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Prevalence, intensity of infestation, and biomarker potential of the nemertean worm, *Carcinonemertes carcinophila* (Kölliker, 1845), on ovigerous blue crabs, *Callinectes sapidus* Rathbun, 1896 (Decapoda: Brachyura: Portunidae), in Chesapeake Bay, USA

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ABSTRACT

The effect of individual and population-level egg mortality is important to quantify to maintain sustainable crustacean fisheries. The nemertean worm *Carcinonemertes carcinophila* (Kölliker, 1845) is an egg predator of the Atlantic blue crab, *Callinectes sapidus* Rathbun, 1896; however, little is known about the impact this nemertean has on the reproduction of the blue crab. We assessed the prevalence and intensity of the infestation of nemerteans in ovigerous blue crabs using a fishery-independent trawl survey. During the primary spawning period of the crab, May–September 2022, 126 ovigerous females were collected and analyzed for worms. Prevalence over this time was 66.6% and mean brood infestation was 53.9 worms per infested crab host. Nemertean egg consumption was quantified with a six-day microcosm experiment. Of the 48 worms in the experiment, 71% actively fed on crab eggs and their consumption ranged 0.16–4.5 eggs day^{-1}. Consumption rates were used to estimate population-level impact of nemertean feeding on crab brood mortality. Modeled proportions of brood loss per crab ranged 0–0.0044%. At the current prevalence and intensity of infestation, egg consumption by nemerteans has a negligible effect on blue crab reproductive output and batch fecundity in Chesapeake Bay. We also investigated the use of mature nemertean worms as a biomarker for establishing the spawning history of ovigerous female blue crabs and determined that the presence of worms in the clutch and in the gills can be used to indicate parity in ovigerous female crabs.

KEY WORDS: Crustacea, egg mortality, egg predation, Nemertea, symbiosis

INTRODUCTION

Many commercially exploited decapods are managed by protecting the female spawning stock in an effort to prevent recruitment overfishing and retain high levels of egg production (Botsford, 1991). Population-level egg production is a more useful metric for management than spawning stock biomass because it accounts for individual differences in egg production related to size or age (Botsford, 1991; Lambert, 2008). Data on population-level egg production may therefore improve stock-recruit relationships and stage-specific population models (Morgan, 2008; Morgan *et al.*, 2011; Kell *et al.*, 2016). If spatial, temporal, or individual-based trends in egg production are identified, management can be tailored to protect the most fecund individuals, the habitats with high egg production, or the times of year with seasonally high productivity. To accurately estimate egg production, sources, and effects of egg mortality must be considered.

Egg mortality can lead to a substantial decrease in viable egg production throughout embryogenesis (Kuris, 1991). Unviable (dead or unfertilized) eggs can be caused by individual factors such as sperm limitation (Ogburn, 2019) or sterile mating (Shields & Wood, 1993). Egg mortality can also be compounded via external factors such as stress endured from capture in fishing pots (Dickinson *et al.*, 2006; Darnell *et al.*, 2009), environmental stressors (Green *et al.*, 2014; Wang *et al.*, 2019), microbes, or symbionts (Kuris, 1991). Various microbes and symbionts cause egg mortality in decapods; however, predatory nemertean worms can be responsible for the majority of symbiont-driven egg mortality at the individual and population level (Shields & Kuris, 1988a; Kuris, 1991).

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Carcinonemertes Coe, 1902 is the primary genus of Nemertea causing egg mortality in decapods. Species of *Carcinonemertes* are specialized symbiotic worms found on brachyuran crabs and, when present at high intensities, are known to consume significant proportions of their hosts' broods (Roe, 1984; Wickham, 1986; Kuris & Wickham, 1987; Shields *et al.*, 1990; Kuris *et al.*, 1991; Santos & Bueno, 2001). Species of *Carcinonemertes* feed, grow to maturity, and lay eggs within the brood of their ovigerous crab hosts (Shields & Overstreet, 2007). They use their stylet-armed proboscis to puncture the chorion of the egg and suck out the yolk (Wickham, 1979a); puncturing the egg membrane kills the embryo regardless of the amount of yolk consumed (Roe, 1984; Shields & Kuris, 1988a). Brood mortality due to nemertean egg predation has been linked to collapse of commercial fisheries, such as the Dungeness crab fishery in California (Shields *et al.*, 1989) and the red king crab fishery in Alaska (Kuris *et al.*, 1991). Nemerteans are therefore a relevant source of egg mortality to consider in decapod fisheries, such as in the Atlantic blue crab, *Callinectes sapidus* Rathbun, 1896.

Callinectes sapidus is an economically, ecologically, and culturally important species in the United States and other regions of North America and represents the most valuable fishery in Chesapeake Bay. *Carcinonemertes carcinophila* Kölliker, 1845 is found within the gills and egg clutches of blue crabs in Chesapeake Bay, Virginia, North Carolina, and the Gulf of Mexico (Humes, 1942; Hopkins, 1947; Messick, 1998; Darnell *et al.*, 2009; Kemberling & Darnell, 2020) and likely infests blue crabs throughout their native range. Planktonic, larval nemerteans recruit to female crabs and settle as juveniles in the gills of non-ovigerous hosts or broods of ovigerous females. Juvenile nemerteans are small (~300 μm), white, and inconspicuous on their host (Humes, 1942). After feeding on crab eggs, the worms grow, mature, and become visibly pink or red in color (Humes, 1942). Mature worms can range 0.5 mm–30 mm in length (Humes, 1942), with females being larger than males (Roe, 1984). At the time of egg eclosion, worms migrate from the brood to the gills. Worms remain encysted in mucous sheaths between the gill lamellae of the host until the female produces her next brood, after which the worms may migrate back to the brood to consume more eggs (Humes, 1942). Mature female crabs continue to accumulate worms during the spawning season through recruitment and maturation of juveniles, leading to high intensities of worms.

The blue crab population in Chesapeake Bay has experienced significant variability over the past thirty years, including a decline in spawning stock abundance since 2017 (Chesapeake Bay Stock Assessment Committee, 2022). To maintain a robust population, all potential drivers of poor stock productivity must be assessed, including egg mortality. As nemerteans have had detrimental effects on other decapod fisheries, determining nemertean intensity of infestation and size on their host will improve our overall understanding of their impact on blue crab egg mortality. The intensity of nemertean infestation (number per infested host) is critical to quantify because the number of mature worms on a female crab determines the level of host egg mortality (Shields & Wood, 1993). Moreover, nemertean size structure may be useful given larger female worms will eat more eggs than smaller male worms (Roe, 1984).

Little is known about the prevalence, intensity or extent of predation of *C. carcinophila* on ovigerous blue crabs at the population level. Our goal was to better understand the impact of nemertean worms on female blue crabs in Chesapeake Bay during the spawning season. Our objectives were to 1) determine the prevalence and intensity of infestation of *C. carcinophila* on the gills and brood of ovigerous female crabs in Chesapeake Bay; 2) assess sizes of worms in the gills and brood on their host crabs; 3) quantify feeding rates of these worms *in vivo*, and 4) examine the impact of nemerteans on egg mortality on the fecundity of blue crabs.

We hypothesized that the prevalence and intensity of infestation of nemerteans would increase over the spawning season as female crabs produce their second and third egg masses and additional worms recruit and mature. We also anticipated that nemertean prevalence and intensity of infestation would be higher in crab eggs in more advanced stages of development, as these nemerteans would have had a longer period to feed and mature compared to worms in recently oviposited eggs. We assumed no difference in the distribution of worm length in hosts with nemertean infestations in their brood and gills, because worms in both infestation sites should have consumed eggs and matured to an adult size distribution. Lastly, we expected nemertean worms to cause egg mortality and have a negative impact on host fecundity, with the additional hypothesis that larger worms would consume more eggs than smaller worms.

MATERIALS AND METHODS

Animal collection and processing

Ovigerous females were collected by the Virginia Institute of Marine Science (VIMS) Juvenile Fish Trawl survey, "trawl survey" herein. The trawl survey is a stratified random fishery-independent survey that samples 39–45 locations monthly in the Virginia portion of the Chesapeake Bay mainstem (Tuckey & Fabrizio, 2022). Collection of ovigerous females occurred in the mainstem of the bay from April to September 2022 to encompass the blue crab's primary spawning grounds and spawning season (Van Engel, 1958; Lipcius *et al.*, 2003). All ovigerous females caught by the trawl survey were tagged by sampling station, kept on ice, and brought to VIMS to be processed within 24–72 h of catch. Ovigerous females were measured for carapace width (CW, from lateral spine to lateral spine) with Vernier calipers to the nearest 0.1 mm. Egg stage was assessed macroscopically, by color, and classified as early development (orange), mid development (brown), and late development (black) as in Van Engel (1958), to be consistent with trawl survey methodology.

Nemertean prevalence, abundance, intensity of infestation, and size

The brood and gills of ovigerous females were inspected for the presence of mature nemerteans. Only mature worms were examined as juvenile worms are ~300 μm long, white, and not visible macroscopically, whereas mature worms are pink or red in coloration and visible macroscopically (Humes, 1942). Infestation thus refers to an infestation of mature nemertean worms and does not consider the potential presence of larval or juvenile worms. For brood inspection, the abdomen, or pleon or "apron," of each crab was removed and crab eggs were stripped from the pleopods and setae. Crab eggs were examined macroscopically, all visible worms were removed,

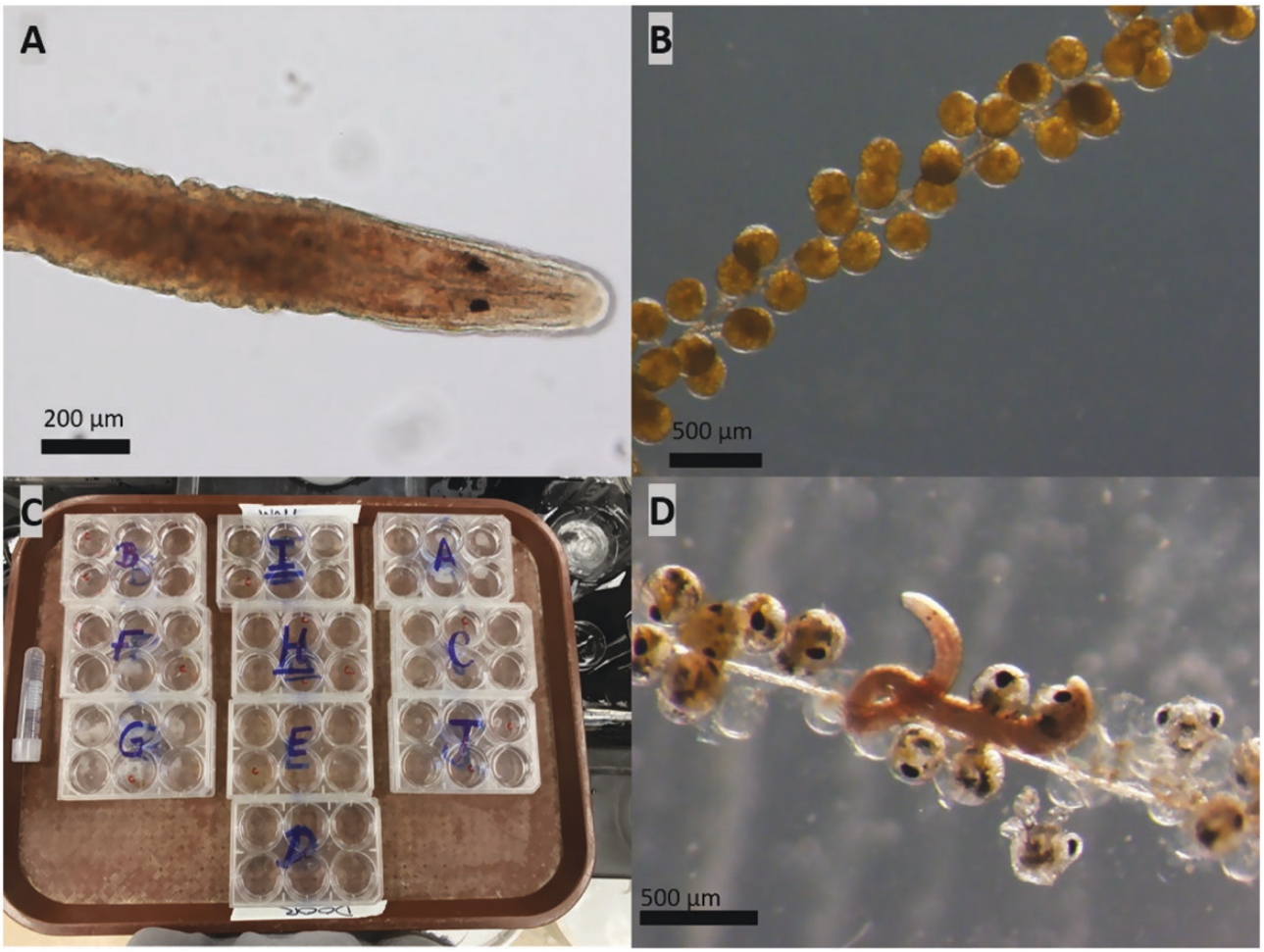

Figure 1. Feeding experiment: nemertean worm *Carcinonemertes carcinophila* (**A**), stage one, eggs in early development (**B**), 6-well plates, individual wells are experimental units, and each containing a worm with a setal strand of 90–129 eggs of *Callinectes sapidus* (**C**), and nemertean interacting with stage four (near hatching) eggs (**D**).

and counted (Fig. 1A). Gills were also assessed macroscopically. The dorsal portion of each crab's carapace was removed to access the gills. The gills were excised from the body and placed in fresh water to facilitate worm removal. Forceps were used to tease apart gill filaments, and remove and count all visible, encysted nemerteans. Given the large number of nemerteans that can be found in individual crabs (Humes, 1942; Shields *et al.*, 1990), the first twenty nemerteans randomly extracted from an egg mass or gills were elongated to their maximum body length using forceps and paintbrushes and measured to the nearest 0.1 mm with Vernier calipers.

Ovigerous females were categorized into four groups according to the site of nemertean infestations: 1) females with nemertean infestations on their gills only, 2) females with nemertean infestations on their brood only, 3) females with nemertean infestations on their gills and broods, and 4) females without nemertean infestations. These site groupings were used for analyzing trends in prevalence, abundance, intensity of infestation, and size.

Feeding experiment

Worms and eggs used in the feeding experiment were obtained from ovigerous females collected in July 2022 by the trawl survey. Ovigerous females were collected from the mouth of the James River due to high nemertean prevalence levels on crabs from the lower James River (A. Schneider, unpublished data). Crabs were placed on ice, transported to VIMS, and processed immediately after their arrival. At the time of collection, the average environmental conditions in the James River were 27 °C and a salinity of 20 psu. Worms and eggs were never collected from the same ovigerous females.

Eggs were obtained from four ovigerous females with embryos in the first stage of development (Fig. 1B, as described by Jivoff *et al.*, 2007). Pleopods were removed from ovigerous females, herein "egg donors," and agitated in seawater to separate setae without damaging eggs. Subsamples of setae from each egg donor were then placed in individual 60 × 15 mm petri dishes with ~4 ml of seawater and acclimated to experimental conditions.

Worms were collected from setae using the nemertean's negative phototaxis (Dunn & Young, 2015). Briefly, pleopods were placed under a direct light and the worms were gently pipetted into experimental units for acclimation when they exited the clutches to move away from the light source. Six worms were collected from each of the eight females, herein worm donors ($N = 48$).

All eggs and worms were maintained at a salinity of 20 psu during crab processing. The eggs and worms were then acclimated separately in a 24 °C incubator for 12 h (precision low temperature

BOD refrigerated incubator; Thermo Fisher Scientific, Waltham, MA, USA). After 12 h, salinity was increased by 2 psu per hour until reaching 25 psu to ensure that salinity was conducive to viable blue crab embryogenesis (Sandoz & Rogers, 1944).

After acclimation, aliquots of 90–120 eggs with a single worm were placed in 6-well plates at 25 psu. Each well (experimental unit) was 35 mm in diameter with a volume of 10 ml filtered seawater (Fig. 1C). Forty-eight replicates and 12 controls were interspersed randomly across plates. Controls were eggs without worms to monitor egg mortality and development that may be related to experimental conditions rather than worm interactions. Well plates were randomly oriented and positioned in the incubator each day to prevent well-plate position effects. The wells were kept in the dark at 24 °C with 25 psu seawater for the six-day period and were only removed from the incubator for brief daily monitoring. Experimental units were monitored daily, for six days.

Egg consumption, worm-egg contact, egg development stage, and relative bacterial growth were assessed every 24 hr with a dissecting microscope (Olympus SZX9; Olympus, Tokyo, Japan). Consumption was indicated by the presence of empty, or partially empty, crab eggs. If egg hatching occurred prior to the end of the experiment, hatched zoea were counted and removed, and the replicate was terminated. Empty eggs due to hatching were omitted from the consumption estimates. Worm contact with eggs was based on whether a worm was intertwined or touching the eggs or seta (Fig. 1D), and if the eggs were entangled in the mucus produced by the worm. Embryo development stage was denoted microscopically as early, mid, late, and near hatching (after Jivoff *et al.*, 2007). We used this more fine-scaled staging for the experiment, as compared to macroscopic classification in the field collections, to ensure that eggs were equally developed at the start of the experiment, and to improve monitoring of development over the course of the experiment. The presence of a fungal oomycete was noted and the development date of hyphal growth was monitored. Eggs were considered to be colonized with microbes when hyphae were clearly visible on the external membrane of the egg. Partial water changes were done daily with sterilized pipettes to prevent growth of the oomycete (presumptively identified as *Lagenidium callinectes*; see Rogers-Talbert 1948). Sterilized forceps were used to gently tease apart egg setae that may have been tangled by nemertean interaction so as to facilitate consumption checks. In some entanglement cases, worms were tightly entwined in the eggs and the daily egg consumption check was skipped to avoid worm injury.

Prior to the experiment, each nemertean was photographed using an Olympus SZX dissecting scope, at 8.3×, with an Olympus DP73 camera. Photographs were taken when the worm's whole body could be easily seen and when it was not coiled onto itself. Photographs were used to measure worm length with the image analysis program FIJI, using the line segment tool (Schindelin *et al.*, 2012).

Data analysis

All statistical analysis and data transformations were performed in R, the statistical computing language (R Core Team, 2020). Linear mixed models (LMM) were run with the nlme package (Pinheiro & Bates, 2000; Pinheiro *et al.*, 2022). Generalized linear mixed models (GLMM) were run using the MASS package (Venables & Ripley, 2002). The significance level was considered as $\alpha = 0.05$.

Nemertean prevalence, abundance, intensity of infestation, and size in field collections

The prevalence, or proportion of females with nemerteans, for each month was estimated as the number of females with infestations in the gills, brood, both, or without infestation divided by the total number of females examined in that month. The standard error was calculated using standard binomial approaches as:

$$((p \times (1\ p))\ /\ (n))^{0.5} \quad (1)$$

where p is the proportion of females infested and n is the total number of blue crabs examined (Fleiss *et al.*, 2003). Mean abundance was calculated as the total number of nemerteans in the sample of ovigerous females, divided by the total number of ovigerous females examined. Mean infestation was calculated as the total number of nemerteans in the sample of ovigerous females, divided by the total number of infested ovigerous females examined.

The number of worms in crab broods was analyzed with a generalized linear model (GLM) and month as a continuous variable, egg stage as a categorical variable (early-, mid-, late-development), and crab infestation site as a categorical variable (broods only or brood and gills). The GLM used a negative binomial log link function, to determine if worm infestation, and therefore potential nemertean feeding, varies by month:

$$y_{ijk} \sim NB(\lambda_{ijk},\ \varphi) \quad (2)$$

$$\ln(\lambda_{ijk}) = \text{Infestation Site}_i + \text{Month}_k + \text{Egg Stage}_j \quad (3)$$

where $\ln(\lambda_{ijk})$ represents the expected count of worms in a crab brood per i^{th} crab infestation site, j^{th} egg stage, and k^{th} month with the negative binomial type I distribution (linear parametrization of variance) and log link. A GLM with the same structure and hypotheses was constructed for counts in the gills, except nemertean location was excluded as not all months had females with gill only infestations. Both models met the relevant assumptions of negative binomial GLMs. Interactions between month and nemertean location, as well as month and egg stage were explored and determined to be inconsequential and therefore excluded from the model. Negative binomial GLMMs with a random effect for trawl tow were explored; however, in all cases the variance explained by the random effect was low (< 0.001) and GLMMs were consistently associated with a higher AIC score than the models without a random effect.

For hosts with infestations in the brood and gills, we compared the size distribution of worms between sites using the LMM:

$$\left(y_{il}^{0.5}\right) = \text{Infestation Site}_i + \text{Crab}_l \quad (4)$$

where y_{il} is square root transformed for normality and represents worm length from the i^{th} site, and l^{th} crab, infestation site represents the effect of infestation site as a categorical variable (gills or brood). Individual "crab" was included as a random effect to

account for multiple worm measurements from the same host. The random effect of trawl tow was explored, but was excluded because of confounding between the random effect of trawl tow and the random effect of individual crab as 55% of trawl tows captured only one ovigerous crab. We used the same model formulation to compare size distributions of worms in the gills and the brood between females that only have infestations in the gills or brood. After the square root transformation of worm length, both size models met the relevant assumptions of normality and homoscedasticity. The estimates for the random effects also met the assumptions of normality.

Feeding experiment

An LMM was also used to quantify consumption rate by nemertean size:

$$\left(y_{mnop}{}^{0.5}\right) = \text{Egg Donor}_m + \text{Length}_n + \text{Worm Donor}_o + \text{Worm Interaction}_p \quad (5)$$

where y_{mnop} is square root transformed for normality and represents daily consumption rate, Egg Donor_m represents the effect of the m^{th} egg donor as a categorical variable, length represents the regression coefficient for the n^{th} worm length as a continuous variable, worm donor represents the random effect of the o^{th} crab the experimental worms originated from, and worm interaction represents the p^{th} number of times worms were observed to interact with the eggs. A simple linear regression was constructed using only nemertean size as a predictor to test the relative importance of egg and worm origin on worm consumption. The two models were compared using AIC (Anderson, 2008).

To test if egg development rate differed between experimental units or egg donors, the developmental rate of the eggs was quantified using an LMM:

$$\left(y_{mrq}\right) = \text{Egg Donor}_m + \text{Days}_q + \left(\text{Egg Donor}_m \times \text{Days}_q\right) + \text{ExpUnit}_r \quad (6)$$

where y_{mrq} represents daily egg stage, Egg Donor_m represents the effect of each m^{th} egg donor as a categorical variable, and days represents the regression coefficient of the q^{th} day (continuous) with an interaction term for egg donor and day. Experimental unit represents a random effect to account for repeated measures on each r^{th} experimental unit.

To examine the growth of *Lagenidium callinectes* on eggs within the feeding experiment, an analysis of variance (ANOVA) was used to test differences in the onset day *L. callinectes* between wells with a nemertean present (worm treatment) compared to wells without a nemertean (control treatment).

Modeling population level egg mortality

To understand the population-level effect of nemertean worms on the reproductive output of blue crabs, individual egg mortality was calculated under a minimum, mean, and maximum feeding scenario using experiment and survey results. Individual egg mortality per individual crab was calculated by multiplying the minimum, mean, and maximum daily feeding rates by the observed number of worms in the crab's brood, by the average length of crab embryogenesis, 14 d (Hines *et al.*, 2003; Jivoff *et al.*, 2007). This produced a maximum, minimum, and mean egg mortality based on the three feeding level scenarios. The egg mortality was then divided by brood size to obtain the proportional loss in reproductive output. Brood size, in millions of eggs, was estimated as:

$$\hat{\mathbf{E}} = -2.248 + 3.77\text{CW} \quad (7)$$

where $\hat{E}$ is the estimated fecundity (in 10^6 of eggs), and CW is carapace width (cm) (Prager *et al.* 1990). The number of eggs consumed and proportion of brood loss due to nemertean consumption was estimated for 124 of the ovigerous females collected. Two individuals were omitted because they were substantially smaller (< 70 CW) than the range of female sizes used to derive the fecundity equation and produced negative fecundities.

RESULTS

Prevalence, abundance, intensity of infestation, and size of nemerteans

A total of 126 ovigerous female crabs were captured by the trawl survey and assessed for nemerteans from April to September (Table 1, Fig. 2). The majority (96%) of egg-bearing crabs were captured in June to August. The proportion of ovigerous females with worms was > 60% in June through September. The proportion of crabs without mature nemerteans was similar from June to September (Table 1). The majority of worms were found in broods, but the distribution of worms varied by month (Fig. 3). The proportion of females with worms only in their broods decreased over the summer whereas those with worms in both the gills and brood increased

Table 1. Summary data for ovigerous females captured in the VIMS Juvenile Fish Trawl Survey from April to September 2022. The proportion of females with worms in their brood includes females with brood only or brood and gill infestation. SE, standard error.

Month	Number of stations sampled	Number of egg-bearing crabs	Proportion of females with worms ± SE	Proportion of females with worms in their broods ± SE
April	39	0	-	-
May	45	1	0.00 ± 0.00	0.00 ± 0.00
June	45	30	0.63 ± 0.04	0.63 ± 0.04
July	45	38	0.66 ± 0.04	0.58 ± 0.04
August	45	53	0.70 ± 0.03	0.62 ± 0.03
September	45	4	0.75 ± 0.09	0.75 ± 0.09

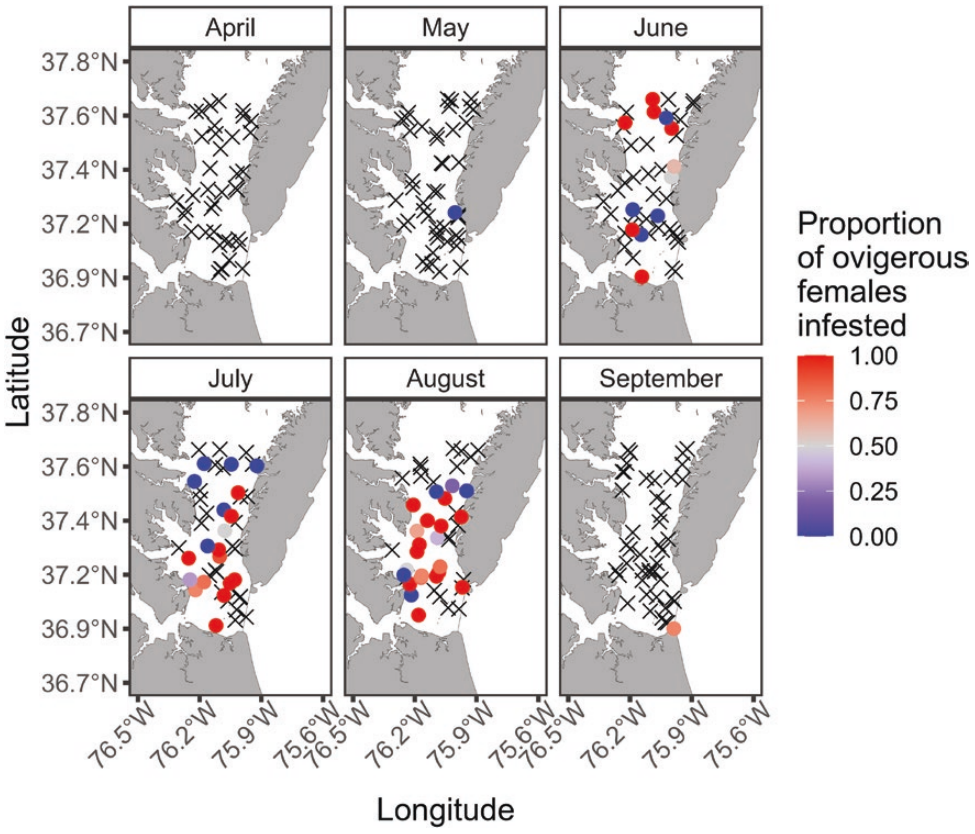

Figure 2. Location of VIMS Trawl Survey sampling locations in the mainstem of Chesapeake Bay from April to September 2022. X, sampling locations where no ovigerous female *Callinectes sapidus* were captured; circles, sampling locations where ovigerous females were captured, with the coloration representing the proportion of the crabs infested with *Carcinonemertes carcinophila*.

(Fig. 3). Nemerteans were uncommon in the gills only, with 7.9% of females in July and 7.5% of females in August having infestations in the gills only. The two smallest crabs (52.3 and 68.3 mm CW) were not infested with worms.

The number of ovigerous females with only gill infestations was consistently low across egg-development stages, unlike the number of females with infestations in their brood, brood and gills, and uninfested females, which varied with egg development stage (Fig. 4). The highest proportion of uninfested hosts were crabs with early stage eggs (47%) compared to those with broods in mid and late stages of development. A large majority of crabs with broods in late-stage development had infestations (96%), but prevalence levels were above 50% for all egg stages.

The intensity of the number of worms in ovigerous females ranged 1–356 worms in the brood and 1–419 worms in the gills. The mean abundance of worms from May to September was 35.9 ± 5.9 worms (SE) in broods and 37.9 ± 6.8 worms in gills. From May to September, the mean intensity was 53.9 ± 8.1 worms in broods and 56.8 ± 9.6 in gills.

The mean number of worms per brood increased significantly with month (GLM, month: χ^2= 13.21, $P < 0.001$; Fig. 3). Exponentiating the coefficient estimate of month, 0.58 (Table 2), indicates that worm abundance increased 1.71 times for each unit increase in month over the summer. The developmental stage of the eggs had a significant effect on the number of worms in a female's brood (GLM, egg stage: $\chi^2 = 6.56$, $P = 0.038$). Estimated marginal means from the model of brood count by egg stage were 65.5 ± 13.0, 32.1 ± 7.4, and 78. 5 ± 18.0 (± SE) for early, mid, and late staged eggs, respectively. Females with late-stage eggs had the most worms, whereas female with mid-stage worms had the fewest worms, although there was an overlap in confidence intervals (Fig. 5). Host infestation site (brood or brood and gills) did not have a strong effect on worm count in the broods (GLM, location: F = 2.84, $P = 0.09$; Table 2). By contrast, the number of nemerteans in the gills did not change significantly over month or egg stage (GLM, month: $\chi^2 = 0.65$, $P = 0.42$; egg stage: $\chi^2 = 2.88$, $P = 0.24$).

Worm length was highly variable, ranging 0.5–43.1 mm (Fig. 5). The mean worm length on the gills was 8.38 ± 4.24 mm (± SD) and the mean worm length in the brood 8.83 ± 5.65 (± SD). There was no effect of site (brood or gills) on worm lengths on females with infestations at both sites (LMM, location: F = 0.28, $P = 0.60$). The length of worms in the brood nonetheless had a greater range, 43.1 mm long compared to those in the gills, up to 29.0 mm (Fig. 6). Individual crab as a random effect accounted for 9.9% of the model variation. Similarly, the LMM of worm length between those in the brood versus those in the gills (only in those locations) indicated no difference in sizes (LMM, location: F = 0.14, $P = 0.71$); however, those in broods once again had a larger range in lengths (Fig. 5). The random effect of crab explained more variation in the worm lengths in this model (17%). Size of worms in broods was consistent among host egg stages in all host groups (Fig. 7).

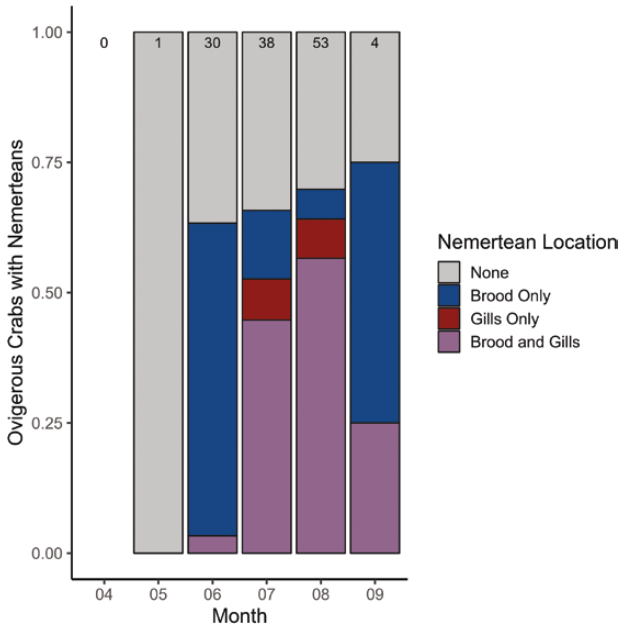

Figure 3. Proportion by month of ovigerous female *Callinectes sapidus* that were infested with *Carcinonemertes carcinophila* and shown by the worms' sites on their host. Numbers at the top of the plot represent the number of ovigerous females assessed each month.

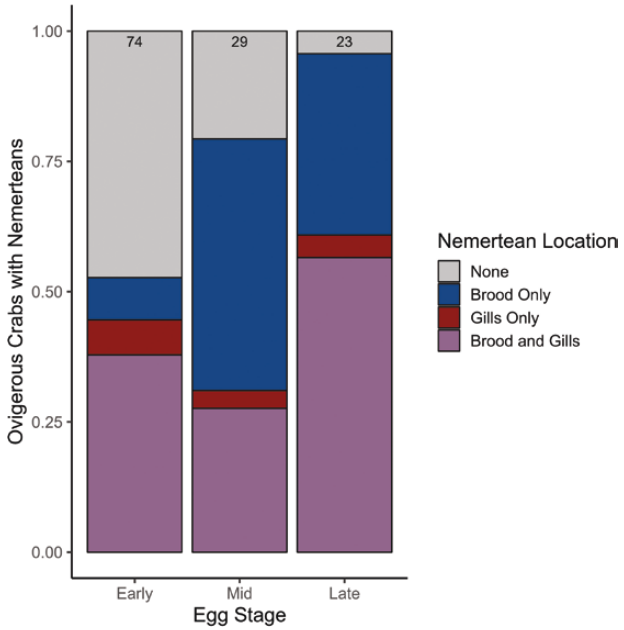

Figure 4. Proportion of ovigerous female *Callinectes sapidus* infested with *Carcinonemertes carcinophila* by the infestation location on their host by egg development stage of the host. Egg development was assessed macroscopically by color (orange, brown, and black, respectively). Numbers at the top of the plot represent the number of ovigerous females assessed per egg development stage.

Nemertean egg consumption

The mean consumption of the 48 worms in the feeding experiment was 3.13 ± 0.74 (± SE) eggs over the 6-d period. The majority (71%) of worms fed during the experiment, consuming a mean 4.41 ± 0.96 (± SE) eggs over the 6-d trial. Total

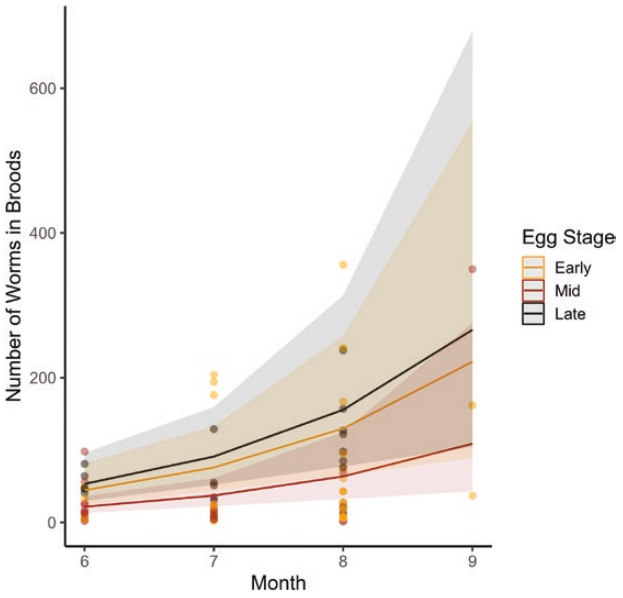

Figure 5. Effect of month and egg stage on the number of *Carcinonemertes carcinophila* in the brood of ovigerous *Callinectes sapidus* from the generalized linear mixed model with a negative binomial distribution of worm counts in broods as a function of egg stage, month, and location of nemertean worms as fixed effects. Egg stages represent early, mid, and late stages of development as indicated by egg mass color (orange, brown, and black, respectively). Bands represent the 95% confidence interval.

Table 2. Parameter estimates for the fixed effect in the generalized linear mixed model with a negative binomial distribution of worm counts in broods. Months include June through September and estimates are in log space. SE, standard error.

Parameter	Estimate ± SE	*z* value	*P* value
Intercept	0.58 ± 1.15	0.50	0.62
Month	0.54 ± 0.16	3.24	0.001
Infestation Site (BG)	–0.58 ± 0.31	–1.65	0.064
Egg Stage (mid)	–0.71 ± 0.31	–2.08	0.023
Egg Stage (late)	0.18 ± 0.29	0.62	0.54

consumption over 6 d ranged 1–27 eggs. The mean consumption rate was 0.62 ± 0.16 (± SE) eggs eaten per day, and ranged 0.167–4.5 eggs eaten per day, for those worms actively feeding during the experiment.

Worm length in the experiment ranged 1.08–8.17 mm. Mean worm length in the trial was 4.57 ± 0.26 mm (± SE). Worm length (mm) and egg donor had no effect on consumption rates (LMM, size: F = 0.022, $P = 0.88$; egg donor: F = 0.99, $P = 0.41$). The random effect of worm donor explained a negligible percentage (6%) of the variation in the model. The number of observed interactions between the worm and the eggs was positively related to consumption rate (LMM: F = 4.82, $P = 0.04$).

Bacterial growth on eggs became visible in microscopy between day two to six (Fig. 7) and appeared significantly earlier in treatments with nemerteans than control treatments without nemerteans (ANOVA: F = 17.17, $P < 0.001$). As expected, egg development stage increased over the 6-d course of the experiment (ANOVA: F = 1208.84, $P < 0.0001$), although development was dependent on

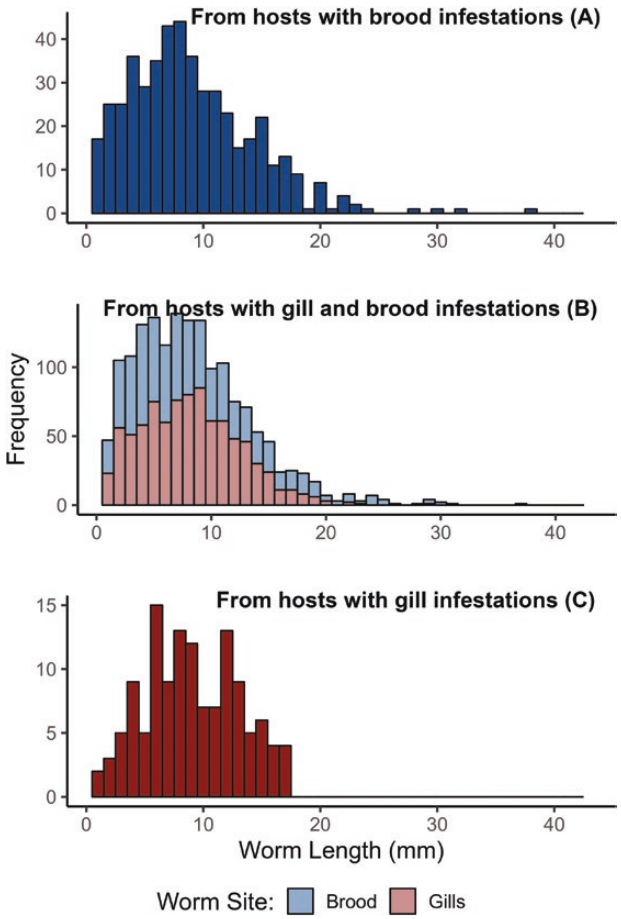

Figure 6. Size distribution of individuals by site, on the brood (blue) and gills (red) of their hosts. Size distributions by location of host infestation brood only (**A**), brood and gills (**B**), and gills only (**C**). Note difference in y axis scale among plots. In B, the maximum worm size was 43.1 mm, however, the y axis scale prevents clear visualization of this individual within the plot.

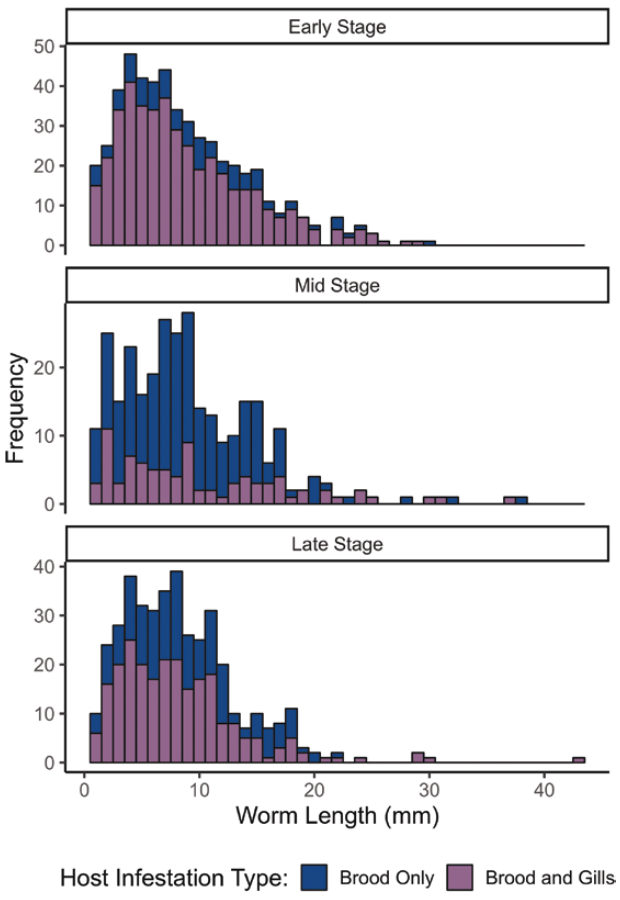

Figure 7. Size distribution of individuals of *Carcinonemertes carcinophila* from host (*Callinectes sapidus*) broods by the development stage of the eggs and the type of host infestation. Egg stages are early, mid, and late stages of development, indicated macroscopically by egg mass color (orange, brown, and black, respectively). Note the difference in y axis scale.

and the rate varied by egg donor (Egg donor: F = 63.30, $p < 0.0001$, interaction: F = 59.81, $P < 0.0001$; Table 3).

Blue crab brood mortality

Brood mortality due to the nemertean was calculated using maximum, mean, and minimum feeding rates. From the estimated maximum number of eggs consumed per day, the largest amount of egg consumption in a brood was calculated at 22,428 eggs, over the course of development, with egg loss ranging 60–22,428 (mean ± SD 3,703.1 ± 543.59). When using the mean number of eggs eaten per day, the egg loss was calculated as 515.36 ± 75.65 (± SD) eggs per clutch, with a range of 8.7–3,121 eggs eaten. In the minimum feeding scenario, egg loss was negligible and ranged 2.33–830.6 eggs (mean ± SD 137.15 ± 20.13). Using these estimates and estimates of brood size (eggs per brood), the maximum proportion of brood loss was 0.000044; the proportion of brood loss in the mean and minimum feeding scenarios were effectively zero.

Use of nemerteans as biomarkers for parity

We extend the hypothesis of Hopkins (1947) using the presence of mature nemerteans by site on the ovigerous host, with the following observations. Females without worms, and females with worms only present in their brood can be classified as primiparous, whereas females with worms in their gills or with worms in their gills and brood can be classified as multiparous females. To this end, uninfested ovigerous females likely did not have worms recruit to their egg masses yet, or are infested with juvenile worms that were not yet detectable macroscopically, and thus, may be

Table 3. Parameter estimates for the fixed effect in the linear mixed model of egg development over the experimental period (6 d). SE, standard error.

Parameter	Estimate ± SE	*t* value	*P* value
Intercept	0.85 ± 0.066	12.86	< 0.0001
Day	0.10 ± 0.016	6.03	< 0.0001
Egg donor (2)	0.10 ± 0.094	1.02	0.3128
Egg donor (3)	–0.09 ± 0.094	–0.94	0.3502
Egg donor (4)	–0.16 ± 0.094	–1.68	0.0985
Day × Egg donor (2)	0.24 ± 0.023	10.40	< 0.0001
Day × Egg donor (3)	0.23 ± 0.023	9.88	< 0.0001
Day × Egg donor (4)	0.28 ± 0.023	12.03	< 0.0001

classified as primiparous spawners. Our data supports these classifications as 47% of females with early stage eggs were uninfested with mature worms, whereas only 4% of females with late stage eggs were uninfested with mature worms. Moreover, no statistical differences in size distributions between worms in the gills and broods in females in our study (Figs. 5, 6) support that worms in the gills fed and grew similarly to those in the brood. These patterns in infestation dynamics support the use of these worms as a biomarker for parity in ovigerous blue crabs.

DISCUSSION

This study is the first to comprehensively document prevalence, intensity of infestation, size, and egg consumption of mature nemertean worms on ovigerous blue crabs. Egg consumption estimates were calculated at the population level as ovigerous females were collected via a fishery-independent survey that randomly sampled the entirety of the blue crab spawning grounds in Chesapeake Bay. Nemertean prevalence was high (67%) among egg-bearing female crabs; however, mean brood infestation of nemerteans was low (~54 worms), compared to other crab-nemertean relationships. Low infestation coupled with the high fecundity and short embryogenesis time of the host resulted in an estimated low consumption of host eggs by nemerteans. In addition, trends in host infestation site and worm lengths support the use of *C. carcinophila* as a biomarker of blue crab spawning history.

Trends in nemertean prevalence and implications for host spawning

The dispersion and infestation patterns of nemertean worms is strongly linked to the reproductive strategy of their respective hosts (Shields, 1993). Blue crabs are multiparous, producing multiple egg masses in a spawning season (Dickinson *et al.*, 2006; Darnell *et al.*, 2009). Worms recruited to female crabs throughout the spawning season as indicated by a general increase in the abundance of mature worms over the summer season. Moreover, because the number of clutches produced by a female crab increases throughout their summer spawning season, the likelihood that nemerteans will be present is higher as indicated by the 1.7 increase in infestation per month.

The seasonal prevalence and temporal patterns of nemerteans in blue crabs in 2022 was similar to the 71% reported for Chesapeake Bay in the 1940s (Hopkins, 1947). Similarly, the prevalence in ovigerous females in 1944 was low in the beginning of the season, and increased to 90% by July (Rogers-Talbert, 1948). Trends in prevalence in May and September 2022 were driven by a smaller number of ovigerous females being present in the crab population, as reflected by the catch of ovigerous females by the trawl in these months. Nonetheless, a commensurate increase was observed in the prevalence of worms present in the gills of crabs in later months, thus indicating a general increase in abundance of the worms in relation to the crab's spawning history.

From previous studies, we expected to find worms in the egg masses and not in the gills of ovigerous females. Contrary to our expectation, 66% of infested ovigerous females had nemerteans in their gills. Nonetheless, the majority of infested females had mature nemerteans in their broods (91%). Worms can be in the gills of ovigerous females for various reasons. Worms may have migrated back into the gills after feeding on the brood or have yet to migrate into recently oviposited egg masses. These hypotheses are supported by our data: 45% of infested females with early-stage eggs had worms in their gills, 31% of infested females with mid-development eggs had worms in their gills, and 61% of infested females with late-stage development eggs had worms in their gills. The higher prevalence of worms in the gills in early development followed by a decline there during mid development supports a lag between host oviposition and worm migration. The subsequent increase in worms in the gills in late development indicates that worms may be migrating back into the gills prior to eclosion. Although, worms present in the gills during mid development of the eggs indicates that a portion of worms already in the gills will remain there during embryogenesis; this is suggestive of a bimodal maturation or feeding cycle in these worms.

Mature nemerteans may remain in the gills due to the rapid embryogenesis of blue crabs. A minimum of 7 d passes between blue crab oviposition and eclosion, and females can produce their next egg mass within one week of the prior brood hatching (Hines *et al.*, 2003). Worms may therefore have limited time or need to migrate and feed on every egg mass produced. This is opposed to nemertean species that infest hosts such as the Dungeness crab, which incubates eggs for longer periods, 65–130 d, with a year in between broods (Rasmuson, 2013). Additionally, post-reproductive male *Carcinonemertes epialti* Coe, 1902 stop feeding on eggs and mature females may stop or reduce feeding for multiple weeks after laying eggs (Roe, 1984), precluding the need to feed on each egg mass. Crabs, such as the portunid, *Portunus pelagicus* (Linnaeus, 1758), with similarly short incubation times (10 d), (Ikhwanunddin *et al.*, 2016) also have nemertean worms (*Carcinonemertes mitsukurii* Takakura, 1910) in their gills mid-way through embryogenesis (Shields & Wood, 1993).

A rapid growth of *C. carcinophila*, aligned with the time of embryogenesis of their host, is supported by the size range of worms that are in early development within broods. Worms in these eggs reached 30 mm in length and had a similar size distribution as worms of mid and late staged eggs. Moreover, 96% of blue crabs with late-stage eggs had a nemertean infestation, indicating juvenile nemerteans can recruit to egg masses, grow, and mature to a visible size and color within 7–14 d.

Impacts of nemerteans on host reproduction

Despite similar feeding rates to other nemerteans, *C. carcinophila* exhibited negligible effects on blue-crab batch fecundity. This condition may be attributed to the higher relative fecundity of blue crabs (Hines, 1982), the short embryogenesis time (Hines *et al.*, 2003), and the lower intensity of infestation of *C. carcinophila*, as opposed to the lower relative fecundity, longer embryogenesis, and epidemic infestations levels of *C. errans* Wickham, 1978 in Dungeness crabs (Wickham, 1979b) and *C. regicides* Shields, Wickham & Kuris, 1989 on red king crabs (Kuris *et al.*, 1991). Other species of *Carcinonemertes* contribute substantially to egg mortality, consuming 20-100% of their host eggs (Shields & Kuris, 1988b; Wickham & Kuris, 1989; Baeza *et al.*, 2016; Simpson *et al.*, 2017) with the degree of mortality a function of nemertean prevalence (Shields & Wood, 1993).

Nemertean infestation may increase over the lifespan of the blue crab, resulting in an increase in mortality with subsequent broods. Female blue crabs have a terminal molt to maturity, and therefore do not shed any nemerteans they may accumulate. As such, females with mature nemerteans in their gills (i.e., multiparous spawners) occur throughout the spawning grounds during winter (Schneider *et al.*, 2023). This condition is opposed to other nemertean decapod symbioses, such as in the Dungeness crab, which loses a small portion of *C. errans* during molting (Wickham *et al.*, 1984), or the American lobster, which mechanically preens its eggs to remove nemerteans (Aiken *et al.*, 1985). Although the terminal molt precludes using size as a proxy for age, decapods with indeterminate growth that grow larger with age show a positive relationship between crab size and the intensity of worm infestation (Kuris, 1978; Santos & Bueno, 2001; Dunn & Young, 2013). An accumulation of worms throughout a lifetime indicates that predation and mortality will be more severe for older crabs, contributing to declines in fecundity with sequential broods (Dickinson *et al.*, 2006; Darnell *et al.*, 2009). Adult female blue crabs exhibit a high mortality rate and a short-lifespan in Chesapeake Bay (Lambert *et al.*, 2006), which may prevent nemertean infestations from reaching the level of longer-lived decapods, such as in the Dungeness crab (Wickham, 1979b) or the American lobster (Brattey *et al.*, 1985).

We did not detect a relationship between worm length and feeding rate, which may be attributable to difficulties measuring worms because nemerteans can expand and contract their bodies in peristaltic movements (Humes, 1942). Our measuring technique was standardized but lethal, and therefore not applied to experimental worms. Rather, we chose to measure size using image analysis techniques, as we were unable to control the body position of the worm (extended or compacted). This may have biased worm length measurements.

Although nemertean feeding may not cause substantial brood loss in blue crabs, they may reduce brood health through introduction of fungi and bacteria (Fisher, 1976; Fisher & Nelson, 1977; Wickham, 1979a). Microbes occur naturally in blue crab eggs, with over 650 genera of bacteria present in their microbiomes (J.S. Koshak, J.D. Shields & A. Magee, unpublished data). Moreover, the feces of the worms provide excess nutrients that allow microbial communities to flourish (Fisher, 1976). Nemerteans can also be a direct source of fouling via the production of their mucous sheaths (Wickham, 1979a). Embryos in our experiment were colonized by an oomycete at a higher rate when paired with worms as compared to control embryos. Water molds and worms often co-occur, with nemertean activity preceding mold infection (Rogers-Talbert, 1948). Oomycota, such as *Lagenidium callinectes,* are common in blue crabs, with up to 87% of females naturally infected, and heavy infections causing up to 25% brood mortality (Rogers-Talbert, 1948). The effect of bacteria and fungi could impact feeding rates of nemerteans (Shields & Kuris, 1988a).

We also found evidence that nemerteans may cause egg mortality indirectly by dislodging embryos. The worms are thigmotactic and thus migrate throughout the host eggs. We observed that worms were frequently entangled in eggs, which caused the egg strands to bind into tight clumps. Such activity broke the funiculi (connections that attach embryos to a female's setae) and dislodged the egg from their setal strand. This would likely worsen over the course of development as embryos increase in size and begin competing for space within the brood, increasing the probability that funiculi may break.

Utility of *Carcinonemertes carcinophila* as a biomarker in ovigerous females

We confirm Hopkins (1947) observation that ovigerous blue crabs with mature *C. carcinophila* in their gills have spawned at least once. He posited that the color and size of the worms in the gills may be indicators of the spawning history of the female crabs. Similarities in size distributions between worms in the gills and broods in females in our study support that worms in the gills fed and grew similarly to those in the brood and that *C. carcinophila* does not regress in size in the gills (Hopkins, 1947), as other species do within other species of *Carcinonemertes* (Kuris, 1978). Although juvenile worms can rely on dissolved organic matter to survive (Crowe *et al.*, 1982), they will not reach maturity without consuming host eggs (Kuris, 1978; Roe, 1979); therefore, the presence of mature worms in the gills indicates the host has produced eggs previously.

Previous studies have used nemerteans as a biomarker of spawning history in non-ovigerous females (Schneider *et al.*, 2023), and ovigerous females (Graham *et al.* 2012). Our classification differs from previous work, which categorized ovigerous females as primiparous if the hosts had small white or pink worms in their gills and multiparous if the ovigerous host had large red worms in her gills or brood (Graham *et al.* 2012). Of the worms we studied, four were less than 2 mm, three of which consumed eggs. Humes (1942) found mature worms as small as 0.5 mm, indicating size may be a poor indicator of worm maturity. Further, experimental worms were predominantly pink in coloration through the duration of the experiment. Lastly, the classification of females with worms in their brood as multiparous may erroneously misclassify primiparous females that were recently infested. Some care is therefore required in interpreting parity based solely on the color of the worms in the gills.

Our classification assumes that all female blue crabs are infested with nemerteans while brooding their first egg mass, since few females are without mature nemerteans by the later stages of their egg development. Primiparous females may be erroneously categorized as multiparous if their nemerteans begin migrating to the gills prior to eclosion. Although we believe this is unlikely given that 35% of females with eggs in late stages of development had infestations in their brood only (i.e., nemerteans had not yet migrated to the gills) and this stage of egg development is the shortest in duration (Jivoff *et al.*, 2007).

Overall, the classification of spawning history by infestation site offers a more robust characterization of the host and may be useful in studying the reproductive ecology of the blue crab. We encourage studies of nemerteans and their hosts to provide prevalence data as a function of the site of infestation on the hosts and worm maturity, rather than in aggregate, to improve our understanding of these symbioses and the utility of nemerteans as a biomarker.

Our study is the first to provide prevalence data of *Carcinophila* in ovigerous blue crabs in Chesapeake Bay at the population level and to identify trends in prevalence by site on the host and worm maturity. The ecology of nemerteans can be used to gain

insight into the reproductive ecology of blue crabs, particularly as markers for spawning. Our data indicates that an ovigerous blue crab with mature worms in her gills is multiparous, whereas an ovigerous female with mature worms in her brood only, or is uninfested, is primiparous. *Carinonemertes carcinophila* in blue crabs contribute little to the overall brood mortality of the host; however, more research is needed to identify if nemerteans degrade the quality of a brood via microbial introductions.

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