Length (in mm; minimum - maximum)	Equations	Fecundity (minimum and maximum)	Citation
Jasus edwardsii	New Zealand	74 - 157	F = 0.1791 CL3.00; F = 0.0889 CL3.11	37,499 - 407,032	Annala and Bycraft 1987
Jasus edwardsii	Tasmania, Australia	60 - 156	F = -1.707 + 2.969 (log CL).	43,918 - 660,156	Green, Gardener, and Kennedy 2009
Jasus edwardsii	South Australia	90 - 141.3	F = 0.0584 X CL3.1642	45,292 - 466,800	Linnane, Penny ,and Ward 2008
Jasus edwardsii	New Zealand	90 - 130	F = 0.169 X CL3.0091	125,000 - 422,000	MacDiarmid 1989
Jasus verreauxi	Spirits Bay, New Zealand	155 - 235	NA	386,611 -2,040,125	Kensler 1967
Palinurus elephas	Western Mediterranean, Spain	71.5 - 135.5	F = 2428 X CL - 148998	23, 483 - 201,549	Goñi et al. 2003
Palinurus gilchristi	South Africa	58.8 - 117.9	NA	36,258 - 125,130	Groeneveld 2005
Panulirus argus	Florida Keys	72 - 141	F = 2.668CL2.709	147, 000 - 1,952,000	Bertelsen and Matthews 2001
Panulirus argus	Cuba	62 - 135	F = 2.668CL2.709	159,000 - 1,727,775	Cruz 1980
Panulirus argus	México Caribbean	77 - 145	F = 2.668CL2.709	280,400 - 1,308,200	Ramírez-Estévez and Briones Fourzán 1996

Panulirus argus	**México**	**76.4 - 137.6**	**F = 3.40 X CL2.5723**	**NA**	**Fonseca-Larios and Briones-Fourzán 1998**
Panulirus argus	**Middle Florida Keys**	**51.3 - 100.4**	**NA**	**105,858 - 757,278 (early); 205,569 - 674, 041 (late)**	**Baeza et al. 2016**
Panulirus argus	**Middle Florida Keys**	**60. 24 - 87.9**	**NA**	**52,189.05 - 397,043.01 (early); 70,151.52 - 348,267.33 (late)**	**Simpson 2018**
Panulirus gracilis	**Gulf of California**	**60 - 80**	**F = 0.6803CL3.1007**	**76, 727 - 1,115,060**	**Pérez-González et al. 2012**
Panulirus homarus	**India**	**52.2 -94.4**	**NA**	**120,544 - 449,585**	**Vijayakumaran et al. 2012**
Panulirus homarus	**Southern Africa**	**50 - 99**	**F = -523,443 -11,905CL**	**100,000 - 900,000**	**Berry 1971**
Panulirus longipipes cygnus	**Western Australia**	**64 - 117.8**	**F = 10430CL - 542940 (newly spawned eggs); F = 9800CL - 581850 (late eggs)**	**61,000 - 682,000**	**Morgan 1972**
Panulirus marginatus	**Necker Island, Northwestern Hawaiian Islands**	**54.3 - 105.4**	**F = 7.9952 CL2.4017**	**109,865 - 590, 530**	**DeMartini, DiNardo, and Williams 2003**
Panulirus ornatus	**Papua New Guinea**	**75.4 - 121.0**	**F = 10,416.68CL - 561,793. 71**	**225,000 - 840,000**	**MacFarlane and Moore 1986**
Panulirus ornatus	**India**	**104.4 - 145.1**	**NA**	**518,181 - 1,979,522**	**Vijayakumaran et al. 2012**
Panulirus penicillatus	**Palau, Western Caroline Islands**	**69 - 131**	**F= -481,347 +85,885CL**	**127,983 -602,807**	**MacDonald 1988**

Panulirus penicillatus	**Philippines**	**46.6 - 92.2**	**F = 0.1694 CL3.27898**	**31,162 - 296,115**	**Juinio 1987**
Panulirus penicillatus	**Saudi Red Sea**	**40 - 130**	**F = 7.43(CL)2.30**	**NA**	**Hogarth and Barratt 1996**
Panulirus penicillatus	**Eilat, Israel**	**51 - 100**	**F = 2.715 X (CL)2.581**	**NA**	**Plaut 1993**
Panulirus polyphagus	**Bombay**	**180- 353**	**NA**	**143,000 - 4,723,000**	**Kagwade 1988**
Panulirus versicolor	**Palau, Western Caroline Islands**	**82 - 128**	**F= -1,169,909 + 185,438CL**	**467,552 - 1,189,324**	**MacDonald 1988**
Panulirus versicolor	**India**	**66.0 - 95.0**	**NA**	**170,212 - 733,752**	**Vijayakumaran et al. 2012**

Table 3. Analysis of Covariance (ANCOVA) summary values for female *P. argus* reproductive performance through individual-level reproduction measures: fecundity and reproductive output. P values in bold indicate statistically significant effects.

Response Variable: Fecundity					
		Sum Sq	Df	F value	Pr(>F)
	Embryo Stage	4.1718e+11	1	20.887	**1.48e-05**
	Carapace length (mm; covariate)	6.8756e+11	1	34.424	**6.593e-08**
	Embryo Stage x Carapace length (mm)	5.9720e+09	1	0.299	0.5858
	Residuals	1.8775e+12	94		
Response Variable: Embryo Mass					
		Sum Sq	Df	F value	Pr(>F)
	Embryo Stage	0.37420	1	12.0824	**0.0007722**
	Female Body Mass	0.64636	1	20.8702	**1.491e-05**
	Embryo Stage x Female Body Mass	0.04830	1	1.5596	0.2148315
	Residuals	2.88958	94		

Table 4. Prevalence of *C. conanobrieni* on *P. argus* across all of *P. argus'* localities sampled in the Caribbean.

Sampling Year	**Sampling Locality**	**Prevalence**	**Number of *P. argus* sampled**	**Citation**
2015	Middle Florida Keys, USA	7.40%	68	Baeza et al. 2016
2018	Middle Florida Keys, USA	93.90%	114	Simpson 2018
2017 - 2019	Saint Kitts and Nevis	87%	31	Atherley et al. 2020a
2019	Magdalena, Colombia	87.78%	90	Berben et al. 2023
2022	Middle Florida Keys, USA	100%	98	This study
2023	Middle, Upper, Lower Florida Keys, USA	100%	127	This study

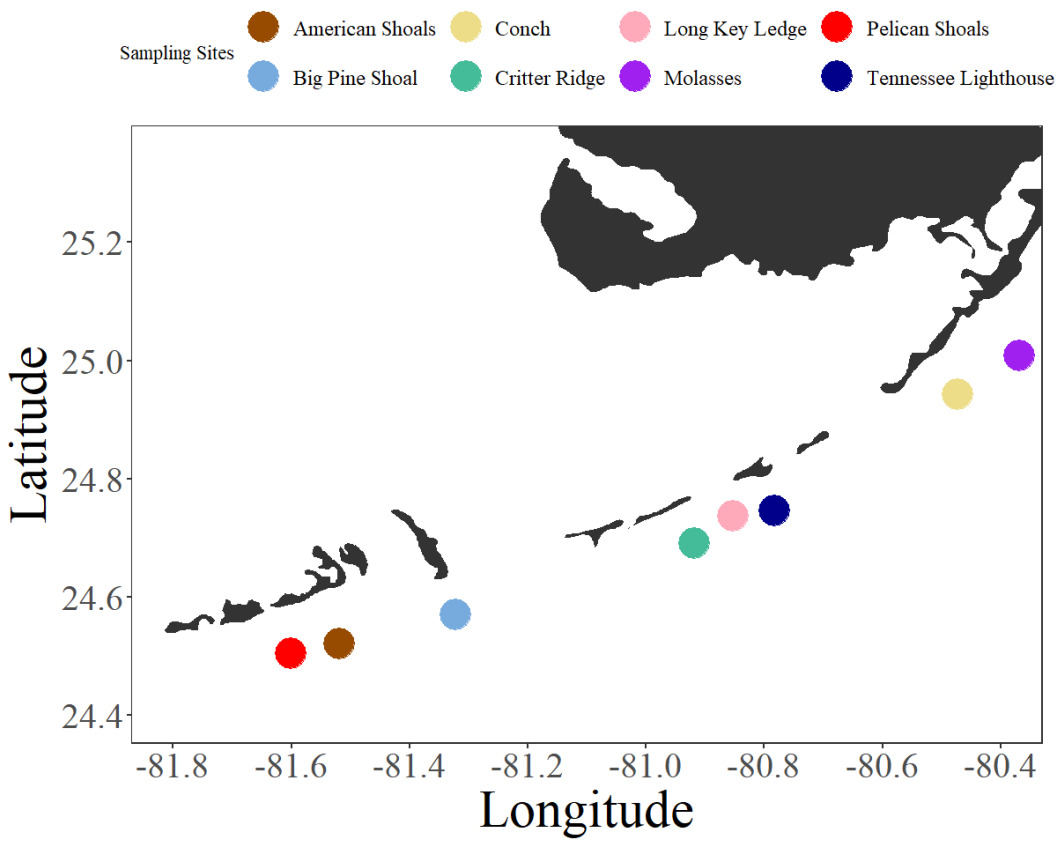


Figure 1. Sampling sites of egg-bearing *Panulirus argus* across the Florida Keys National Marine Sanctuary, USA across two years: 2022 and 2023. To account for potential geographic differences in the prevalence of *C. conanobrieni*, we collected egg-bearing *P. argus* from three geographic regions along the Florida Keys reef tract, the Upper, Middle, and Lower Florida Keys. In the Middle Keys, female *P. argus* were collected from Tennessee Reef (N 24°44.726', W 080°46.915'), Critter Ridge Reef (N 24° 43.95', W 80° 54.726'), and Long Key Ledge (N 24° 44.2667', W 80° 51.1333'). In the Upper Keys, females were collected from Molasses Reef outside of the sanctuary preservation area (SPA) (N 25°00.528', W 080°22.590') and Conch Reef outside of the SPA (N 24°56.541', W 080°28.525'). In the Lower Keys, females were collected from American Shoals (N 24° 31.400', W 081° 31.266'), Big Pine Shoals (N 24°34.222', W 081°19.300'), and Pelican Shoals (N 24°29.986', W081°37.883').

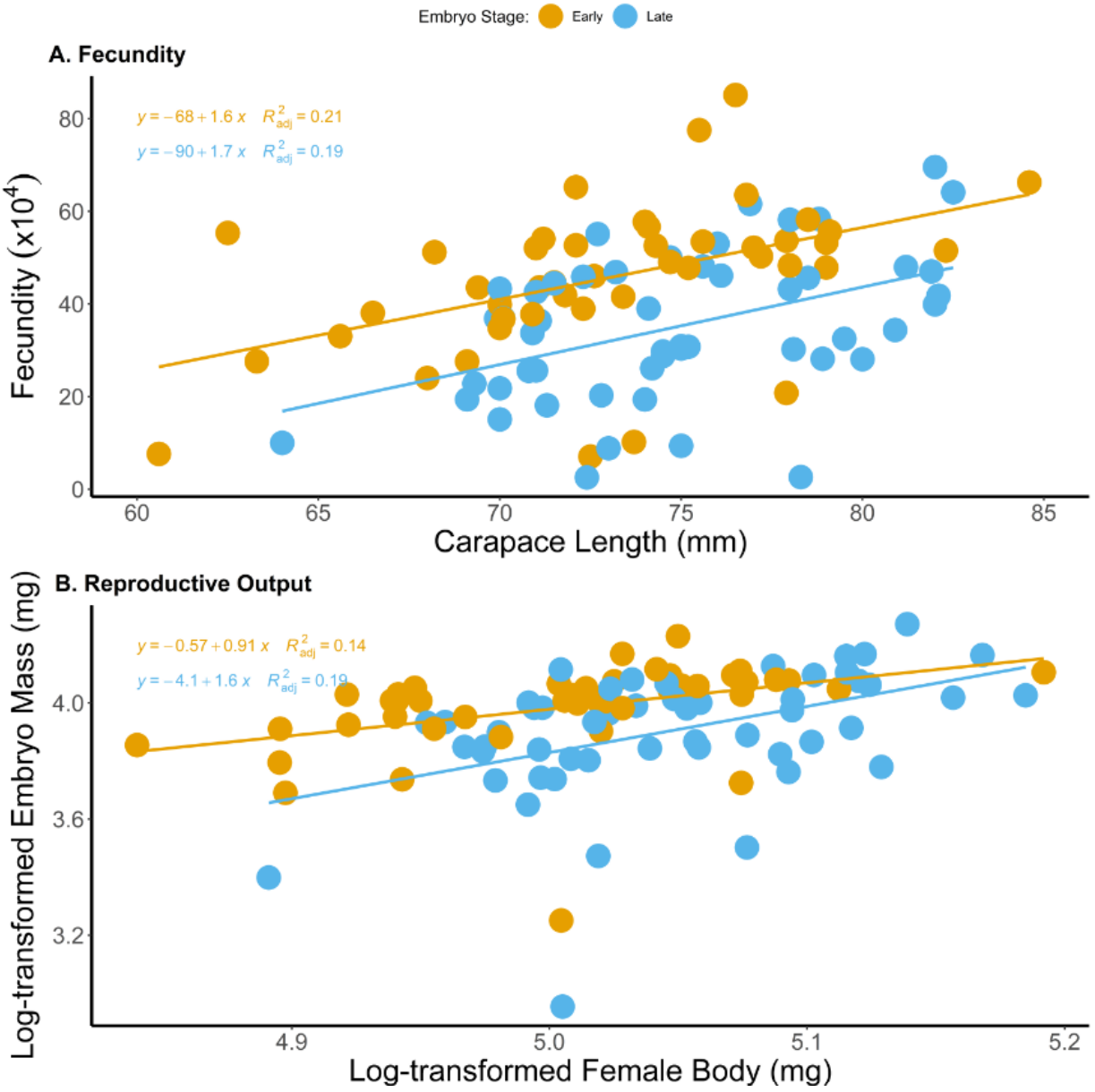


Figure 2. The reproductive performance of egg-bearing *P. argus*. A) Fecundity estimates are higher for egg-bearing lobsters carrying early-stage embryos compared to those carrying late-stage embryos. Fecundity is displayed as fecundity times 10^5 embryos and carapace length (CL) is measured in mm. B) Reproductive output (RO) is higher for egg-bearing lobsters carrying early-stage embryos compared to those carrying late-stage embryos. Female body mass and embryo mass were both measured in mg and both were log-transformed. Early-stage embryos are depicted in orange, while late-stage embryos are depicted in blue. Regression equations and R^2 values are displayed for each reproductive parameter.

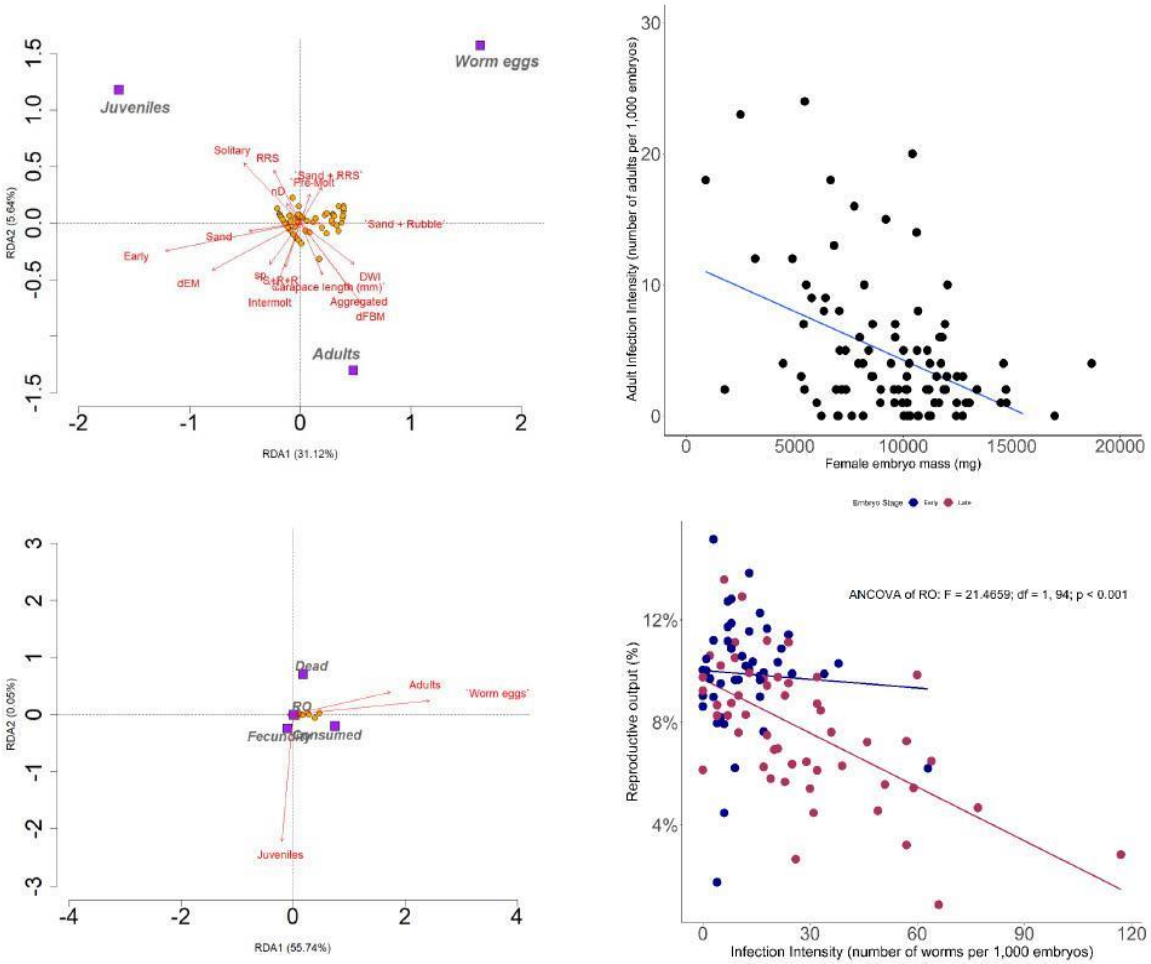


Figure 3. A) Triplot of my first redundancy analysis exploring the relationship between host (*P. argus*) traits and *C. conanobrieni* abundance in the host's brood mass. Triplot showing first and second ordination axes. Scaling two is focused on the effects of the host traits (explanatory variables) on *C. conanobrieni* abundance. Orange circles = female *P. argus* individuals. Purple squares = *C. conanobrieni* abundances (adults, juveniles, egg masses). Red arrows = female *P. argus* traits. B) RDA 1 relationship between female brood weight (in mg) to adult *C. conanobrieni* infestation intensity. Adult *C. conanobrieni* infestation intensity is represented as the number of adults per 1,000 embryos. C) Triplot of my second redundancy analysis exploring how *C. conanobrieni* abundance in the host's brood mass explains female *P. argus* reproductive performance. Triplot showing first and second ordination axes. Scaling two is focused on the effects of *C. conanobrieni* abundances at different life stages on female *P. argus* reproductive performance measures. Orange circles = female *P. argus* individuals. Purple squares = Female *P. argus* reproductive performance measures (fecundity, reproductive output, dead and consumed lobster embryos). Red arrows = *C. conanobrieni* abundances (juveniles, adults, egg masses). D) RDA 2 relationship between female *P. argus* reproductive output (%) and *C. conanobrieni* infestation intensity (number of worms (all life stages) per 1,000 embryos. Colors represent female *P. argus* embryo stage. Reproductive output (RO) ANCOVA results are displayed on the graph.

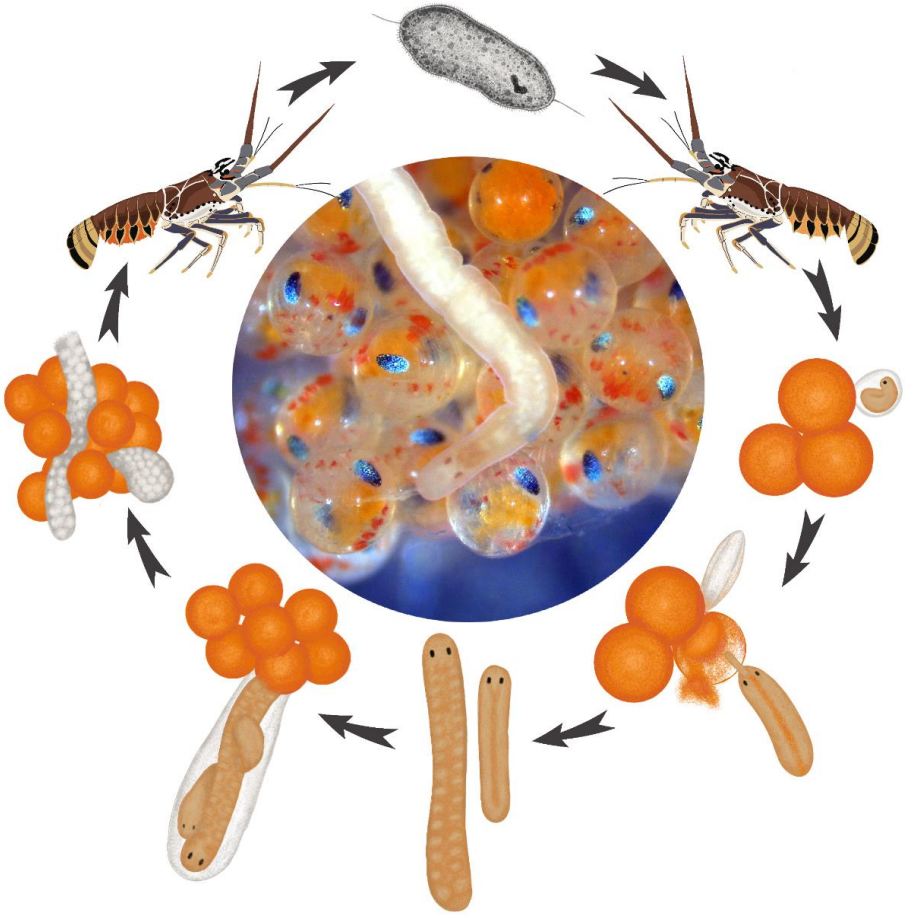
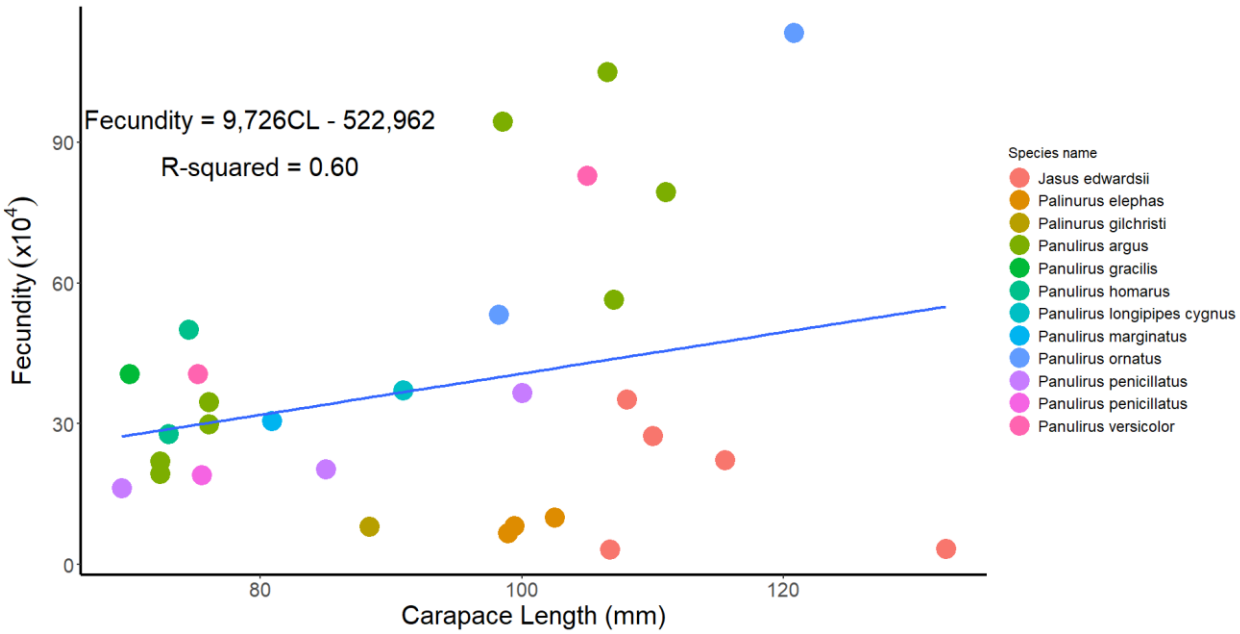


Figure 4. Proposed model for the life cycle of *Carcinonemertes conanobrieni* on egg-bearing female *Panulirus argus*. The life cycle of *C. conanobrieni* begins with a hoplonemertean planuliform larva that hatches from nemertean egg strands deposited among host embryos. These larvae are ovoid in shape and settle among the brood mass of early-stage female *P. argus*. Upon settlement, larvae metamorphose into juveniles that can adhere onto individual *P. argus* eggs in a mucus sheath. At this stage, *C. conanobrieni* actively feeds on embryos by manipulating its stylet and everted proboscis to extract yolk using suctorial feeding. This occurs until the embryo is fully consumed, leaving behind an empty oval-shaped capsule devoid of yolk. Feeding *C. conanobrieni* juveniles grow into adults that exhibit minimal sexual dimorphism and once these adults reach sexual maturity, seen as the visualization of gametes through the body wall, male and female worms begin mating within the embryo mass of their host. During mating, female and male worms enter a shared mucus sheath and once the male leaves, the female stays behind to lay eggs within her mucus sheath. Female *C. conanobrieni* egg mass organization can range from spherical to spiral strings encased in mucus and wrapped around clumps of late-stage *P. argus* embryos. Once female host embryos are close to hatching, *C. conanobrieni* embryos typically hatch in synchrony with the host's embryo mass and enter an unknown, swimming larval period within the water column. Illustration by Heather Bruck.



Supplementary Figure 1. Fecundity-carapace length relationships of all spiny lobster species with data available from the literature. I eliminated two data points, *P. polyphagus and Jasus verrauxi* as they were outliers and skewed the slope line and equation. Each species' fecundity estimate corresponds to a single point (or multiple points if multiple reports were available). Computed average fecundity-carapace length for all spiny lobster species was calculated, including the R^2 of the equation. Fecundity is displayed as fecundity times 10^4 embryos. Carapace length (CL) was measured in mm for all species.

Chapter III:

The effect of the nemertean egg predator *Carcinonemertes conanobrieni* on Caribbean spiny lobster *Panulirus argus* active parental care

Abstract

Caribbean spiny lobsters *Panulirus argus* are host to a nemertean egg predator, *Carcinonemertes conanobrieni* that causes significant embryo mortality, and consequently lowered reproductive performance in the brood masses of females. Female *P. argus* engage in behaviors, including pleopod fanning and specialized embryo cleaning, likely to supplement oxygen across the entire brood mass and prevent excessive epizoic growth on embryos. These active parental care behaviors may secondarily be adapted by this host to aid in behavioral mitigation strategies that limit the negative effects of this egg-eating micro-egg predator. In this study, I investigated the effect of *C. conanobrieni* infestation on female *P. argus* active parental care behaviors. Specifically, I tested whether females use grooming bouts as an explicit mode of mitigation in light of *C. conanobrieni* infestation across early and/or late-stages of embryo development and secondarily if these hosts can sense and react to the presence of these nemerteans in their brood. If *C. conanobrieni* is present, it is expected that egg-bearing females will groom their embryos more frequently to limit the negative effects associated with infestation, including increased embryo mortality, thereby boosting the number of healthy embryos that survive during incubation. All female *P. argus* collected during this study were infested with *C. conanobrieni* (100% prevalence). Laboratory behavioral assays revealed that, against expectations, there were no differences in the frequency of grooming, using the 4th and 5th pereopods, between different *C. conanobrieni* infestation loads (low versus high). However, egg-bearing *P. argus* with early-stage embryos significantly increased pleopod fanning bouts while sustaining high *C. conanobrieni* infestation loads, which could indicate they are sensing juvenile and adult nemertean feeding activity in their brood masses. A manipulative experiment did not provide evidence that *P. argus* is capable of chemically sensing *C. conanobrieni*, as females did not significantly alter the frequency in which they groomed when directly exposed to nemerteans. The use of mitigation behaviors by female *P. argus*, such as grooming, to limit *C. conanobrieni's* negative effects on their reproductive success and offspring survival requires further investigation.

Keywords: behavioral mitigation; avoidance; active parental care

Introduction

The interface between the evolution of host behavior in response to pathogens and parasites has been thoroughly studied across a variety of host-pathogen systems (Ezenwa et al. 2016; Trumbo 2012; Arundell et al. 2014; Stott and Poulin 1996; Tewksbury et al. 2002; Field and Brace 2004; Gross 2005; Balshine 2012; Mooring et al. 2004). Parasite infection can lead to negative consequences, such as decreased metabolic energy, nutritional condition, and reproductive performance, which could ultimately lead to outbreaks and increased mortality of hosts or their offspring (Hart 1990; Berben et al. 2023; Fernàndez et al. 2020; Herrera-Salvatierra et al. 2019). The constant exposure to parasites has resulted in numerous host behavioral adaptations that reduce the direct and indirect costs associated with parasitism (behavioral adaptation in *Dendroica petechia* infected by the brood parasite *Molothrus ater*- Tewksbury et al. 2002; behavioral adaptation in *Cheilodipterus quinquelineatus* infected by Cymothoid isopod parasites *Anilocra apogonae* - Östlund-Nilsson et al. 2005; behavioral adaptation in *Hylobius abietis* infected by nematode parasites *Steinernema carpocapsae* and *Heterorhabditis downesi* - Ennis et al. 2010; behavioral adaptation in *Bufo americanus* tadpoles infected by cercariae life stage of the trematode parasite *Echinostoma trivolvis*- Rohr et al. 2009). A preliminary defense mechanism against endo- or ecto-parasites and pathogens is avoidance of infected conspecifics, pathogens, and/or habitats with high infection risk to restrict initial contact with pathogens (Hart 1990; Behringer et al. 2018). The evolution of avoidance behaviors only occurs if the net benefits to host fitness outweighs the associated costs of performing them, which may include, among others, increased vulnerability to predators and time spent foraging (Hart 1992; Poulin 1995; Råberg et al. 2009.

If avoidance is unsuccessful and parasites become established on their host, mitigation behaviors are expected to help mediate the negative effects of parasite infection and, thereafter, be maintained by selection for removing parasites when detected (Poulin 1995; Bauer 2013; Moore 2013; Vale et al. 2018; Gibson and Amoroso 2020). Behaviors aimed at inhibiting parasite survival and reproduction, post-contact, can help hosts directly improve their fitness, reproductive success, and boost the survival of their offspring to the next generation (Hart 1990; Moore 2013). Examples of parasite mitigation strategies employed by hosts include but are not limited to migration, selective foraging, altered activity levels, lethargy, or imitating the behaviors of sick individuals (Barber et al. 2000; Vale et al. 2018; Hart 1990; Moore 2013; Daly and Johnson 2011). Due to the complexity of marine environments, visual cues are coupled with chemical and mechanosensory cues to provide hosts with an optimal strategy to limit infection when facing increasing parasite infestations (Behringer et al. 2018). While some host species require physical contact with parasites before any parasite-mitigation strategy is triggered, others only need to sense chemical cues emanating from the parasite itself (Taylor et al. 2004; Poulin 1995; Bauer 2013; Daly and Johnson 2011; Rohr et al. 2009). Discriminating the chemical cues associated with parasites, before or after sensing them, becomes essential for species in environments where the agents that cause disease rapidly spread from host to host. Explicitly, the connectivity of marine systems, via ocean currents and open recruitment, permits increased parasite spread and has provided numerous case studies in disease dynamics, especially for species in social groups that interact with conspecifics regularly, as seen for the large marine decapod crustacean, the Caribbean spiny lobster (Behringer et al. 2018).

The Caribbean spiny lobster *Panulirus argus* has a distribution that spans the Greater Caribbean (Cruz et al. 2015) and a complex life cycle with an oceanic planktonic larvae,

swimming puerulus larvae, and early benthic juvenile that transitions from being a small, solitary juvenile to a gregarious, larger juvenile and subsequent adult (Holthuis 1991; Booth and Phillips 1994; Herrnkind et al. 1994; Maxwell et al. 2009; Phillips et al. 2013; Baeza et al. 2016; Goldstein et al. 2008; Butler and Herrnkind 2000; Briones-Fourzán and Lozano-Álvarez 2001; Cox et al. 2008). This developmental shift from being an asocial juvenile to social adult also coincides with heightened exposure to parasites and pathogens through increased conspecific interactions (Butler et al. 2015; Behringer et al. 2006). In response to disease, behavioral avoidance has been described as a successful strategy used by adult *P. argus* to limit infection spread. Specifically, *P. argus* exhibits the ability to detect chemical signals transmitted by conspecifics to engage in viral pathogen detection in social settings and, concomitantly, limit their risk of becoming infected in the first place (Candia-Zulbarán et al. 2015; Behringer et al. 2006). Healthy juvenile and adult *P. argus* use chemically-mediated cues present in hemolymph and urine to successfully avoid spiny lobsters infected with *Panulirus argus* virus 1 (PaV1), a species-specific virus that causes lethargy and in some cases, mortality (Candia-Zulbarán et al. 2015; Behringer et al. 2018; Ross et al. 2019; Butler et al. 2015; Behringer et al. 2006; Behringer et al. 2010). *Panulirus argus*' ability to avoid viral pathogens is well documented, but no studies have explored female spiny lobster behavioral mitigation in light of heightened infestation by an emerging threat, the nemertean egg predator *Carcinonemertes conanobrieni.*

The nemertean worm *Carcinonemertes conanobrieni* is found exclusively in the brood masses of female *P. argus* throughout their Caribbean distribution, where most of the life stages of this egg predator's life cycle (juvenile, adult, egg masses) have been observed throughout spiny lobster embryo development (Baeza et al. 2016; Gonzalez-Cueto and Quiroga 2018; Atherley et al. 2020a; Berben et al. 2023; Simpson 2018). These nemerteans contribute to

significant lobster embryo mortality, which ultimately causes lowered female reproductive performance, namely, decreased fecundity and reproductive output (Simpson 2018; Atherley et al. 2020a; Berben et al. 2023). Egg-bearing *P. argus* exhibit active parental care behaviors, which includes grooming and pleopod fanning, while carrying large, compact masses with thousands of embryos underneath their abdomen for 3-4 weeks (Baeza et al. 2016; Maxwell et al. 2009). Female lobsters utilize active parental care likely to provision oxygen across the entire embryo mass throughout incubation (Baeza et al. 2016). In response to egg-mass infestation, they may also utilize these specialized behaviors to mitigate egg predators that feed and reproduce in their embryo mass. Grooming may be adapted by hosts to remove parasites, especially those with free-living larvae stages that infect host eggs, go through their life cycle in the brood mass, and contribute to increased embryo mortality at juvenile and adult stages through their feeding behavior such as nemertean egg predators (Baeza et al. 2016; Daly and Johnson 2011; MacIntosh et al. 2012). Juvenile and adult *P. argus* use behavioral avoidance to limit viral pathogen spread; therefore I expect the adoption of mitigation strategies to occur when *P. argus* are infested by *C. conanobrieni* given the significant damage these nemerteans cause to host broods (Berben et al. 2023).

The overarching aim of this study was to explore the effect of *C. conanobrieni* infestation on egg-bearing *P. argus* active parental care. To accomplish this, (1) I performed laboratory behavioral assays on lobsters infested with *C. conanobrieni* and recorded the frequency and duration of active parental care behaviors during the night, (2) I explored if these hosts have an explicit mode of behavioral mitigation in light of infestations across different stages of embryo development, and (3) I tested whether females sense and react to the presence of *C. conanobrieni* in their brood mass. Specifically, I predicted that there would be an increase in the time spent

grooming, to limit the negative effects of egg predator intensities, in heavily infested females compared to low *C. conanobrieni* infested females as a direct result of increased egg predator loads. Other active parental care behaviors, precisely pleopod fanning, standing, abdominal flapping, and abdominal extension, performed by female *P. argus* were expected to be performed at a constant level between high and low *C. conanobrieni* loads and not change given egg predator presence due to their importance for embryo oxygen requirements throughout incubation. Active parental care is expected to significantly decrease the negative effects of *C. conanobrieni* on female reproductive performance, therefore optimizing the number of healthy embryos. Without behavioral intervention by this host, *C. conanobrieni* infestation will proliferate in female brood masses, leading to heightened embryo mortality and lowered host fitness, as suggested in Chapter 2.

Methods

Field collections of egg-bearing female *Panulirus argus* across the Florida Keys National Marine Sanctuary and determining *Carcinonemertes conanobrieni* prevalence

All female Caribbean spiny lobsters collected for this study were made under the Special Activity License (SAL-21-1674B-SR) granted by the Florida Fish and Wildlife Conservation Commission. Over the course of June 7th 2023 - July 30th 2023, egg-bearing *P. argus* were collected using SCUBA, with the aid of a tickle stick and hand net, across the Florida Keys National Marine Sanctuary reef tract (USA). Lobsters were caught across three localities in the Florida Keys (Upper, Middle, Lower). In the Middle Florida Keys, female *P. argus* were all collected from Tennessee Reef (N 24°44.726', W 080°46.915') and attempts to collect females from other reefs in the Middle Florida Keys did not yield results. In the Upper Florida Keys,

female *P. argus* for this study were found at Molasses Reef outside of the sanctuary preservation area (SPA) (N 25°00.528', W 080°22.590') and Conch Reef outside of the SPA (N 24°56.541', W 080°28.525'). From the Lower Florida Keys, females were collected from American Shoals (N 24° 31.400', W 081° 31.266'), Big Pine Shoals (N 24°34.222', W 081°19.300'), and Pelican Shoals (N 24°29.986', W081°37.883') (see Figure 1).

Upon capture, egg-bearing females were placed in individual, aerated coolers or buckets to account for any *C. conanobrieni* transference between female brood masses. Female *P. argus* were transported, alive, to the Keys Marine Laboratory (Layton, Florida, USA). In the laboratory, I recorded carapace length (CL) using a dial caliper (precision = 0.1 mm), female weight, to the nearest 0.01 mg, using an analytical balance (OHAUS Discovery, DV215CD, USA), and any notable carapace features (e.g. barnacles on the mouthparts, missing appendages, scratched spermatophore). To determine the female's molt status at the time of collection (pre-molt, intermolt, post-molt), I snipped a pleopod to observe its developmental features (changes in setae) under a Leica S8AP0 stereomicroscope in accordance with Lyle & MacDonald (1983). Lastly, female *P. argus* embryo stage of development at time of collection was recorded by looking at a subset of embryos from each female's brood mass under a Leica S8AP0 stereomicroscope or Wild M5-97874 dissecting scope. In this study, I used parameters of the four embryo stages defined in Baeza et al. (2016): Stage I are defined as embryos with uniformly distributed yolks; Stage II embryos are those that possess cell differentiation and yolk clusters; Stage III embryos begin to show chromatophore features and well-developed eyes; Stage IV embryos contain all features including thoracic appendages, eyes, and chromatophores. For simplicity during data collection and analyses, I defined all females carrying stage I and II embryos as early-stage and all females carrying stage III and IV into late-stage.

To assess *C. conanobrieni* prevalence and infestation intensity in my collected female *P. argus*, I used fine-tip forceps to haphazardly select two, approximately ~1,000 embryo subsamples from each female to look at under a Leica S8AP0 stereomicroscope or Wild M5-97874 dissecting scope. Specifically, within each subsample I made counts of the number of live *P. argus* embryos, dead embryos (misshapen, neon orange, or cloudy in coloration), and consumed embryos (empty egg capsules devoid of yolk, oval-shaped and clear in coloration) (Baeza et al. 2016). Additionally, I counted the number of *C. conanobrieni* juveniles, adults, and egg masses found within the two subsamples, separately. Juvenile *C. conanobrieni* are usually found encysted onto individual *P. argus* embryos, while adults can be found free-roaming the embryo mass or in a mucus sheath. This egg predator's embryo masses can vary between spherical or spiral in shape, encased in mucus, and laid among *P. argus* embryos. In my previous chapter, I reported 100% prevalence of *C. conanobrieni* on this host across the Florida Keys reef tract. Given that I did not collect any female lobsters that were free of *C. conanobrieni* infestation, for this study's behavioral comparisons I decided to categorize *P. argus* that were heavily infested with *C. conanobrieni* compared to lobsters with low intensity nemertean loads. Heavy nemertean worm densities were categorized as females with ≥10 worms and/or with ≥10 dead or consumed embryos in the two, ~1000 embryo subsamples. I chose to classify females as low density *C. conanobrieni*/absent because some *P. argus* had <10 or no live worms present in the subsamples that I counted, however did show limited evidence of *C. conanobrieni*, including few consumed embryos, in the subsamples, in the female's embryo mass and/or pleopods.

Experiment One: *Panulirus argus* active parental care behavioral assays

I formally tested whether *C. conanobrieni* infestation level has an effect on egg-bearing *P. argus* active parental care behaviors. Separation for the behavioral assays included classifying female *P. argus* based on their embryo stage of development (early or late) and egg predator prevalence (*C. conanobrieni* present (high density) or absent (low density)). All behavioral assays were performed inside a wet lab at the Keys Marine Laboratory (Layton, FL, USA) to minimize any human disruption during recordings. Egg-bearing *P. argus* were placed in individual, transparent 30-gallon acrylic aquaria with a bifold flow through system of seawater collected from the bayside near the Keys Marine Laboratory that was filtered (down to 40 microns), sterilized (UV), and kept at 27-28 degrees Celsius (ambient reef temperatures) with constant aeration. Infrared Illuminators (A14 No Hot Spot Wide Angle Infrared Light, 90° flood light spread, Tendelux) were placed on the side and top of the aquaria to allow for recording female lobster behavior at night. I cut sheets of black, waterproof and UV resistant fabric and taped them to the back of the aquarium to limit the amount of reflectance seen during recordings. Individual tanks were also set up with a HDR Time-Lapse camera, TLC200 Pro (Brinno) with a 6-15 mm lens (M12Lenses, model M12PT-0615A) on a tripod placed directly in front of the aquarium with a time-lapse video set at ≥ 5 frames s^{-1}. The lab set-up allowed for four lobsters to be filmed at a time, per night (Figure 2). Lobsters were given 2-4 hours of acclimation time to the tanks before recordings began.

Active parental care behaviors in female *P. argus* have been previously classified as pereopod probing, standing, abdominal extension, pleopod fanning, and abdominal fanning (Baeza et al. 2016). From herein, grooming will be exclusively described as probing using the 4th

pereopod, while pleopod fanning and abdominal fanning will be described as behaviors occurring after standing and abdominal extension. Active parental care behaviors were distinguished based on whether they were considered events or states. Behaviors considered 'events' performed by the host were pleopod fanning and 4th and 5th pereopod probing (grooming) because they are performed in discrete bouts, usually lasting <1 minute (Baeza et al. 2016). Conversely, behavioral 'states' such as standing and abdominal extension were performed by the lobsters over longer durations of time, generally >1 minute (Baeza et al. 2016, Bateson and Martin 2021). Recordings began immediately after sunset (~7PM) and ended at sunrise (~7AM). I only recorded at night based on previous research indicating that female *P. argus* are more active with parental care during the evening (Baeza et al. 2016). I recorded and identified behaviors that happened in the aquarium by haphazardly selecting two time blocks, each 1 hour long (both at night). Time blocks were selected, at random using a random number generator, based on the camera's placement and if the female lobster is facing the camera laterally, diagonally, or frontally to ensure the behavior is fully recorded (Baeza et al. 2016).

Experiment One: Statistical analysis of female *P. argus* active parental care behavior

For the statistical analyses evaluating active parental care behaviors of egg-bearing *P. argus*, I compared the time or frequency that a female was grooming, pleopod fanning, standing, or performing abdominal extensions for each time block using a mixed nested ANOVA. Significant differences in the time or frequency spent performing each active parental care behavior during two randomly selected time blocks (1 hr each) were analyzed while examining the effect of *C. conanobrieni* prevalence (*C. conanobrieni* present (high density) versus absent (low density)) and female *P. argus* embryo stage of development (early versus late) on brood care. Female *P. argus* embryo stage of development, *C. conanobrieni* infestation status, and hour block were

treated as fixed effects, while female identity (ID) was treated as a random effect in my mixed nested ANOVA. To ensure adequate representation across all factors for experiment one, I collected and recorded 46 egg-bearing female *P. argus* (11 females with early stage embryos and 35 females with late-stage embryos; 16 females classified as *C. conanobrieni* present (high density) and 30 females classified as *C. conanobrieni* absent (low density)).

The frequency of occurrence of each behavioral event (n° event bouts h^{-1}) for each time block and the proportion of time that females spent in each behavioral state (% time h^{-1}) for each time block was documented. To avoid any bias while viewing lobster footage, this study used a double-blind video footage procedure that ensured that behaviors were scored without knowing the lobster's embryo stage or *C. conanobrieni* infestation status. I employed this procedure because previous research has shown that *P. argus* parental care behaviors change drastically to meet the differing needs of embryos as they develop from early to late stages (Baeza et al. 2016). Video footage analysis was conducted by an undergraduate creative inquiry student. I tested that my data met statistical assumptions of the independence of dependent variables, normality of residuals, and homogeneity of variances by looking at residual plots, performing a Shapiro-Wilk normality test on my model residuals, and testing for homogeneity of variance using a Levene's test. In order for the data to meet the assumptions of normality, I log-transformed grooming, standing, and abdominal extension while fanning was square-root transformed. All statistical analyses were conducted in RStudio v. 2022.2.0.443 (R Development Core Team 2021). I used packages lme4 (Douglas Bates et al. 2015) and lmerTest (Kuznetsova et al. 2017) for my mixed nested ANOVA analyses in R. Nested ANOVA models were analyzed using Satterthwaite's approximation method, which is useful in unbalanced designs and/or small

sample sizes for calculating denominator degrees of freedom and *p* values (Kuznetsova et al. 2017).

I predicted that there will be an increase in the time spent grooming, using the 4th and 5th pereopods, in heavily infested females compared to low *C. conanobrieni* infestation intensity females as a direct result of increased nemertean egg predator loads. Females are expected to intensify grooming efforts to limit the negative effects of egg predator infestation, e.g., decreases in reproductive performance (Chapter 2) caused by increasing nemertean loads. I hypothesized that heavy *C. conanobrieni* loads would not have an effect on other active parental behaviors, like pleopod fanning and abdominal extension, because a previous study on this species (Baeza et al. 2016) revealed that these behaviors significantly increase in frequency and duration as embryos develop, with later stages requiring more oxygen before hatching.

Experiment Two: Host sensing egg predator experiment

For my second experiment, I investigated whether egg-bearing *P. argus* are able to sense *C. conanobrieni* loads on their brood mass and if the presence of *C. conanobrieni* triggers female grooming behavior, specifically probing using the 4^{th} or 5^{th} pereopod. To accomplish this, I collected and assessed egg-bearing lobsters using the same protocols as experiment one. However, after recording all relevant body condition parameters and identifying *C. conanobrieni* prevalence, I glued a 3 mm x 5mm C-Flex plastic tube onto the females near the sixth abdominal segment using cyanoacrylate and dental wax. Lobsters were then placed, individually, in their 30-gallon acrylic aquaria filled with aerated seawater (same water conditions/treatments as experiment one). Once the tube was glued onto the lobster's abdomen, the lobsters were given four hours of acclimation time before the recording of behaviors took place. The other end of the

tube was connected to an analytical peristaltic liquid dosing pump (INTLLAB). To address my experimental factor, egg predator prevalence, there were two separate water treatments directed into the females' brood mass through the peristaltic pump. One consisted of ½ L of seawater stocked with 100 whole, live *C. conanobrieni* worms collected off of individuals with the egg predator present in their embryo mass and the other contained ½ L of seawater with no *C. conanobrieni* worms present. The different experimental seawater treatments were pumped into the female abdomen at a flow rate of 30 ml min^{-1} from the ½ L containers filled with seawater treated according to the experimental design for a total of one hour, chosen at random. For experiment two, my replicates included 12 female *P. argus* (6 with early-stage embryos and 6 with late-stage embryos) being exposed to the ½ L of seawater stocked with 100 whole, live *C. conanobrieni* and 19 lobsters (7 with early-stage embryos and 12 with late-stage embryos) being exposed to ½ L of seawater with no *C. conanobrieni* worms present (N total = 31). During the one hour of female lobsters being exposed to the different water treatments, my analysis of grooming behavior focused on females probing their egg masses with their 4^{th} and 5^{th} pereopod. I recorded the frequency of occurrence of grooming (n° event bouts h^{-1}) for each time block.

Experiment Two: Statistical analyses

I performed a mixed nested ANOVA to test for differences in grooming behavior during the time that females were exposed to my two different water treatments (seawater with 100 live *C. conanobrieni* and seawater with no *C. conanobrieni*). Grooming frequencies during the one hour females were exposed to either treatment (n° event bouts h^{-1}) were square-root transformed to meet the assumptions of normality. My three fixed effects included female *P. argus* egg predator prevalence (*C. conanobrieni* present (high density) or absent (low density)), dosing pump

treatment (with or without *C. conanobrieni*), and *P. argus* embryo stage (early or late). I treated each individual lobster as a random effect. When executing my second experiment, I did not collect any *P. argus* carrying early-stage embryos with high *C. conanobrieni* loads to use as a replicate in my dosing pump treatment without 100 live *C. conanobrieni*. Therefore, when conducting my nested ANOVA I had a rank deficiency error, which occurred due to the missing interaction between these three fixed effects (early-stage *P. argus* embryos x high *C. conanobrieni* infestation load x treatment without 100 *C. conanobrieni*). I anticipated that this test would show that egg-bearing *P. argus* increase grooming as a direct result of sensing nemertean worm presence in the ½ L seawater treatments with 100 *C. conanobrieni*, indicating that females are actively sensing these egg predators and trying to minimize infestation loads in their brood mass to optimize the number of healthy embryos.

Results

Experiment One: *Panulirus argus* active parental care behavioral assays

In egg-bearing *P. argus*, I recorded four active parental care behaviors including pleopod fanning, grooming (or probing using the 4th and/or 5th pereopod), standing, and abdominal extension. Females would raise their body from a crouched position by skidding all of their pereopods at the bottom of the aquarium to gain enough traction to stand up. After standing up, female lobsters would engage in bouts of pleopod fanning by thrusting all of their pleopods, synchronously, back and forth over their embryo mass. This would be performed either stationary or while walking the entire area of the tank. While standing and performing pleopod fanning bouts, females often fully extended their entire abdomen to line up flush with the rest of their body or keep their abdomen furled up to fully encapsulate the embryos underneath their

abdomen. Once settled in one spot again, egg-bearing *P. argus* would groom the embryos in their clutch while crouched at the bottom of the aquarium. Grooming typically started by the female probing with her pereopods at the posterior end of the clutch, near the uropods. As the female groomed, the 4th and 5th pereopods worked their way up towards the anterior end of her clutch near the cephalothorax. Once the female groomed through all of her eggs, she started back again at the posterior end and continued repeated cycles of grooming until the cessation of this behavior. Sometimes grooming events would extend beyond the embryo mass and females would use their pereopods to groom other external structures such as their antennae, cephalothorax, and near their eyes.

During my investigation into *P. argus* active parental care, I observed two egg-bearing females (both carrying late-stage embryos) releasing their brood in the aquarium. Prior to releasing embryos, the females would engage in frequent bouts of pleopod fanning. The females would be standing near the edges of one side of the tank, arch their telson inwards, and then fully extend their abdomen to begin rapidly expelling all of her embryos through quick and forceful bursts of tail and pleopod fanning. Visually, the releasing of embryos appeared as a cloudy mass and this would occur until the female had little, if any, embryos left underneath her abdomen. I did not observe any swimming *P. argus* larvae in the tanks post-hatching events. Additionally, when quantifying *C. conanobrieni* infestation among embryo subsamples of one of my late-stage female *P. argus*, I saw hatched nemerteans engaging in fast paced, erratic swimming behavior among clumps of *P. argus* embryos (before the female released her own embryos).

The time span, per hour, that egg-bearing female *P. argus* performed abdominal extension varied significantly between females with early versus late-stage embryos (mixed

nested ANOVA, embryo stage effect: F = 7.9109; df = 1, 47; p = 0.007149; Table 1). Female *P. argus* carrying late-stage embryos engaged in abdominal extension behaviors for longer durations than those carrying early-stage embryos (Figure 3). In turn, female *C. conanobrieni* infestation status (low versus high) did not have a statistically significant effect on abdominal extension duration (mixed nested ANOVA, infestation level effect: F = 0.0064 ; df = 1,47; p = 0.936605). The interaction between *P. argus* embryo stage and *C. conanobrieni* infestation status was not statistically significant (mixed nested ANOVA, infestation level x embryo stage effect: F = 0.0281; df = 1, 47; p = 0.867537).

There were no statistically significant differences in standing durations explained by the embryo stage of egg-bearing *P. argus* (mixed nested ANOVA, embryo stage: F = 1.9401; df = 1, 47; p = 0.1702; Table 2) or the females' infestation status (mixed nested ANOVA, infestation level: F = 0.1553; df = 1,47; p = 0.6953). Additionally, the interaction between *P. argus* embryo stage and *C. conanobrieni* infestation status was not significant (mixed nested ANOVA, infestation level x embryo stage effect: F = 0.0185; df = 1,47; p = 0.8924; Figure 3).

There were statistically significant differences in pleopod fanning bouts between females with different *C. conanobrieni* infestation loads (mixed nested ANOVA, *C. conanobrieni* infestation effect: F = 4.7403; df = 1, 47; p = 0.03452; Table 3). Embryo stage did not affect the frequency of pleopod fanning in egg-bearing females (mixed nested ANOVA, embryo stage effect: F = 0.5128; df = 1, 47; p = 0.47748). Importantly, I found a statistically significant interaction between *C. conanobrieni* infestation status and *P. argus* embryo stage (mixed nested ANOVA, infestation level x embryo stage: F = 5.4053; df = 1,47; p = 0.02445). Specifically, female *P. argus* with high *C. conanobrieni* infestation loads and carrying early-stage eggs

significantly increased the frequency of pleopod fanning bouts, while there was no significant change in pleopod fanning between the different nemertean loads for females carrying late-stage embryos (Figure 4).

When examining grooming behavior, *P. argus* embryo stage did not have a statistically significant effect on the frequency of grooming bouts (mixed nested ANOVA, embryo stage effect: F = 0.5596 ; df = 1, 47 ; p = 0.4582; Table 4). Grooming bouts were not significantly different between females with low versus high *C. conanobrieni* infestation loads (mixed nested ANOVA, infestation level effect: F = 1.0383; df = 1,47; p = 0. 3134) and the interaction between *P. argus* embryo stage and *C. conanobrieni* infestation status was also not significant (mixed nested ANOVA: embryo stage x infestation level interaction: F = 0.0051; df = 1, 47; p = 0.9433; Figure 4).

Experiment Two: Host sensing egg predator experiment

A mixed nested ANOVA found no statistically significant effect of my different water pumping treatments on grooming frequency (mixed nested ANOVA, treatment effect: F = 0.4013 , df = 1, 24, p = 0.5324; Table 5). Furthermore, there were no detectable differences in grooming behavior during experimental manipulation for females carrying early versus late-stage embryos (mixed nested ANOVA, embryo stage effect: F = 0.0042 , df = 1, 24, p = 0.9488) or between females with high versus low *C. conanobrieni* infestation loads (mixed nested ANOVA, infestation load effect: F = 0.0398 , df = 1, 24, p = 0.8436). Neither interaction between embryo stage or *C. conanobrieni* infestation load with water dosing treatments were significant (Figure 5). A three-way interaction between the three fixed effects could not be calculated due to a missing row of data. Specifically, I had a combination of treatments without replicates (early

stage embryo, high *C. conanobrieni* infestation load, treatment without *C. conanobrieni*), leading to rank deficiency in the nested ANOVA model.

Discussion

Active parental care in Panulirus argus

I observed a suite of behaviors that comprise active parental care in egg-bearing *P. argus*: pleopod fanning, abdominal extension, standing, and 4th/5th pereopod probing (herein grooming)**.** In agreement with Baeza et al. (2016), I observed this species perform the aforementioned series of behaviors in a specific sequence. Females began the pattern of brood care by transitioning from a crouched position to standing up in the tank. This was immediately followed by the flexing of their abdomen until it was fully extended, as this enabled them to initiate rapid bursts of pleopod fanning or grooming bouts using their 4th and 5th pereopods. My study goes into a new detailed description about the recurring cycles of grooming that a given female would perform during a grooming bout that previous studies on this species (Baeza et al. 2016) did not characterize. Explicitly, a grooming cycle typically started with pereopod grooming at the posterior end of the clutch, near the uropods. As the female groomed the embryos, her pereopods worked up towards the anterior end of the clutch near the cephalothorax. Once the female had groomed through all of her eggs, she started back again at the posterior end of her brood mass and this cycle happened repeatedly, up to 10 times. The longest grooming bout consisted of 12 cycles that transpired over 53 minutes.

The presence of active parental care in brooding females has been described for a variety of aquatic invertebrates, from small brooding amphipods (Arundell et al. 2014) and caridean shrimps (Baeza et al. 2019) to larger crustaceans including brachyuran crabs (Fernàndez and

Baeza 2002; Fernàndez et al. 2000; Naylor et al. 1999; Ruiz-Tagle et al. 2002), clawed lobsters (Eriksson et al. 2006), and spiny lobsters (Baeza et al. 2016). Similar to *P. argus*, brachyuran crabs, including female *Cancer setosus*, perform brood care in a specific sequence: 1) females begin the sequence by standing with their abdomen fully extended, 2) females rhythmically flap their abdomen, 3) females probe their embryo mass using their pereopods or chela, and 4) females beat their maxillipeds (Baeza and Fernàndez 2002; Baeza et al. 2016). Baeza et al. (2019) observed an almost identical active parental care series to *C. setosus* and *P. argus* in egg-bearing peppermint shrimp *Lysmata boggessi*, however they described probing and grooming as two distinct behaviors. Like *P. argus*, these crustaceans have behaviors that appear to be tailored exclusively to embryo oxygen demands (pleopod fanning) or for detecting brood mass conditions (grooming/probing) (Baeza et al. 2016, 2019; Baeza and Fernàndez 2002). These similarities highlight that crustaceans have adapted their behavior to combat the constraints they face in their changing marine environments to facilitate embryo survival to hatching. These include, but are not limited to, lowered viability from fouling since bacteria and fungi are leading causes of embryo mortality, and oxygen constraints that if not met result in reduced growth rates, small sizes at hatching, or death (Baeza and Fernàndez 2002; Fernàndez et al. 2020; Strathmann and Strathmann 1995). Additionally, all of these egg-bearing crustaceans incur significant increases in metabolic costs that accompany the execution of these active care behaviors, which ultimately may reduce female condition for future reproduction, increase their visibility to predation, among others (Fernàndez et al. 2020).

The effect of **Carcinonemertes conanobrieni** *on female* **Panulirus argus** *active parental care*

There are additional parallels and dissimilarities between other studies that investigated active parental care in brooding crustaceans and this species. The biggest difference in my study is the investigation of active parental care behaviors as an explicit mode of mitigation in light of *C. conanobrieni* infestations across female *P. argus* brood masses. I investigated the effect of the nemertean egg predator, *Carcinonemertes conanobrieni*, on egg-bearing *Panulirus argus* active parental care, specifically focusing on those behaviors that may limit the number of *C. conanobrieni* among host embryos. I anticipated to observe an increase in grooming frequencies for female *P. argus* with heavy *C. conanobrieni* loads. This behavior was expected to discriminate and remove *C. conanobrieni* to limit the negative effects of nemertean infestation (e.g., egg mortality), and thus increase host overall reproductive performance. In disagreement with my expectations, I found that female *P. argus* with high *C. conanobrieni* loads spent the same effort grooming embryos in their clutch as females with low *C. conanobrieni* intensities. Although females, regardless of embryo stage, engaged in regular grooming bouts as previous studies on this species (Baeza et al. 2016) and other species have shown (*P. violaceus*-Förster and Baeza 2001), this behavior did not increase in frequency with *C. conanobrieni* prevalence as I originally hypothesized. These results in combination with the lack of any significant differences in grooming frequency between my experimental treatments (with and without *C. conanobrieni* being pumped into female embryo masses; see subsection below) highlight that this behavior's assumed function in nemertean mitigation requires additional studies that focus solely on grooming in females with *C. conanobrieni* present.

Counter to grooming, during my first experiment I found a significant interaction between *C. conanobrieni* infestation status and *P. argus* embryo stage. Specifically, female *P. argus* with high *C. conanobrieni* infestation loads and carrying early-stage eggs significantly increased the frequency of pleopod fanning bouts, while there was no significant change in pleopod fanning between low or high nemertean loads for females carrying late-stage embryos. This is inconsistent with previous observations in this same species by Baeza et al. (2016) and additionally disagrees with previous studies that have quantified active parental care behaviors in other decapod crustaceans, including egg-bearing *L. boggessi* (Baeza et al. 2019), *C. setosus* (Baeza and Fernández 2002), and *P. violaceus* (Förster and Baeza 2001). These studies have shown that oxygen demands increase dramatically from early to late-stages of embryo development, which is frequently accompanied by elevated frequencies in pleopod fanning (see Baeza et al. 2016; Baeza and Fernández 2002; Arundell et al. 2014; Fernández and Brante 2003). While the primary function of pleopod fanning in brooding females has largely been hypothesized to be for oxygen provision, studies have demonstrated that female American lobsters, *Homarus americanus*, use this behavior to generate water currents that may transmit chemical cues through the formation of odor plumes (Atema 1986). Indeed, increased fanning by female *P. argus* carrying early-stage embryos may create odor plumes that help with transmitting chemical cues of the different life stages of *C. conanobrieni* present (feeding juveniles and adults) during this period of lobster embryo development. However in my study, female *P. argus* failed to display the ability to successfully sense the cues associated with *C. conanobrieni* infestation (see below).

Female **P. argus** *behavioral response to sensing chemical cues of* **C. conanobrieni**

In my second experiment, I tested the ability of female *P. argus* to sense the chemical cues of *C. conanobrieni* in their embryo masses and anticipated that egg-bearing lobsters would respond by increasing grooming. In disagreement with my prediction, there were no significant changes in host grooming frequencies during exposure to treatments of seawater with *C. conanobrieni*. Previous work in early-benthic juveniles has highlighted *P. argus*' ability to sense cues related to conspecifics infected with PaV1 (Candia-Zulbarán et al. 2015; Behringer et al. 2006; Behringer and Butler 2010; Anderson and Behringer 2013). Although I failed to observe egg-bearing females sense *C. conanobrieni* in this study, this species has demonstrated its attraction to chemical cues. *Panulirus argus* pueruli can sense chemically-mediated signals from their environment, specifically a mixture of metabolites produced by red algae *Laurencia* spp. and healthy or PaV1 infected conspecifics, when choosing where to settle (Ambrosio and Baeza 2023; Baeza et al. 2018). Additionally, adult egg-bearing *P. argus* are able to sense pheromones released by their embryos as signals for larval release, which in turn induces heavy bouts of pleopod fanning that facilitate the breaking of embryo cases so females can release their clutch (Ziegler and Forward Jr. 2007a; Ziegler and Forward Jr. 2007b). This species clearly exhibits the ability to sense chemical cues, including those linked to disease, but the lack of response to *C. conanobrieni* cues by female *P. argus* may suggest that this nemertean is successful at going undetected by this host in the brood mass, which requires further experimental manipulation of this host-egg predator system. My results also suggest that my *C. conanobrieni* treatment was not strong enough to elicit a grooming response by *P. argus*, which I discuss in my limitations section (see below).

Similarly to *P. argus*, pheromones released by embryos have been sensed by other crustaceans to signal timing of hatching and are cues met by rapid, spontaneous increases in pleopod fanning behaviors. For instance, when egg-bearing shrimps, *Palaemon serratus* and *Crangon crangon*, were exposed to low concentrations of the pheromone, bradykinin, associated with larval release events, female shrimps in experimental treatments doubled their pleopod ventilation frequencies (Reinsel et al. 2014). These shrimps had the same response, i.e. increased embryo ventilation, when also exposed to their own crushed eggs (Reinsel et al. 2014). Indeed, brooding *C. setosus* responded to the cues of water with late-stage embryos with more pleopod fanning than water with just early-stage embryos (Tankersley et al. 2002). These case studies demonstrate that hatching eggs release a chemical that acts as a signal to the female that embryos in her clutch are ready to be dislodged from the abdomen and released into the environment (Ziegler and Forward Jr. 2007a). Late-stage embryos have the highest concentration of these pheromones, however my results showed an increase in pleopod fanning frequencies in lobsters carrying early-stage embryos nowhere near ready to hatch. This could indicate that *C. conanobrieni* are altering the normal cues between embryos and females. Indeed, feeding *C. conanobrieni* adults and juveniles use their proboscis to puncture a hole in *P. argus* embryo cases, often releasing crushed egg extract during the process (Simpson 2018). These cues may induce female *P. argus* pleopod fanning prematurely. Future experimental studies should address whether *P. argus* are indirectly sensing *C. conanobrieni* from their feeding behavior releasing lobster egg extract.

Whether *P. argus* are able to sense *C. conanobrieni* once they establish in the brood mass remains to be investigated, however my results, in agreement with other crustaceans infected with brood parasites/predators (Arundell et al. 2014; Stone and Moore 2014), may indicate that

active parental care behaviors are solely for tending to their embryos developmental needs before hatching. For example, egg-bearing amphipods, *Gammarus* sp., infected by the acanthocephalan *Leptorhynchoides thecatus* in the host's haemocoel did not change their brood care behaviors when exposed to chemical cues of infected conspecifics, leading to the conclusion that these amphipods are unable to sense stimuli during parasite infestation (Stone and Moore 2014). Brooding *Crangonyx pseudogracilis* showed no alteration in active brood care behaviors when infected by microsporidian parasites, nor did microsporidian infection in the host's ovaries have an effect on juvenile release in *G. duebeni* (Arundell et al. 2014; Ironside et al. 2003). While there are cases of brooding crustaceans modifying their parental care during parasite infections (for example, *Synalpheus elizabethae* parasitized by branchial and abdominal isopods groomed less than their healthy counterparts; see McGrew and Hultgren 2011), I conclude that *P. argus* are more similar to those who do not change active parental care for the purpose of infestation minimization and instead use them for ensuring adequate conditions for embryos to survive and grow in.

As an alternative, I argue that the increases in pleopod fanning recorded in female *P. argus* with early-stage embryos could be their behavioral response to sensing chemical cues emanating from live, feeding juvenile and adult *C. conanobrieni*. The chemical cues of juveniles and adults could be stronger than the chemical cues of nemertean egg masses, which comprised the majority of infestation in females carrying late-stage embryos. Evidence from my study (Chapter 2) and prior studies investigating *C. conanobrieni* infestation on female *P. argus* (Berben et al. 2023) found mostly juvenile and adult *C. conanobrieni*, either encysted or free-roaming the brood mass, in lobsters carrying early-stage embryos. Contradictorily, females carrying late-stage clutches only had either *C. conanobrieni* egg masses intertwined within the

lobsters embryos or adults in mucus sheaths on the host's pleopods. The feeding activity of *C. conanobrieni* juveniles and adults, especially through the releasing of lobster egg extract explained above, could contribute to *P. argus'* sensing these stages.

Evolutionary rationale of active parental care and parasite mitigation behaviors

Predation, shifting environmental conditions, resource competition, and body fouling are just some of the ecological pressures that have led to the adaptation of parental care behaviors that provide protection and alleviate the severity of these problems on brooding crustaceans (Trumbo 2012). While parental care is somewhat rare in marine environments compared to its terrestrial counterpart, where brooding extends to the juvenile stages, behaviors that clean brooding embryos and supplement oxygen are performed until larvae hatch (Fernández et al. 2020). Oxygen limitations across the compacted embryo masses of female decapods has resulted in the evolution of behaviors, like pleopod fanning, to ventilate young throughout the entire duration of incubation (Fernández et al. 2002). Indeed, pleopod fanning is considered the most essential of brood care behaviors because it is performed at all stages of embryo development in both small and large crustaceans (Dick et al. 1998; Fernández et al. 2000; Fernández et al. 2020). The fouling of appendages and structures associated with important respiratory, sensory, and locomotory functions by microbial and ectoparasites has led to the diversification of these structures through morphological additions, such as setae, that allow hosts to perform grooming bouts, thereby limiting excessive growth (Bauer 2013). Brood infestations, from nemertean egg predators such as those in the genus *Carcinonemertes*, can lead to the modification of grooming behaviors even further for mediating the associated negative effects they cause (Trumbo 2012). In these cases, grooming removes dead embryos that lower reproductive performance through

clutch contamination (Fernández et al. 2020). Both of these behaviors are favored for their positive effect on increasing embryo viability (Fernández et al. 2020), however, when additionally performing these behaviors to eliminate egg predator infestation, the host incurs other costs which could explain the barriers to *P. argus* using parental care in my study.

In response to parasitism, brooding females must balance adopting behaviors that aid them in mitigating parasites at the expense of current or future reproductive success (Arundell et al. 2014). Namely, resources invested into parasite defense cannot be secondarily used by these hosts for reproduction but may indirectly benefit fitness given the demand to perform these behaviors to ensure host survival during infection (Bryan-Walker et al. 2007; Sheldon and Verhulst 1996). The costs of parasite infection therefore accumulate with those associated with the execution of parental care, such as direct mechanical losses during ventilation, higher oxygen requirements with increased temperature, among others (Fernández et al. 2020). Despite this, modifying host behavior is considered the most cost-effective strategy when compared to non-behavioral immunity responses since the use of the immune system to fight infection may impose greater costs than the direct negative effects of the parasite itself (Bryan-Walker et al. 2007; Moret and Schmid-Hempel 2000). Indeed, the initiation and subsequent activation of the immune system from parasite infection can reduce host reproductive performance through reductions in fertility for snails, *Biomphalaria glabrata*, who mount resistance to the parasite *Schistosoma mansoni* (Webster and Woolhouse 1999) and delayed maturation as seen in the *Biomphalaria glabrata* (host)/*Echi nostoma caproni* (parasite) system (Langand et al. 1998). Therefore, selection for investment into an immune response would only be favorable for hosts frequently infected by parasites with loads that cause severe negative impacts (Bryan-Walker et al. 2007).

While host behaviors can successfully limit infection at times, it is unlikely that these strategies eradicate parasitism completely, and in some examples these behaviors can achieve the opposite effect by positively contributing to the success and survival of the parasite on its host (Hart 1990). Indeed, the coevolution of host behaviors and parasites can shape virulence patterns leading to a constant feedback loop between host behaviors and parasite traits that result in plastic behavioral responses (Ashby and Boots 2015; Ezenwa et al. 2016). In some cases of trait coevolution, parasites are able to engage in egg mimicry to evade being groomed by their host. For example, the parasitic rhizocephalan *Lernaeodiscus porcellanae* infects the embryo masses of the porcelain crab *Petrolisthes cabrilloi* and by morphologically appearing the same as its hosts eggs, this parasite is groomed and ventilated while it lives and mates on its host's clutch, which subsequently aids in rhizocephalan larvae release (Ritchie and Høeg 1981). *Carcinonemertes conanobrieni*, including their egg masses laid between *P. argus* late-stage embryos, may similarly blend in with this host's embryo clutch and provide an explanation for the lack of increased grooming behaviors seen in my collected females. I also observed the erratic swimming behavior of hatched nemerteans under the microscope after late-stage females rapidly fanned their pleopods to release their embryos, therein presenting further evidence that supports the role of parental care behaviors in the release of parasite/egg predator larvae in addition to their own. This nemerteans ability to live and reproduce successfully in *P. argus* embryo masses and consequently benefit from host active parental care behaviors reflects the intricate link between this host-egg predator system and the behavioral mitigation that this host adopts, or fails to, in response to growing infestation.

Limitations of this study and future research considerations

A significant limitation to my study on the effect of *C. conanobrieni* on female *P. argus* active parental care behavior was my inability to compare infested females to spiny lobsters completely free of nemertean worms. I attempted to find brooding females that lacked any evidence of *C. conanobrieni* infestation by sampling across the entire Florida Keys reef tract, at multiple coral reef sites, but my efforts were unsuccessful. I acknowledge that the ability to discern significant differences in behavior for egg-bearing *P. argus* with *C. conanobrieni* was restricted due to my sampling of only infested individuals, however my distinction between low versus high *C. conanobrieni* loads (see Methods) tried to minimize this limitation. Problems with sample size for my second experiment, especially the inability to collect early-stage females for all treatment combinations, resulted in inconclusive results for some of the interactions and limited the model's statistical power. Other problems with the performed experiments that may have contributed to the negative results is the stress of being confined to small tanks in the laboratory and the associated consequences of manipulating the hosts in my second experiment (via gluing a tube to their abdomen). While I gave lobsters in experiment two an additional few hours of acclimation time compared to my first experiment, a majority of their time with the tube glued on their abdomen was spent trying to remove the tube via flapping, grooming, or scaling the tank walls. Additionally, having to re-attach tubes to females who lost them because of improper seal or physical removal by the lobster resulted in increased interaction between me and animals in the behavioral assays. To limit this interaction time, future studies should test various materials out and compare their feasibility e.g. staying attached to the lobsters embryo mass throughout the dosing pump experimental assay.

For future replication of my second experiment, I recommend a factorial experimental design incorporating other cues like crushed *P. argus* embryos. Namely, embryo condition could consist of water treatments with healthy embryos, as examples highlighted earlier showed that hosts can sense embryo cues, and with consumed *P. argus* embryos. Supplementing treatments with consumed or partially consumed *P. argus* embryos, due to the feeding behavior of *C. conanobrieni*, could give us insight into the ability of females to indirectly sense *C. conanobrieni* infestation since my study concluded they do not directly sense these egg predators in their embryo mass. A future prediction could be that *P. argus* indirectly senses these egg predators through detecting consumed or partially consumed embryos given their previously described sloppy feeding behavior that sometimes leaves yolk inside the eaten lobster egg capsule. By looking at both the egg predators and their feeding as a cue to the behavioral response performed by *P. argus*, future studies can learn if the egg predator or signals inadvertently produced by it lead to host sensing and thereafter, mitigation behavior.

In my second experiment testing *P. argus*' ability to chemically sense *C. conanobrieni*, 100 live nemerteans were placed in treatments of seawater. The lack of any significant changes in *P. argus* grooming bouts while testing *P. argus* sensitivity to nemertean chemical cues suggests that the *C. conanobrieni* treatment was not strong enough. Other experiments have tested host sensitivity to chemical cues and observed a behavioral response, albeit not related to parasite/egg predator infestation (De Vries et al. 1991; Tankersley et al. 2002; Derby and Sorensen 2008). Indeed, in a similar chemical sensing experiment performed on the crab *Neopanope sayi* by De Vries et al. (1991), each water treatment was concentrated with an estimated 20-50 larvae/mL which resulted in females significantly increasing their abdomen pumping behaviors. Additionally, prior to testing female *C. sapidus'* response to larval chemical

cues, Tankersley et al. (2002) homogenized and diluted crushed eggs (both early and late-stage) before filtering the water through a 100-μm filter. During this experiment, female *C. sapidus* showed increased responsiveness when exposed to the homogenized egg treatments (Tankersley et al. 2002). In comparison to these examples, I used seawater that was filtered to 40 microns as my stock solution prior to the addition, or not, of *C. conanobrieni*, however I did not manipulate live *C. conanobrieni* before placing them into the containers with seawater. In agreement with the conclusions drawn in Baeza et al. (2018) when *P. argus* pueruli failed to sense chemical cues, differences in treatment preparations can significantly alter study results. Future research should test different compositions of chemical cues (e.g. increasing the number of *C. conanobrieni* per ml seawater) to assess the minimum concentration of this egg predator that elicits a host behavioral response and try different preparations of the chemical cues similar to the examples highlighted above (e.g. that homogenized and diluted the chemical cues). Additionally, there is a severe absence of studies investigating brooding decapod crustacean sensitivity to chemical cues of parasites/egg predators for direct comparison to my study. Therefore, I recommend that future work aim to improve our understanding of the relationship between infestation and host perception of parasite/egg predator cues.

Conclusions

In this study, I investigated the effect of *C. conanobrieni* on female *P. argus* active parental care behaviors. Of the four behaviors I measured in my first experiment, pleopod fanning was the only one to change between low and high *C. conanobrieni* loads. Specifically, females carrying early-stage embryos with high *C. conanobrieni* loads significantly increased their frequency of pleopod fanning bouts, which is in stark contrast with the increase in oxygen provisioning seen

with embryo development (from early to late) in other brooding crustaceans. I argue that this difference could be explained by the presence of more actively roaming and feeding adult and juvenile *C. conanobrieni* in the embryo masses of early-stage females. For the host sensing experiment, my initial prediction identified grooming as the behavioral response brooding *P. argus* use when they sense *C. conanobrieni* in their clutch. However, my results disagree with that prediction as there were no significant changes in *P. argus* grooming bouts during direct exposure to treatments of seawater with live *C. conanobrieni* being pumped into their embryo masses. I suggest additional studies into *P. argus*' ability to sense *C. conanobrieni* through the use of indirect infestation cues, such as partially or fully consumed lobster embryos. While more research into female *P. argus* behavioral sensing and subsequent mitigation in light of *C. conanobrieni* infestation is warranted, this study explored how active parental care behaviors might be one strategy that may limit the negative effects of nemertean infestation on spiny lobster embryo clutches.

Tables and Figures

Table 1. Type III Mixed Nested Analysis of Variance Table (ANOVA) to test the effect of *C. conanobrieni* infestation status (high vs. low) and *P. argus* embryo stage (early vs. late) on abdominal extension duration, an active brood care behavior performed by egg-bearing *P. argus*. In a mixed nested ANOVA, I used Satterthwaite's method, which calculated the denominator degrees of freedom and *p* values given my uneven sampling design/small sample size. Statistical *P* values in bold indicate statistically significant effects. Abdominal extension durations were log-transformed prior to being fitted in the nested ANOVA.

Type III Analysis of Variance Table with Satterthwaite's method

Response Variable: Abdominal extension (log-transformed)							
		d.f.	$d.f._{denominator}$	Sum Sq	Mean Sq	F Value	Pr(>F)
	C. conanobrieni Infestation	1	47	0.000161	0.00161	0.0064	0.936605
	Embryo Stage	1	47	0.199213	0.199213	7.9109	**0.007149**
	Hour Block	1	50	0.005988	0.005988	0.2378	0.627939
	Infestation x Embryo Stage	1	47	0.000708	0.000708	0.0281	0.867537

Table 2. Type III Mixed Nested Analysis of Variance Table (ANOVA) to test the effect of *C. conanobrieni* infestation status (high vs. low) and *P. argus* embryo stage (early vs. late) on standing duration, an active brood care behavior performed by egg-bearing *P. argus*. In a mixed nested ANOVA, I used Satterthwaite's method, which calculated the denominator degrees of freedom and *p* values given my uneven sampling design/small sample size. Statistical *P* values in bold indicate statistically significant effects. Standing durations were log-transformed prior to being fitted in the nested ANOVA.

Type III Analysis of Variance Table with Satterthwaite's method

Response Variable: Standing (log-transformed)							
		d.f.	$d.f._{denominator}$	Sum Sq	Mean Sq	F Value	Pr(>F)
	C. conanobrieni Infestation	1	47	0.002960	0.002960	0.1553	0.6953
	Embryo Stage	1	47	0.036985	0.036985	1.9401	0.1702
	Hour Block	1	50	0.009618	0.009618	0.5045	0.4808
	Infestation x Embryo Stage	1	47	0.000352	0.000352	0.0185	0.8924

Table 3. Type III Mixed Nested Analysis of Variance Table (ANOVA) to test the effect of *C. conanobrieni* infestation status (high vs. low) and *P. argus* embryo stage (early vs. late) on the frequency of pleopod fanning, an active brood care behavior performed by egg-bearing *P. argus*. In a mixed nested ANOVA, I used Satterthwaite's method, which calculated the denominator degrees of freedom and *p* values given my uneven sampling design/small sample size. Statistical *P* values in bold indicate statistically significant effects. Pleopod fanning frequencies were square-root transformed prior to being fitted in the nested ANOVA.

Type III Analysis of Variance Table with Satterthwaite's method

Response Variable: Pleopod fanning (square-root transformed)							
		d.f.	$d.f._{denominator}$	Sum Sq	Mean Sq	F Value	Pr(>F)
	C. conanobrieni Infestation	1	47	1.94167	1.94167	4.7403	**0.03452**
	Embryo Stage	1	47	0.21004	0.21004	0.5128	0.47748
	Hour Block	1	50	0.16532	0.16532	0.4036	0.52813
	Infestation x Embryo Stage	1	47	2.21403	2.21403	5.4053	**0.02445**

Table 4. Type III Mixed Nested Analysis of Variance Table (ANOVA) to test the effect of *C. conanobrieni* infestation status (high vs. low) and *P. argus* embryo stage (early vs. late) on the frequency of grooming, an active brood care behavior performed by egg-bearing *P. argus*. In a mixed nested ANOVA, I used Satterthwaite's method, which calculated the denominator degrees of freedom and *p* values given my uneven sampling design/small sample size. Statistical *P* values in bold indicate statistically significant effects. Grooming frequencies were log-transformed prior to being fitted in the nested ANOVA.

Type III Analysis of Variance Table with Satterthwaite's method

Response Variable: Grooming (log-transformed)							
		d.f.	$d.f._{denominator}$	Sum Sq	Mean Sq	F Value	Pr(>F)
	C. conanobrieni Infestation	1	47	0.26145	0.26145	1.0383	0.3134
	Embryo Stage	1	47	0.14089	0.14089	0.5596	0.4582
	Hour Block	1	50	0.46759	0.46759	1.8570	0.1791
	Infestation x Embryo Stage	1	47	0.00129	0.00129	0.0051	0.9433

Table 5. Type III Mixed Analysis of Variance Table (ANOVA) to test the effect of *C. conanobrieni* infestation status (high vs. low), water dosing treatment (seawater with 100 *C. conanobrieni* or seawater without 100 *C. conanobrieni*), and *P. argus* embryo stage (early vs. late) on the frequency of grooming behaviors. In a mixed nested ANOVA, I used Satterthwaite's method, which calculated the denominator degrees of freedom and *p* values given my uneven sampling design/small sample size. Statistical *P* values in bold indicate statistically significant effects. Grooming bouts were square-root transformed prior to being fitted in the nested ANOVA. A three-way interaction between the three fixed effects (Embryo Stage x Treatment x *C. conanobrieni* infestation) was not assessed due to the rank deficiency of my model from the low number of replicates (see Supplementary Table 5).

Type III Analysis of Variance Table with Satterthwaite's method

Response Variable: Grooming bouts (square-root transformed)							
		d.f.	$d.f._{denominator}$	Sum Sq	Mean Sq	F Value	Pr(>F)
	C. conanobrieni Infestation	1	24	0.07784	0.07784	0.0398	0.8436
	Embryo Stage	1	24	0.00823	0.00823	0.0042	0.9488
	Treatment	1	24	0.78576	0.78576	0.4013	0.5324
	Embryo Stage x *C. conanobrieni* Infestation	1	24	0.49745	0.49745	0.2540	0.6188
	C. conanobrieni Infestation x Treatment	1	24	0.166650	0.16650	0.0850	0.7731
	Embryo Stage x Treatment	1	24	0.00009	0.00009	0.000	0.9945
	Embryo Stage x Treatment x *C. conanobrieni* Infestation	0	0	NA	NA	NA	NA

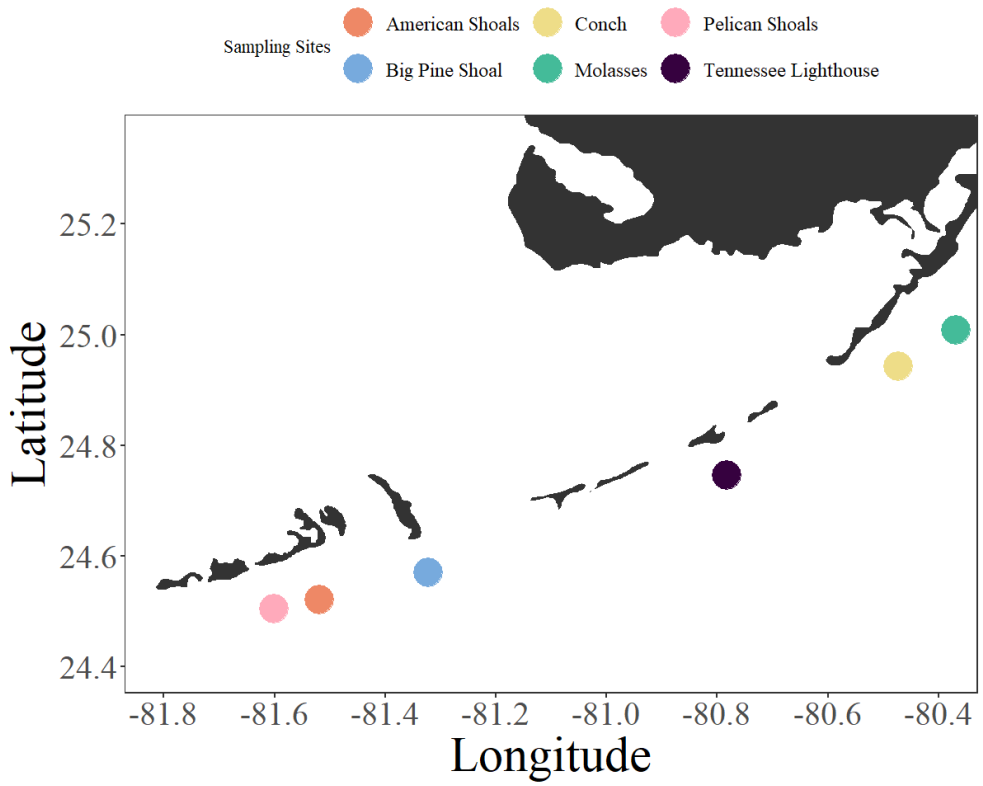


Figure 1. Geographic localities where collections of egg-bearing *Panulirus argus* were collected across the Florida Keys reef tract for laboratory behavioral assays. Over the course of two months, *P. argus* were collected using SCUBA, with the aid of a tickle stick and hand net. Lobsters were caught across three localities in the Florida Keys (Upper, Middle, Lower). Coral reef sites include Tennessee Reef in the Middle Florida Keys, Molasses Reef (outside of the sanctuary preservation area (SPA)) and Conch Reef (outside of the SPA) in the Upper Florida Keys, and American Shoals, Big Pine Shoals, and Pelican Shoals in the Lower Florida Keys.

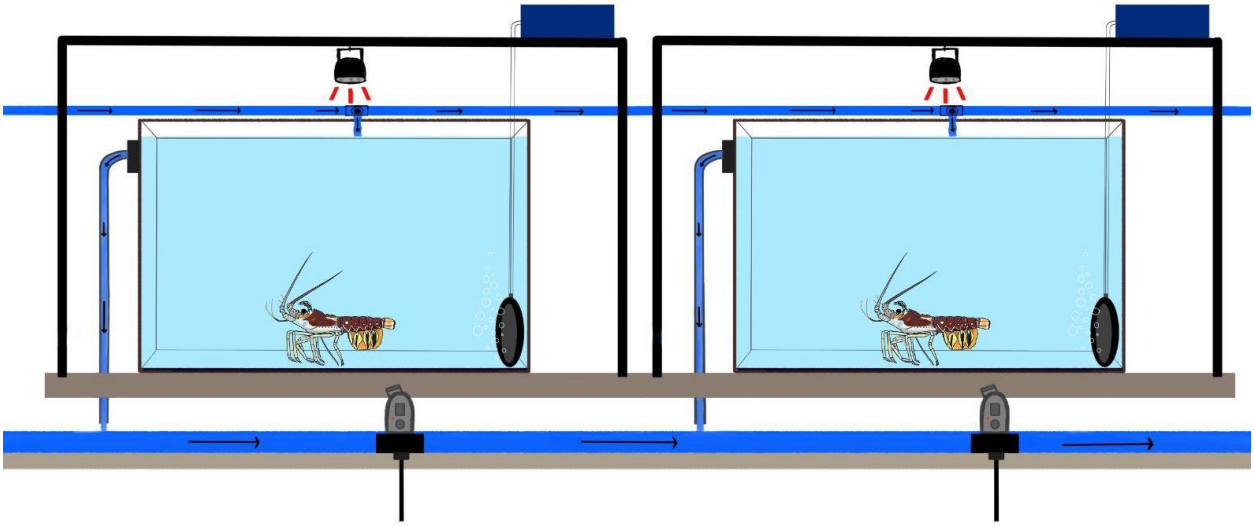


Figure 2. Experimental lab set-up of egg-bearing *P. argus* behavior recording experiments at Keys Marine Laboratory, Layton, Florida. Over the course of behavior recording (sunset to sunrise), I could record four lobsters at a time in a small laboratory room. Individual, 30-gallon acrylic aquaria with a bifold flow through system of seawater (filtered, sterilized, and kept at 27-28 degrees Celsius) were equipped with Infrared Illuminators on the sides and top of the aquaria to record lobster behavior at night, constant aeration, and black UV resistant fabric on the back of the tanks to limit reflectance. Directly positioned in front of each tank was a HDR Time-Lapse camera (TLC200 Pro, Brinno) on a tripod. See Methods for further specifications. Illustration by Rose Porter.

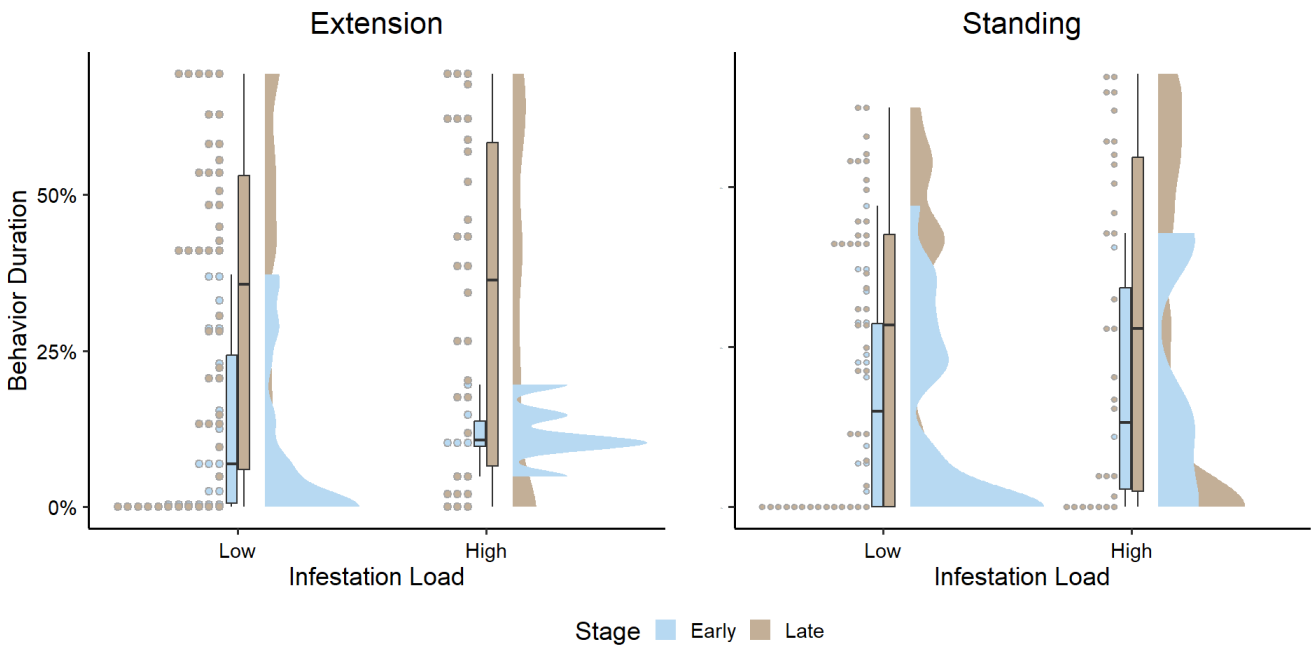


Figure 3. Raincloud plot displaying the effect of *P. argus* embryo stage and *C. conanobrieni* infestation status on active parental care behaviors classified as states (standing and abdominal extension). Raincloud plots include a halved violin plot, box plot, and the raw data as individual dots displaying the distribution of the dataset. Female lobster embryo stage (early versus late) was classified on each individual. *Carcinonemertes conanobrieni* infestation load (low versus high) is plotted on the x-axis, while behavior duration (% time per hour) is on the y-axis.

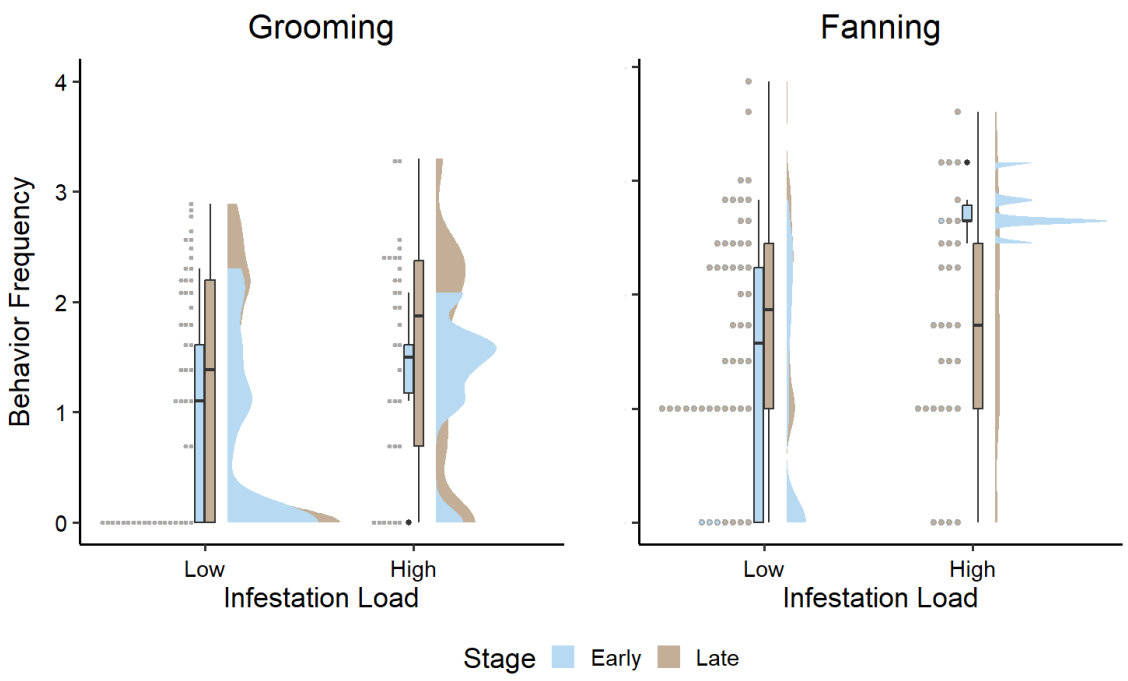


Figure 4. Raincloud plot displaying the effect of *P. argus* embryo stage and *C. conanobrieni* infestation status on active parental care behaviors classified as events (pleopod fanning and grooming). Raincloud plots include a halved violin plot, box plot, and the raw data as individual dots displaying the distribution of the dataset. Female lobster embryo stage (early versus late) was classified on each individual. *Carcinonemertes conanobrieni* infestation load (low versus high) is plotted on the x-axis, while behavior frequency (# of times performed per hour) is on the y-axis.

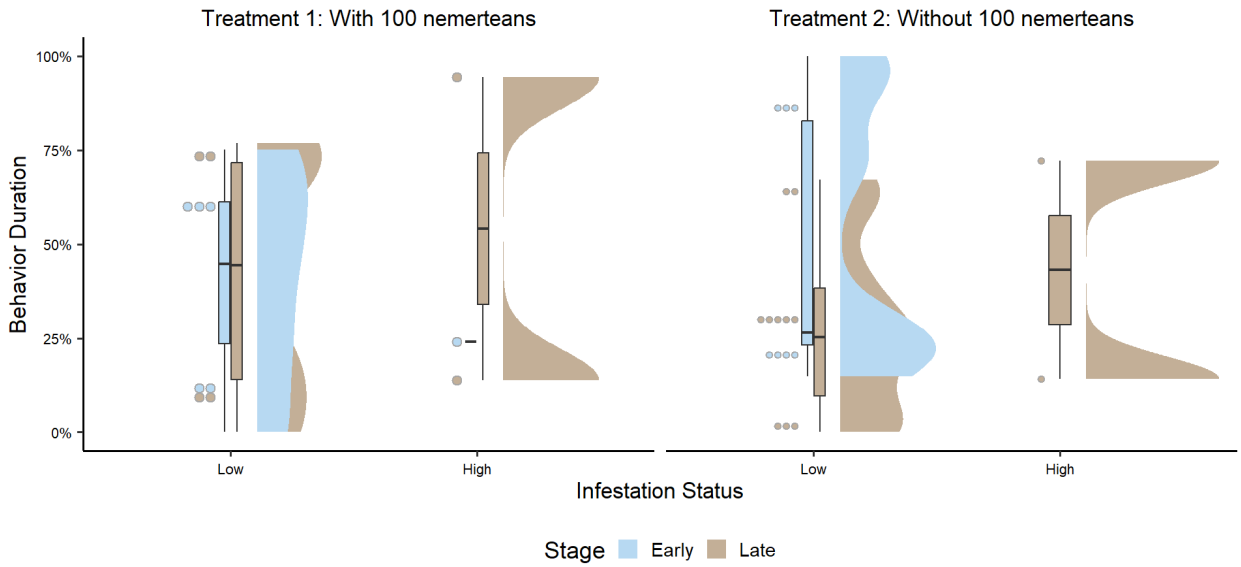


Figure 5. Raincloud plots comparing the grooming behavior of female *P. argus* experimentally exposed to water with and without 100 live *C. conanobrieni*. Raincloud plots include a halved violin plot, box plot, and the raw data as individual dots displaying the distribution of the dataset. Female lobster embryo stage (early versus late) was classified for each individual. *Carcinonemertes conanobrieni* infestation load (low versus high) is plotted on the x-axis, while behavior duration (% time performed per hour) is on the y-axis.

Supplementary Materials

Supplementary Table 1. Summary statistics of the mean ± standard deviation (SD) of the % time per hour (duration) standing behavior was performed. Behavior duration (Output) is separated by female *P. argus* embryo stage of development (early vs. late) and *C. conanobrieni* infestation status (high vs. low). Standing durations were log-transformed prior to conducting any analyses.

Infestation	Stage	variable	n	mean	sd
High	Early	logOutput_plus1	6	0.183	0.191
High	Late	logOutput_plus1	30	0.296	0.258
Low	Early	logOutput_plus1	20	0.164	0.155
Low	Late	logOutput_plus1	46	0.257	0.219

Supplementary Table 2. Summary statistics of the mean ± standard deviation (SD) of the % time per hour abdominal extension was performed. Behavior duration (Output) is separated by female *P. argus* embryo stage of development (early vs. late) and *C. conanobrieni* infestation status (high vs. low). Extension durations were log-transformed prior to conducting any analyses.

Infestation	Stage	variable	n	mean	sd
High	Early	logOutput_plus1	6	0.117	0.050
High	Late	logOutput_plus1	30	0.336	0.254
Low	Early	logOutput_plus1	20	0.123	0.136
Low	Late	logOutput_plus1	46	0.318	0.249

Supplementary Table 3. Summary statistics of the mean ± standard deviation (SD) of the number of pleopod fanning bouts (frequency) performed per hour (Output). Behavior frequency output is separated by female *P. argus* embryo stage of development (early vs. late) and *C. conanobrieni* infestation status (high vs. low). Fanning bouts were square-root transformed prior to conducting analyses.

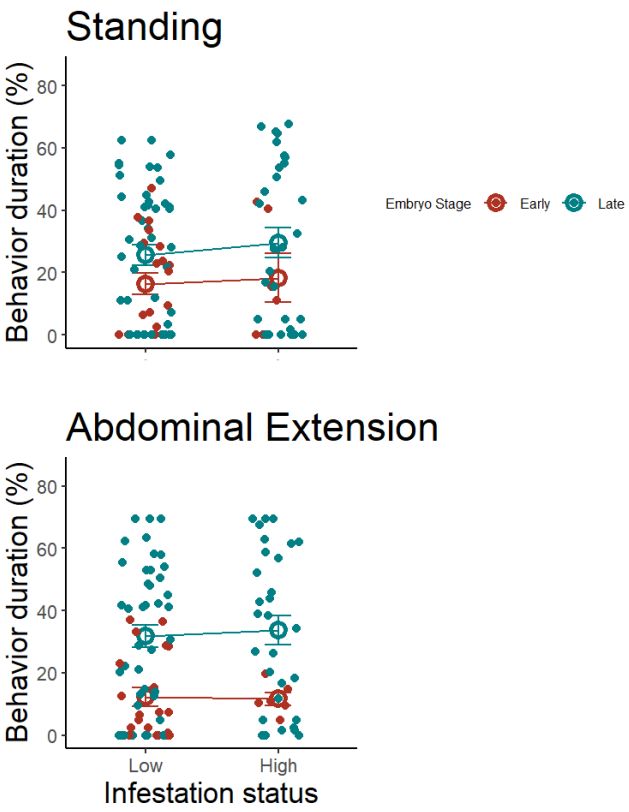
Infestation	Stage	variable	n	mean	sd
High	Early	sqroot	6	2.730	0.244
High	Late	sqroot	30	1.748	1.023
Low	Early	sqroot	20	1.276	1.062
Low	Late	sqroot	46	1.796	0.951

Supplementary Table 4. Summary statistics of the mean ± standard deviation (SD) of the number of grooming bouts (frequency) performed per hour (Output). Behavior frequency output is separated by female *P. argus* embryo stage of development (early vs. late) and *C. conanobrieni* infestation status (high vs. low). Grooming bouts were log-transformed prior to conducting analyses.

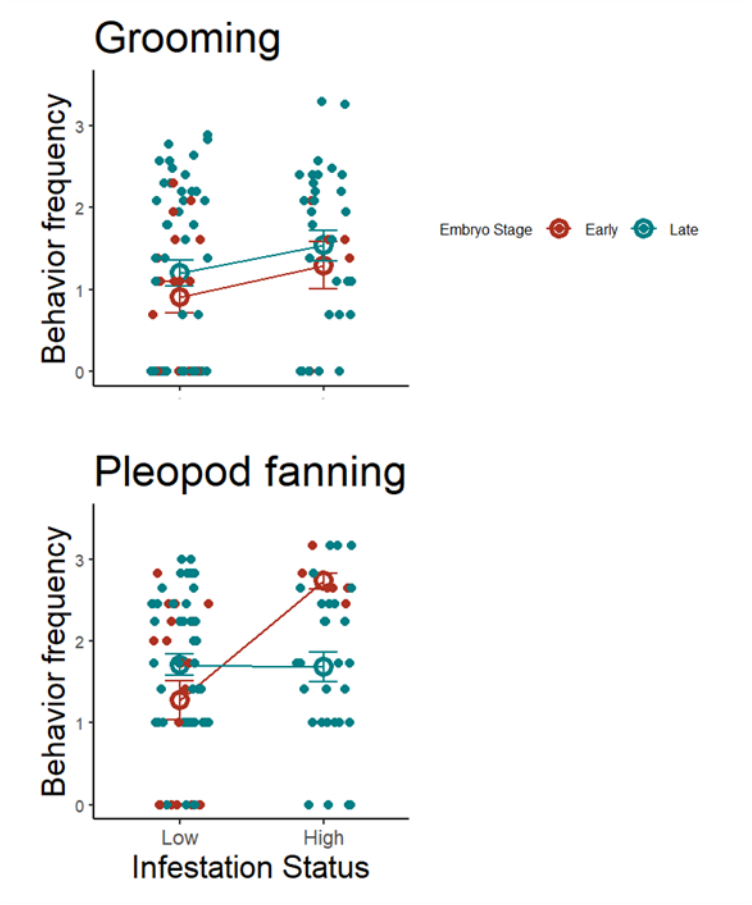
Infestation	Stage	variable	n	mean	sd
High	Early	logOutput_plus1	6	1.297	0.712
High	Late	logOutput_plus1	30	1.537	1.023
Low	Early	logOutput_plus1	20	0.911	0.860
Low	Late	logOutput_plus1	46	1.201	1.085

Supplementary Table 5. Summary statistics of the mean ± standard deviation (SD) of the number of times per hour (frequency) grooming was performed. Behavior bouts are separated by female *P. argus* embryo stage of development (Early vs. Late), dosing pump treatment (With 100 *C. conanobrieni* worms or Without 100 *C. conanobrieni* worms), and *C. conanobrieni* infestation status (High vs. Low).

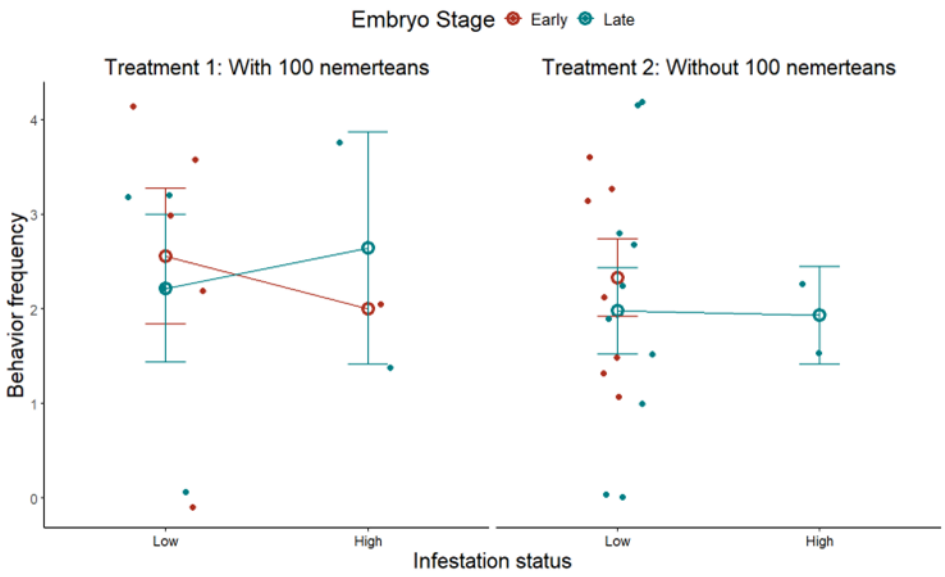
Embryo	Worm	Treatment	variable	n	mean	sd
Early	HIGH	WITH	sqrootbouts	1	2.000	NA
Late	HIGH	WITH	sqrootbouts	2	2.644	1.739
Early	LOW	WITH	sqrootbouts	5	2.559	1.602
Late	LOW	WITH	sqrootbouts	4	2.217	1.564
Late	HIGH	WITHOUT	sqrootbouts	2	1.932	0.732
Early	LOW	WITHOUT	sqrootbouts	7	2.329	1.082
Late	LOW	WITHOUT	sqrootbouts	10	1.980	1.446



Supplementary Figure 1. Multi-panel line graphs with box plots looking at the effect of *P. argus* embryo stage and *C. conanobrieni* infestation status on active parental care behavior states (standing and abdominal extension). Female lobster embryo stage (early versus late) was classified on each individual. *Carcinonemertes conanobrieni* infestation load (low versus high) is plotted on the x-axis, while behavior duration (% time per hour) is on the y-axis. Mean values of each behavior's duration were plotted.



Supplementary Figure 2. Multi-panel line graphs with box plots looking at the effect of *P. argus* embryo stage and *C. conanobrieni* infestation status on active parental care behavior events (grooming and pleopod fanning). Female lobster embryo stage (early versus late) was classified on each individual. *Carcinonemertes conanobrieni* infestation load (low versus high) is plotted on the x-axis, while behavior frequency (# of times performed per hour) is on the y-axis. Mean values of each behavior's frequency were plotted.



Supplementary Figure 3. Side-by-side line graphs with box plots comparing the grooming frequency of female *P. argus* experimentally exposed to water with and without 100 live *C. conanobrieni*. Female lobster embryo stage (early versus late) was classified for each individual and colored points (red = early-stage and blue = late-stage) represent lobsters that went through experiment two. *Carcinonemertes conanobrieni* infestation load (low versus high) is plotted on the x-axis, while behavior frequency (# of times performed per hour) is on the y-axis. Mean values of each behavior's frequency were plotted.

Chapter IV:

Conclusions and Outlook

Nemertean worms in the family Carcinonemertidae are voracious egg predators that infest a variety of decapod crustaceans. In this thesis, I explored the intricate relationship between the *Panulirus argus*/*Carcinonemertes conanobrieni* host-egg predator system. Initially only discovered in the brood masses of female *P. argus* in the Middle Florida Keys (Florida, USA), the prevalence of this nemertean across its host's Caribbean distribution has grown significantly over the course of this and other studies conducted since 2017. Across two field summers (May-August 2022 and 2023) in the Florida Keys, I discovered 100% prevalence of *C. conanobrieni* across brooding female *P. argus* in three geographic regions along the Florida Keys reef tract: the Upper, Middle, and Lower Florida Keys. This is the highest prevalence estimate to date for this host-egg predator system, which allowed for both the quantitative and qualitative investigations of the effect of this micro egg-predator on female Caribbean spiny lobsters.

In detail, I described the effect of this nemertean worm on female Caribbean spiny lobster reproduction. From this investigation, *C. conanobrieni* can be directly tied to significant declines in female *P. argus* reproductive performance. Infestation by *C. conanobrieni* persists throughout *P. argus* embryo incubation, which highlights the synchrony of this nemerteans life cycle to its host's brooding pattern as noted in other *Carcinonemertes* spp. *Carcinonemertes conanobrieni*, similarly to other species in genus *Carcinonemertes*, causes significant embryo mortality in host brood masses. Although I did not directly observe this nemerteans feeding behavior that contributes to embryo mortality, Lunden (2018) described its suctorial feeding mechanisms. In addition to significant embryo mortality, I noted a significant decline in host fecundity and

reproductive output as a result of *C. conanobrieni* prevalence. I observed that the majority of my collected female *P. argus* carrying early-stage embryos had encysted juvenile *C. conanobrieni*, while females bearing late-stage embryos primarily had *C. conanobrieni* adult worms and nemertean eggs among the host's own embryos. These observations ultimately corresponded with late-stage females having lower fecundity and reproductive output, coupled with higher embryo mortality, compared to females with early-stage embryos. Multivariate analyses (i.e., redundancy analyses (RDA)) further supported my hypotheses' that female *P. argus* reproduction was negatively affected by *C. conanobrieni* infestation. The RDA confirmed that the presence of *C. conanobrieni* juveniles in females with early-stage embryos contributed to decreases in fecundity and reproductive output (RO), but that the most notable declines in these reproductive measures occurred in late-stage females with nemertean adults and their egg masses. This indicates that all life stages of *C. conanobrieni* contribute to significant losses in host fecundity and reproductive output, coupled with increased embryo mortality, but the negative effect accumulates and progresses as both *P. argus* embryos and *C. conanobrieni* undergo development.

Based on my observations, I also proposed a model for the life cycle of *C. conanobrieni* when found in *P. argus* clutches. The life stages of *C. conanobrieni* (larvae, juvenile, adult) have distinct behaviors that correspond with feeding, mating, and moving among the host's embryos. *Carcinonemertes conanobrieni's* life cycle aligns with *P. argus'* embryo incubation period and it exhibits a similar lifestyle to other nemertean worms with crustacean hosts, as described for *C. errans* and *C. epialti*, among others (Wickham 1980; Kuris 1978). However, unlike other *Carcinonemertes* sp. that have been observed on male and female hosts in the gill lamellae, pleopods, and brood mass (Santos and Bueno 2001; McDermott and Gibson 1993), I only ever

found *C. conanobrieni* in the embryo mass and pleopods of female *P. argus*. Specifically, *C. conanobrieni* juveniles, adults, and their egg masses infest gravid females throughout embryo incubation and as *P. argus* embryos get closer to hatching, adult *C. conanobrieni* were found in mucus sheaths lining the host's pleopods. This suggests that if *C. conanobrieni* are able to temporarily adhere to the pleopods, using their mucus sheath with external hooks, after lobsters release their clutch through forceful pleopod fanning then they are at an advantage to re-infest their host during its next clutch. Indeed, individual nemerteans that were isolated from the brood mass survived, without eating, for 1-2 weeks (Stephens *pers obs*). Future observational studies of infested *P. argus* throughout embryogenesis would help us fill in the gaps of this nemerteans life cycle, especially to investigate if it infests the host when no longer egg-bearing.

Decapod crustaceans, including *P. argus*, have utilized behavioral responses to surmount the costs associated with pathogens and parasites. Behavior is considered the first line of defense, opposed to an immune system response, with avoidance acting as a barrier to stop parasite establishment on the host. As a preliminary defense mechanism against endo- or ecto-parasites, hosts may directly avoid infected conspecifics, pathogens, and/or habitats with high infection risk to restrict initial contact (Hart 1990; Behringer et al. 2018). Healthy juvenile and adult *P. argus* avoid PaV1 conspecifics using chemical cues transmitted in the urine of infected individuals (Candia-Zulbarán et al. 2015; Behringer et al. 2018; Ross, Behringer, and Bojko 2019; Butler et al. 2015; Behringer, Butler, and Shields 2006; Behringer, Butler, and Shields 2010). When avoidance fails and the parasite establishes on the host, mitigation occurs in the form of migration, selective foraging, altered activity levels, lethargy, or imitating the behaviors of sick individuals (Barber et al. 2000; Vale et al. 2018; Hart 1990; Moore 2013). Active parental care behaviors have previously been demonstrated by egg-bearing decapods to assist with

embryo development, namely oxygen provision and removal of foreign debris (Baeza et al. 2019; Baeza et al. 2019; Baeza and Fernández 2002; Förster and Baeza 2001). Secondarily they have been used for behavioral mitigation in some host-parasite systems (McGrew and Hultgren 2011).

Active parental care in female *P. argus* includes a suite of behaviors performed in a specific sequence: standing, abdominal extension, pleopod fanning, and 4th/5th pereopod probing (herein grooming). The series of behaviors, performed in a sequence, agreed with Baeza et al.'s (2016) description in the same species and similarly matched observations of other egg-bearing crustaceans including *C. setosus* (Baeza and Fernàndez 2002) and *L. boggessi* (Baeza et al. 2019). In this study, however, I investigated the importance of these behaviors beyond basic embryo maintenance and focused on whether they limit and mediate the negative effects associated with *C. conanobrieni* infestations in female *P. argus* brood masses. Contrary to my prediction, grooming bouts did not increase in frequency in infested females (neither low or high *C. conanobrieni* loads). While I found this surprising, the lack of change in active parental care of females has been reported in the literature for egg-bearing amphipods with microsporidian infections (Arundell, Weddell, Dunn 2014). However, my results did record significant changes in pleopod fanning bouts for early-stage females with high *C. conanobrieni* loads. Previous studies have demonstrated that this behavior increases as embryos progress from early to late stages of development (Baeza et al. 2016; Baeza and Fernández 2002; Arundell, Wedell, Dunn 2014; Fernández and Brante 2003), which is in opposition with my results in Chapter 3. My results may suggest that the use of this behavior in early-stage females indicates their sensing of roaming and feeding juvenile and adult *C. conanobrieni* in their brood masses, however this prediction requires further experimental behavioral assays throughout the entirety of incubation.

While there are cases of brooding crustaceans modifying their parental care during parasite infections (for example, see McGrew and Hultgren 2011), these results show that *P. argus* are more similar to those who do not change these behaviors for the purpose of infection/infestation minimization and instead uses them for ensuring adequate conditions for embryos to survive and grow in.

Considering *P. argus'* ability to detect and avoid signals of PaV1 (Candia-Zulbarán et al. 2015; Behringer et al. 2018; Ross, Behringer, and Bojko 2019; Butler et al. 2015; Behringer, Butler, and Shields 2006; Behringer, Butler, and Shields 2010), I expected to see a similar response to sensing cues associated with *C. conanobrieni* infestation and anticipated that egg-bearing lobsters would respond by increasing grooming. In contradiction to my hypothesis, there were no significant changes in host grooming frequencies during exposure to treatments of seawater with *C. conanobrieni*. *Panulirus argus* has clearly exhibited its attraction and subsequent detection of chemical cues at larval (Ambrosio and Baeza 2023), juvenile and adult life stages (Candia-Zulbarán et al. 2015), therefore my results could highlight these nemerteans potential to remain undetected while infesting *P. argus* clutches. As female *P. argus* perform rapid pleopod fanning bouts to release their own embryos adhered to the pleopods, this behavior may aid with nemertean larval release as they presumably get released alongside host eggs. Indeed, some brood parasites, such as the rhizocephalan *Lernaeodiscus porcellanae*, engage in egg mimicry by morphologically appearing identical to host embryos, which allows the parasites to blend in and gain from the behaviors the hosts perform to their own eggs including grooming and ventilation throughout development (Ritchie and Høeg 1981). I argue that the synchrony of *C. conanobrieni's* life cycle with *P. argus* embryo development requires further long-term studies that examine the entire incubation period.

In summary, this thesis has identified the effects of *C. conanobrieni* on female fecundity, reproductive output, embryo mortality, and spiny lobster active parental care behaviors. While I uncovered many findings that will advance what we know about this host-egg predator system and more broadly what we know about decapod crustaceans infested with *Carcinonemertes* sp., there are plenty of avenues left to investigate. Namely, *C. conanobrieni*'s survival during this host's non-reproductive months, e.g. with no access to embryos for feeding, and what traits contribute to this egg predator's success in remaining undetected in *P. argus* brood masses throughout infestation remain to be explored. I provide the framework for future studies to improve the description of *C. conoanobrieni*'s life cycle when in *P. argus* brood masses, as described in Chapter 2. I encourage researchers to focus on the active parental care behaviors that I did find to be significantly affected by *C. conanobrieni* infestation (Chapter 3) as a starting point for identifying if active parental care is indeed helpful in egg predator mitigation, or if it is only for facilitating embryonic development as I suggested. By continuing to monitor *C. conanobrieni* prevalence across the Florida Keys reef tract, we can document the spread of egg predator infestation in this host species and make suggestions for the management of this heavily fished spiny lobster to proactively limit fishing efforts when nemertean outbreaks are severely impacting local host populations.

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