



Co-infesting symbionts on a threatened marine host: evaluating correlations between an introduced parasitic isopod and a native symbiotic clam

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ABSTRACT: In marine ecosystems, increased global-scale transportation creates opportunities for rapid introduction of invasive parasitic species that, in some cases, result in dramatic shifts within the native communities. A lack of detailed knowledge regarding the ecology of invasive marine parasites hinders our ability to develop effective conservation strategies and avoid unforeseen ecological consequences. We examined co-infestation patterns of a highly pathogenic, introduced parasitic isopod (*Orthione griffenis*) and a native symbiotic clam (*Neaeromya rugifera*) on the North American native blue mud shrimp *Upogebia pugettensis*. Our comparisons included infestations of *O. griffenis* and *N. rugifera* among 447 *U. pugettensis* hosts over 3 study years and were designed to statistically assess whether the 2 symbionts exhibited significant associations with one another. Our results indicate that infestations by the 2 symbiont species are positively correlated, such that the presence of one symbiont is a strong, positive predictor for the presence of the other. For both symbionts, host size is an important factor that drives the observed correlation. Host sex is also influential for *O. griffenis*. Interestingly, even after accounting for these host attributes, the infestations by the 2 symbionts continue to correlate positively, particularly among older (second-year and beyond) symbionts, highlighting the likely influence of additional host and environmental factors in driving the symbiont correlation post-settlement. We consider potential mechanisms, including differential energetic reserves and longevities between infested and co-infested hosts, in detail. These results offer insights into the ecological drivers of symbiont co-infestation, which have important implications for understanding host–parasite interactions and future conservation measures.

KEY WORDS: Introduced parasite · Host–parasite interactions · Coinfection · *Upogebia pugettensis* · *Orthione griffenis* · *Neaeromya rugifera*

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1. INTRODUCTION

Coastal estuarine systems are among the most invaded ecosystems on earth (Cohen & Carlton 1998, Grosholz 2002). Parasites are frequently entrained in human-driven dispersal mechanisms that cross previous natural dispersal barriers (Ruiz et al. 2000). The resulting infections can cause mass mortalities among native populations, change community inter-

actions, and catalyze trophic cascades throughout food webs (Goedknecht et al. 2016, Pascal et al. 2019). Assessing the ecology of invasive marine parasites can be challenging due to their cryptic nature, episodic dynamics, complex life cycles, and wide geographic distributions (Torchin et al. 2002, Kuris et al. 2008), leaving dispersal and establishment mechanisms of invasive parasites underexplored (Zenetos et al. 2005, Goedknecht et al. 2016, Poulin 2017, Bous-

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sellaa et al. 2018). The poor understanding of marine invasive parasite ecology hinders conservation strategies and increases the risks of ecological consequences. We assessed co-infestation patterns of an introduced invasive parasite and a native symbiont across host populations, with the aim of offering insight into mechanisms that impact host selection and survival of symbionts.

In estuaries along the Pacific coast of North America, a well-documented introduced parasite is the bopyrid isopod *Orthione griffenis* Markham, 2004 (Smith et al. 2008, Dumbauld et al. 2011, Chapman et al. 2012). *O. griffenis* infests the gill chambers of reproductive-sized mud shrimp (*Upogebia* and *Austinogebia* species), in which they feed on hemolymph. The first settling isopod larvae (cryptoniscans) on a host develop into females (bopyridans), and later settlers become males (Chapman et al. 2012; Fig. 1A). In North America, *O. griffenis* has established on the native burrowing blue mud shrimp *U. pugettensis* (Dana, 1852) (Fig. 1A) and has spread along the North American coast in nearshore currents (Chapman et al. 2012). *O. griffenis* appears to threaten *U. pugettensis* over the entire geographic range of the mud shrimp (Chapman et al. 2021) that extends between Prince William Sound, Alaska, and Morro Bay, California, USA (Williams 1986).

High-density *U. pugettensis* populations are classified as 'ecosystem engineers' in estuaries due to their

substantial contributions to total macroinvertebrate biomass, species diversity, sediment surface area (Chapman & Carter 2014, Dumbauld et al. 2021), increased nutrient cycling, sediment remineralization (D'Andrea & DeWitt 2009), and as prey for shorebirds (Stenzel et al. 1976) and sturgeon (Dumbauld et al. 2008). *U. pugettensis* live in solitary, Y-shaped burrows and aggregate into dense beds, where their tube galleries can increase the mudflat surface areas by at least 7-fold (Chapman & Carter 2014, Dumbauld et al. 2021). The tube galleries provide unique and productive habitats for dozens of obligate native symbiotic species (Chapman et al. 2012, Li & Ó Foighil 2012). *U. pugettensis* were abundant in intertidal mudflats prior to the 1980s, but population collapses associated with infestation by *O. griffenis* since the 1980s have been extensive (Dumbauld et al. 2011, Chapman & Carter 2014, Asson et al. 2017, Dumbauld et al. 2021). Hemolymph losses to *O. griffenis* effectively castrate *U. pugettensis* females and cause reproductive declines by up to 80% in some populations (Dumbauld et al. 2011). The declining *U. pugettensis* populations are likely to produce major indirect effects on entire estuary ecosystems where they occur.

Despite the severe ecological consequences of *O. griffenis* invasion, the recruitment patterns and host recognition mechanisms of this parasite are poorly understood. Host recognition is likely to be selective; for example, *O. griffenis* infestations among *U. pugettensis* are at least 9 times greater than among its original Asian host, *U. major* (De Haan, 1841), co-occurring on the same North American mudflats (Chapman et al. 2021). *O. griffenis* are approximately twice as frequent among female than male hosts (Asson et al. 2017). In addition, repeated preliminary attempts to infest *U. pugettensis* by injecting cryptoniscan larvae, the final dispersal stage of *O. griffenis*, into the gill chamber of reproductive-size host individuals have not been successful (J. Chapman pers. obs.).

The experimentally injected cryptoniscans (via pipetting in 1 ml of sea water), which are visible through the transparent *U. pugettensis* carapace, are invariably carried passively through and washed out in the *U. pugettensis* respiratory flow within seconds or minutes of introduction. We

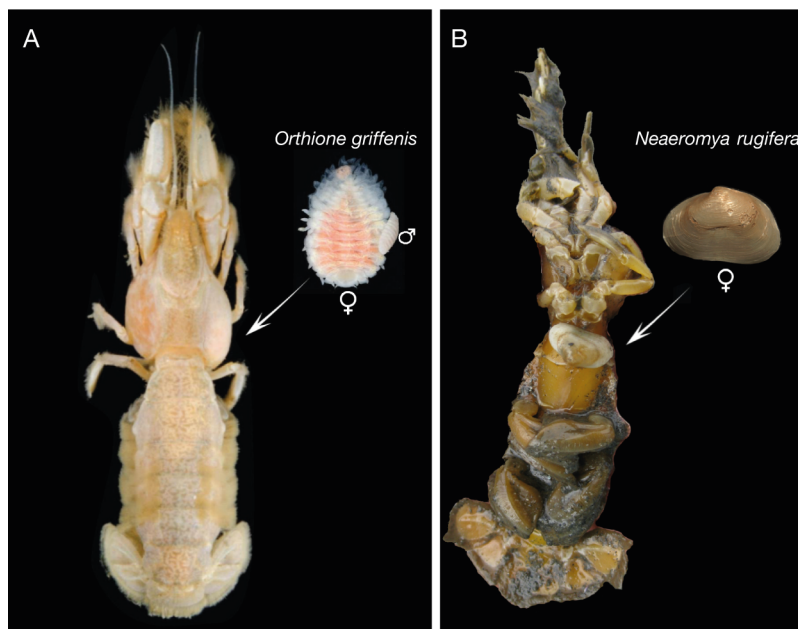


Fig. 1. Native burrowing blue mud shrimp *Upogebia pugettensis* associated with (A) the parasitic isopod *Orthione griffenis* in its gill chambers and (B) the symbiotic bivalve *Neaeromya rugifera* on the abdomen. Photo credits: Gustav Paulay, Jingchun Li

have found no sign that these implanted cryptoniscans recognize their hosts, or vice versa, in the laboratory.

The settling *O. griffenis* cryptoniscans thus may require more than simple host detection. Settlement cues from the host or host environments, that do not appear to occur under experimental conditions, may be required. *O. griffenis* is nevertheless highly effective in colonizing hosts. *O. griffenis* transmission is insensitive to even the most extreme low *U. pugettensis* densities (Dumbauld et al. 2011, Chapman et al. 2012). Critical information gaps thus include which host or environmental factor(s) drive *O. griffenis* larvae to settle on particular hosts.

Interspecific symbiont interactions are a crucial component of host ecology that may influence parasite transmission and host population dynamics. *U. pugettensis* is associated with several native macro-symbionts, including isopods, bivalves, and copepods (Campos et al. 2009, Li & Ó Foighil 2012). Comparing infestation patterns of the native symbionts and *O. griffenis* may inform host-selection mechanisms of this invasive parasite. When a host species is associated with multiple symbiont taxa, aggregation is sometimes observed, i.e. multiple symbiont species co-occur on some host individuals or populations more than expected (Cox 2001, Johnson & Buller 2011, Beugnet et al. 2014, Khokhlova et al. 2015, Deus et al. 2016). This pattern can form through diverse mechanisms (Telfer et al. 2008). Certain host individuals possess biological or behavioral characteristics that can increase their exposure or susceptibility to the infectious stages of multiple parasite taxa. These characteristics can include variations in activity, immunity, body size, sex, developmental stage, and microhabitat use (Beugnet et al. 2014). Prior infestations by one symbiont can increase the likelihood of colonization by other symbionts, resulting in aggregation (Khokhlova et al. 2015). Multiple symbionts can also correlate negatively in their occurrences among hosts due to resource competition, cross-reactive immune responses by the host, symbiont predation, or elevated mortality among co-infected hosts (Downes 1986, Jackson et al. 2006, Roe et al. 2011). Preliminary analyses by dissection and external examinations of thousands of *U. pugettensis* have not revealed any other external or internal symbionts other than *O. griffenis*, the rare native bopyridan *Phyllodurus abdominalis* Stimpson, 1857 and the native bivalve *Neaeromya rugifera* (Carpenter, 1864). Currently, it is unclear how *O. griffenis* infestations are associated with other symbionts, which is an important aspect of its invasive biology.

N. rugifera is a common obligate native symbiont of *U. pugettensis*. *N. rugifera* disperses to new hosts via free-swimming pelagic trochophore larvae that morph into veligers, which initially settle under the *U. pugettensis* carapace. The settling veligers then use byssal threads to securely attach to the ventral host surface (Fig. 1B). *N. rugifera* is a protandrous hermaphrodite. Individuals >6 mm in length become females. *N. rugifera* that settle onto shrimp already hosting female clams become dwarf males (<1.25 mm) that are taken inside the mantle cavity of the female clams (Ó Foighil 1985). *N. rugifera* live only with their host and depend entirely on suspended food supplied from the water-pumping activities of the mud shrimp. *N. rugifera* are unlikely to significantly impact the physiological functions of the host and thus are commonly classified as a commensal species (Lafferty 1993). Given that the impact of *N. rugifera* on host fitness is poorly resolved, we nevertheless refer to it as a symbiont instead of as a commensal from here on. The prevalence of *N. rugifera* on different *U. pugettensis* populations is poorly known. Also, whether the presence of *N. rugifera* affects the settlement of *O. griffenis* has never been examined.

We addressed these information gaps by examining natural co-infestation patterns of *O. griffenis* and *N. rugifera* among 447 *U. pugettensis* hosts and using statistical models to test whether the probability of co-infestation is impacted by host size, host sex, or parasite development stages. A simple null hypothesis is that the bivalve and the isopod have minimal interactions with each other. Under this hypothesis, we predict that the distributions of these 2 symbionts are not significantly correlated. However, the greater prevalence of *O. griffenis* observed on large reproductive hosts indicates that these *U. pugettensis* individuals have a greater probability of supporting symbionts. Thus, if specific host traits positively influence the probability of infestation for both symbionts, we predict that infestations of *O. griffenis* and *N. rugifera* will be aggregated and that host traits are more likely to account for the correlation. Similarly, if these symbiont species attract each other, we predict aggregation of the 2 symbionts, although in this situation host traits alone will not be sufficient to explain the correlated presence. Alternatively, if symbionts have differing host preferences, or co-infested hosts suffer higher mortality, *O. griffenis* and *N. rugifera* infestations could be negatively correlated. Collectively, our analyses offer valuable insights into the environmental- and host-level drivers of co-infestation that are required for understanding patterns of host pathology and parasite transmission.

2. MATERIALS AND METHODS

2.1. Collections and specimen processing

We surveyed *Upogebia pugettensis* and associated *Orthione griffenis* and *Neaeromya rugifera* collected from a dense *U. pugettensis* population in the Idaho mudflat of Yaquina Bay, Oregon, USA (44.619°N, 124.041°W). Collections were made using a hand-held 15 cm diameter by 40 cm length corer. Coring first to a 40 cm depth followed by coring again at the bottom of the initial core hole permitted sampling to approximately 80 cm depths.

We sampled the Idaho flat population between 5 and 22 July 2016; between 9 May and 3 July 2018; and on 8 July 2019. Recovered shrimp were rinsed and purged of adhering sediments, placed into individual plastic bags with unique labels, and frozen for at least 24 h to fix attached commensals and parasites, and prevent them from escaping. The thawed shrimp were processed within less than 24 h and refrozen or preserved in 70% ethanol. Every shrimp was inspected directly for large and external symbionts and then under 6–10× magnification for small symbionts. The gill chambers and external surfaces were then vigorously flushed using a squirt bottle into a 100 mm petri dish. All organisms washed out by this process were counted, identified under 6–40× magnification, measured using a calibrated microscope, and then preserved in a sample vial using 70% ethanol. Shrimp were then refrozen or transferred into 70–95% ethanol for long-term preservation at the Hatfield Marine Science Center, Oregon State University.

We distinguished *U. pugettensis* sexes by the first pleopods (present on mature females and absent on mature males), and from female gonopore (on the distal, medial female pereopod 3 coxa) and male gonopore (on the distal, medial corner of the male pereopod 5 coxa). Hermaphroditic *U. pugettensis* (bearing male and female gonopores) were not found. We estimated shrimp size from carapace length (CL) measured to the nearest 0.5 mm from the distal tip of the rostrum to the posterior carapace edge. Body lengths and widths of large *O. griffenis* and shell lengths of large *N. rugifera* were measured to the nearest 0.1 mm using calipers. Body lengths of small *O. griffenis* and *N. rugifera* were measured through a calibrated microscope grid.

We did not dissect *N. rugifera* to find and count dwarf males within females. However, the dwarf males always coexist with the females, and we used only the presence or absence of *N. rugifera* in our co-infestation models. The omissions of *N. rugifera* males from our analyses are therefore unlikely to have affected our main results.

2.2. Statistical modeling

We tested for associations between symbionts using generalized linear mixed effects models (GLMMs) with a binomial distribution. These analyses considered only the presence of each parasite for predicting the occurrence of the other, rather than the number of symbionts per host, because there is little variation in the number of symbionts per host. The infested host either harbors 1 female *N. rugifera*, a pair (male and female) of *O. griffenis*, or both symbiont species. We subsequently incorporated host-level characteristics, including size (CL, log₁₀-transformed) and host sex. Year of sampling was included as a random intercept term to account for non-independence in time, for which we used the 'glmer' function in the R package 'lme4' (Bates et al. 2014).

To further explore how symbiont co-infestation varied as a function of symbiont age, we examined size frequencies of both symbionts and identified age cohorts based on body size. We assumed that first-year *N. rugifera* age classes were <2 mm in length and that first-year *O. griffenis* age classes were <4 mm in length (Fig. 2) based on their size-frequency distributions (see also Section 3). By separating out first-year symbionts from those that were older (second year or beyond), we examined whether associations differed between newly colonizing sym-

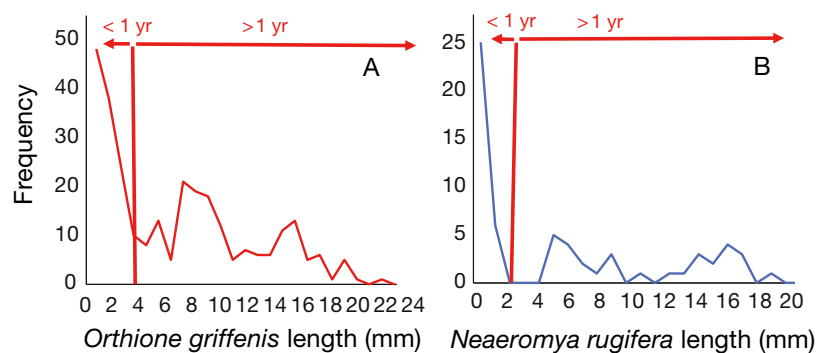


Fig. 2. Length frequencies of (A) *Orthione griffenis* and (B) *Neaeromya rugifera* among all hosts. *O. griffenis* <4 mm and *N. rugifera* individuals <2 mm in length are classified as first-year cohorts

bionts versus older more established individuals, thereby offering insights into how patterns of co-infestation initiated and ultimately shifted through time.

Our analyses were based on 70 first-year and 67 larger (second-year or older) *O. griffenis* individuals (up to 23.6 mm length). For *N. rugifera*, the dataset included 23 recently established individuals and 25 older individuals (up to 21 mm length). We tested how infestation by each symbiont size class (small vs. large) covaried with the presence of the other symbiont using GLMMs. Once again, host body size and sex were included as fixed effects while sampling year was a random intercept term. Likelihood-ratio tests were used to assess the significance of individual predictors and guide model simplification.

Among infected hosts, we further evaluated how the sizes of each symbiont species varied with host body size, infestation load (within each symbiont species), and the presence of the other symbiont. We specifically tested how the size of a given symbiont related to the CL of the host, the number of conspecifics on the same host, and whether the other symbiont species co-occurred. Host individual was incorporated as a random intercept term to account for multiple symbiont observations per host. We used linear mixed effects models with a Gaussian distribution in the package 'lme4' to construct the models, followed by likelihood-ratio tests to evaluate the significance of individual predictors relative to a reduced model without that predictor. All analyses were conducted in the R Computing Environment (Version 3.6.3, R Core Team 2019).

3. RESULTS

3.1. Symbiont distributions

Our host sample consisted of 447 *Upogebia pugettensis* (218 females and 229 males) collected in 3 years (2016, $n = 209$; 2018, $n = 152$; 2019, $n = 86$). Of these *U. pugettensis*, 48 (10.7%) supported *Neaeromya rugifera*, and 137 (30.6%) were infested by *Orthione griffenis*. Thirty-one hosts (6.9%) were co-infested with both symbionts. The maximum and average number of *O. griffenis* per host were 8 and 0.63, respectively, while the maximum and average *N. rugifera* were 6 and 0.14, respectively. Among female hosts, 71 were infested by *O. griffenis* only, 10 were infested by *N. rugifera* only, and 20 were co-infested. For male hosts, the numbers were 35, 7, and 11, respectively.

The symbiont size-frequency plots are shown in Fig. 2. The mean \pm SE sizes of male and female hosts were 18.6 ± 0.41 and 22.3 ± 0.38 mm, respectively. For both *O. griffenis* and *N. rugifera* populations, a first-year settling cohort is apparent within a striking first-year size-frequency peak. *N. rugifera* <2 mm in length and *O. griffenis* <4 mm in length are considered first-year settlers. Older symbionts occur within less well-defined larger size-frequency modes and are all defined as 'beyond 1 yr old'.

3.2. Co-infestation models

The 2 symbionts correlated positively in their occurrence (Fig. 3), such that the presence of one symbiont was a strong, positive predictor for the presence of the other (GLMM: coefficient_{clam infestation} = 1.727 ± 0.334 , $z = 5.168$, $p < 0.00001$). Exponentiating the coefficient to derive the odds ratio, we found that mud shrimp supporting *N. rugifera* were 5.6 \times more likely to also be infested with 1 or more *O. griffenis*.

This relationship persisted when additional host-level predictors were incorporated. Host body size

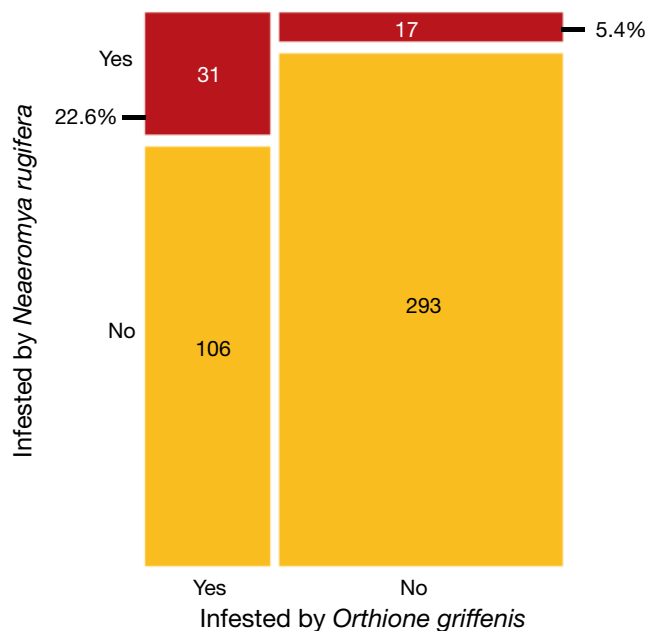


Fig. 3. Mosaic plot illustrating the positive association between infestation of blue mud shrimp *Upogebia pugettensis* by *Orthione griffenis* and *Neaeromya rugifera*. The numbers on the rectangles and rectangle sizes indicate number of host individuals infested or uninfested by the 2 symbionts, respectively. If the 2 symbiont infections were independent, the 2 red rectangles would be roughly the same size. However, we see a higher proportion [$31/(31+106) = 22.6\%$] of *N. rugifera* associated with hosts already infested by *O. griffenis*, compared to the proportion [$17/(17+293) = 5.4\%$] of *N. rugifera* on mud shrimp that are not infested by *O. griffenis*.

(CL) was a consistently positive predictor of infestation status for both symbionts, while female shrimp were more likely to be infected with isopods (Fig. 4; Table A1 in the Appendix). For each 1 mm increase in CL, the odds of infestation increased by a factor of approximately 1.2× for both isopods and clams. However, even with terms for host size and sex in the model, co-infested hosts occurred significantly more often than expected. Thus, after accounting for CL and host sex, the presence of one symbiont species increased the likelihood of detecting the other by ~3-fold.

When we categorized symbionts into size classes (i.e. small or first-year symbionts versus larger, more established individuals), the non-random association between symbionts was greatest for the larger size classes (Fig. 4; Table A2), i.e. co-infestation positively predicted the presence of large symbionts specifically, even after inclusion of host CL (Fig. 4). No such relationship was detected for small (first-year) symbionts, suggesting that initial colonization by symbionts occurred independently of one another after accounting for host size.

For both symbionts, host body size was a positive predictor of infestation for both size classes (Fig. 4). The strength of this relationship was greater for larger, more established symbionts relatively to newly colonizing individuals, i.e. larger symbionts occurred primarily on larger hosts, whereas small symbionts occurred on hosts across a range of sizes (see ‘ceiling effect’ in Section 3.3). Indeed, the estimated coefficient for host size was approximately 2.7× greater for large clams relative to small clams. Female mud shrimp were also more likely to be infested with large isopods, whereas sex was generally non-significant for other models.

3.3. Symbiont–host size relationship

Among the 283 examined isopods and 62 clams with measured lengths, host body size had a broadly positive influence on symbiont size. Interestingly, both symbionts showed a strong ‘ceiling effect,’ such that large hosts supported both small and large symbionts, whereas small hosts only supported small symbionts (Fig. 5). This suggests a constraint on symbiont body sizes in relation to host body size (or age); while colonizing symbionts can likely settle on hosts of any size, larger (and older) symbionts will only be detected upon larger hosts.

For *O. griffenis*, host body size had a positive effect on isopod body size, while total parasite load (number of *O. griffenis* per host) had a negative effect (GLMM: coefficient_{host size} = 0.112 ± 0.021, $z = 5.321$, $p < 0.00001$; coefficient_{isopod load} = -0.053 ± 0.0139, $z = -3.833$, $p = 0.0005$). However, given that *O. griffenis* typically occur in male–female pairs, and the male is significantly smaller than the female, this pattern is likely due to more male/immature isopods on the same host, rather than to density-dependent isopod growth. There were no significant effects of co-infestation by *N. rugifera* or host sex ($p > 0.4$). We did not observe significant interactions between parasite load and host body size. The overall mean ± SE isopod size was 7.39 ± 0.354 mm.

For *N. rugifera*, the best-fitting model included only a positive effect of host body size on clam size (GLMM: coefficient_{host size} = 0.188 ± 0.0555, $z = 3.396$, $p = 0.0014$). We did not detect any effects associated with clam load, the presence of *O. griffenis*, host sex, or the interaction between host size and clam load ($p > 0.1$). The overall mean clam size was 6.37 ± 0.864 mm.

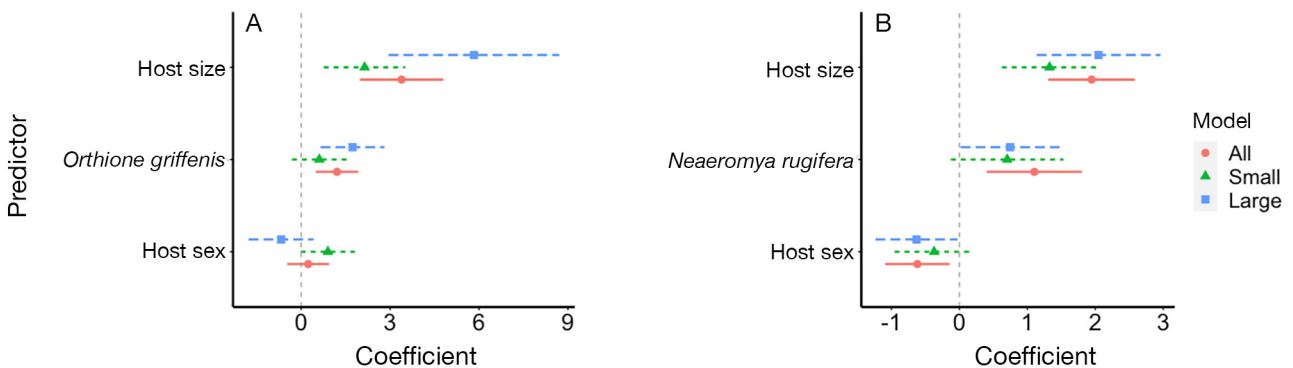


Fig. 4. Coefficient plots of different predictors from the generalized linear mixed models. (A) Coefficients of host size, sex, and presence of *Orthione griffenis* on infestation by all, small, and large *Neaeromya rugifera*. (B) Coefficients of host size, sex, and presence of *N. rugifera* on infestation by all, small, and large *O. griffenis*. Coefficients with error values that do not overlap 0 are significant

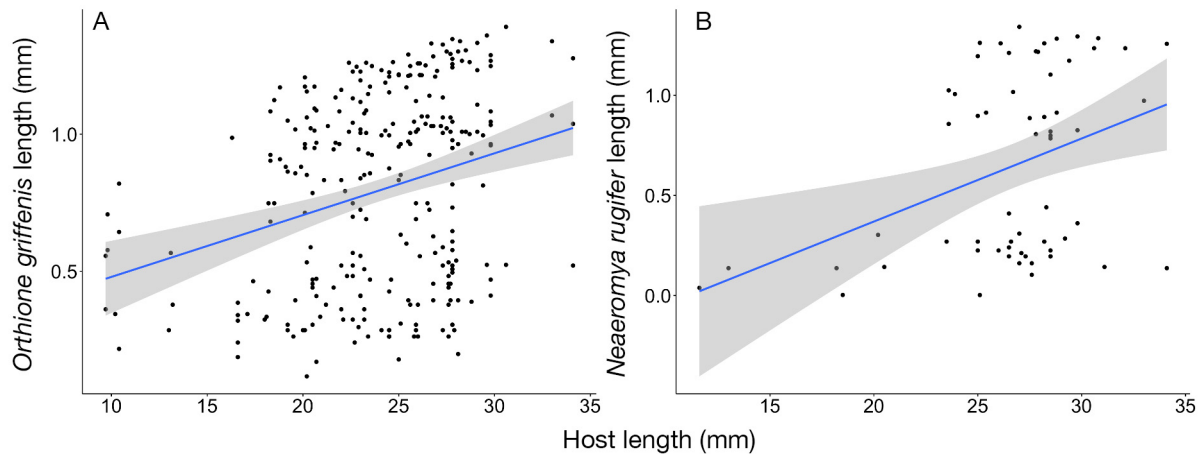


Fig. 5. Relationships between host body size (carapace length) and sizes of (A) *Orthione griffenis* and (B) *Neaeromya rugifera*. Note a strong 'ceiling effect,' whereby large hosts supported both small and large symbionts, whereas small hosts only supported small symbionts

4. DISCUSSION

Growing research interest is being focused on the ecological implications of symbiont co-occurrence and co-infection (Pedersen & Fenton 2007, Johnson & Hoverman 2012, Viney & Graham 2013). Most naturally occurring hosts are concurrently infected by multiple parasitic or commensal organisms, and interactions within this symbiont community can have important implications for host pathology, pathogen transmission, and evolution (Viney & Graham 2013). Our examination of 447 mud shrimp *Upogebia pugettensis* hosts indicated that the presence of the parasitic isopod *Orthione griffenis* and that of the commensal clam *Neaeromya rugifera* were strongly positively correlated; the 2 symbiont species co-occurred on the same host $\sim 5.6\times$ more often than expected based on a random distribution of symbionts across hosts. This pattern was broadly consistent across the 3 sampling years.

The aggregation of *N. rugifera* with *O. griffenis* is all the more remarkable because increased mortality, which we expect of *O. griffenis*-infested hosts, could only reduce the co-occurrences of *N. rugifera*. To better understand the potential drivers and implications of *U. pugettensis* symbiont co-infestation, we incorporated host-level covariates (CL, sex) to evaluate whether attributes of the host might jointly drive symbiont infestations, independent of co-infestation. For both symbionts, host size was a strong predictor for infestation, while host sex was also influential for isopods, with females tending to support a higher probability of infestation. Interestingly, even after accounting for these host attributes, we continued to

detect a significant signal of co-infestation among larger (presumed older) symbionts, such that the presence of one symbiont was positively predicted by the other.

The strong, positive influence of host body size on the probability of infestation (and co-infestation) is broadly consistent with previous research. Numerous analyses of diverse systems have demonstrated that host body size is an important variable that impacts host-parasite interactions. Examples include a meta-analysis incorporating 62 studies in which host size was strongly positively correlated with parasite species richness among host species (Kamiya et al. 2014). Host size is also positively correlated with parasite diversity per host (Guégan & Huguény 1994, Patterson et al. 2008). These patterns are likely results of multiple, species-specific, underlying mechanisms. For example, larger hosts may generate stronger chemical cues or represent more prominent physical targets for symbionts, increasing the probability of multiple symbiont species settling on the same host (Sikkel et al. 2011, Cook & Munguia 2013). Larger hosts also tend to be older, resulting in more time to accumulate infestations. Larger shrimps also pump more water through their burrows and thus increase the chances of symbiont encounter (Griffen 2009). Lastly, greater tolerances of larger hosts to multiple symbionts could also contribute to their over-representation on those hosts.

The isopod and clam association remained even after accounting for host size, emphasizing the importance of additional factors in explaining co-infestation patterns. This continued positive association could stem from 2 mechanisms: (1) both sym-

bionts selectively settle on particular hosts due to ecological factors other than host size or sex, i.e. differential symbiont settlement; or (2) hosts with particular traits or living in specific habitats can maintain more symbiont individuals, leading to an aggregated infestation, i.e. differential symbiont survival. These 2 driver categories are not mutually exclusive and are likely to require experimental manipulations, detailed longitudinal data, and information on host and symbiont ages to resolve.

We partially addressed how the above 2 mechanisms could affect the observed co-infestations by comparing symbiont cohort sizes. If symbiont aggregation is caused by enhanced larval settlement on preferred hosts/habitats, then first-year-sized isopods and clams would show signs of aggregation after host size has been accounted for. Alternatively, if the aggregation is mainly caused by differential host/parasite persistence post-establishment, then settlement by new symbionts should be relatively random after accounting for host size. Our results supported the second scenario. The first-year symbiont aggregation was driven by host body size only, with no residual influence on co-infestation. For older symbionts, however, infestation continued to be positively predicted by the presence of the other symbiont species. This suggests that the non-random symbiont aggregation develops post-settlement (although differential symbiont growth rates could bias our age estimates, which depended solely on size).

At least 2 possible explanations may help explain the post-settlement aggregation. The first option is that there are additional host-related attributes that we did not capture in our surveys, which could influence the survival of the 2 symbionts. For example, the biomass of a 28 mm *U. pugettensis* is 8 times greater than that of a 14 mm shrimp (J. Chapman pers. obs.). Host energetic reserve may therefore play important roles in host and symbiont survival following infestation. Unhealthy, immune-compromised, or starved hosts may not have the ability to support multiple symbionts or even *O. griffenis* infestations alone. Although poorly resolved, *O. griffenis* generate heavy metabolic costs that are sufficient to effectively castrate their hosts. Hosts with lower energetic reserves may also pump less water (Pascal et al. 2019, 2020), and thus reduce food resources for the filter-feeding *N. rugifera*. This might also explain why for larger (older) *O. griffenis*, female hosts, which tend to have higher energetic reserves relative to males, were more likely to support infestation, even after controlling for host size and *N. rugifera* presence. Energetic reserves in the

host burrow microbial communities could also influence the ability of hosts to support symbionts. The *U. pugettensis* burrow includes a unique microbiome (Li et al. 2020), for which mutualistic metabolic relationships could provide host nutritional benefits and indirectly support more symbionts.

Direct and indirect interactions between the symbionts can also affect aggregation. We do not have evidence supporting direct symbiont interactions, and tests for direct interactions without experimental manipulations will be challenging (Fenton et al. 2014). In addition, the 2 symbionts have not interacted evolutionarily until the recent introduction of *O. griffenis*. It is unlikely that they have evolved mechanisms to attract or avoid each other. The shared preferences of certain host types are likely a result of convergent evolution. Nevertheless, comparisons of associations among symbionts through time can help to generate hypotheses into indirect interactions between co-infesting symbionts. Indirect interaction could involve changes in host survival or growth in response to infestation. Reduction in host growth by *O. griffenis* could, for example, increase aggregation of *N. rugifera* among host size classes that include shrimp older than their CLs might indicate. Parasite-induced changes in host longevity could be another mechanism for indirect symbiont interactions. Some parasites are likely to increase host mortality, while other parasites may extend host life spans by diverting energy allocations away from stressful activities such as reproductive effort (Hurd et al. 2001). Effective castration by *O. griffenis* could thus lead to longer host life spans and greater chances of being colonized by *N. rugifera*. Additionally, hosts infested with one symbiont might change their behavior or chemical cues that enhance the probability of settlement by the other co-infesting symbiont. Experimental manipulations could facilitate distinctions among these possibilities.

Symbionts could potentially increase aggregation by switching to a more desirable host. However, there is no direct evidence of host switching. Settled *N. rugifera* and *O. griffenis* have limited mobilities. *U. pugettensis* live in solitary burrows that are interconnected only for mating or, possibly, conflict. Switching hosts after attachment requires the clam or isopod to recognize a new 'preferred' host (energy reserve, microbiome composition, or other factors) and then to quickly detach from an existing host and reattach to the new host. In preliminary observations, directly transplanted *O. griffenis* were immediately removed by the *U. pugettensis* pereopods. Detached *N. rugifera* reintroduced to their original hosts, which were confined within vinyl tubing in a flow through

seawater system, failed to reattach in a 24 h observation period (J. Chapman pers. obs.). This observation is consistent with a study of an Asian commensal bivalve (*Peregrinamor ohshimai*) on *Upogebia* (Itani et al. 2002). Itani et al. (2002) performed a detached bivalve experiment with 20 replicates and found that the detached bivalves were not able to reattach to *Upogebia*. The host consistently picked up the bivalve and cast it away from the tube. It was concluded that 'migration of a bivalve to another host individual is not likely in nature' (Itani et al. 2002, p. 75). Given that lab experimental conditions do not resemble the natural habitat, more extensive observations are needed before the 'host-switching' mechanism for aggregation can be entirely discounted, but we currently have no evidence that it occurs.

O. griffenis and *N. rugifera* sizes are positively correlated with host CL. Thus, larger hosts were more likely to be infected, and the symbionts they supported were typically larger in size. Both symbionts also showed strong evidence of a 'ceiling effect', for which small symbionts could be found on small or large hosts but larger symbionts occurred predominantly on larger hosts. The *U. pugettensis* age and size relationship remains poorly resolved (Dumbauld & Bosley 2018). However, the simplest explanation for the aggregation patterns we have resolved is likely to depend on host age, i.e. symbionts on larger/older hosts are likely to have had more time to grow. However, it is also possible that the energetic resources necessary for symbiont growth are limited on small hosts, or that symbionts have a greater chance of killing the host if they grow too fast/large, either of which could generate a similar ceiling effect. These lines of evidence are consistent with differential symbiont survival mechanisms affecting *U. pugettensis* symbiont infestation patterns.

Understanding host-symbiont interactions in *U. pugettensis* has important conservation implications for this species. This native mud shrimp is at risk of being replaced by the invasive introduced species, *U. major*. *U. major* is a native host of *O. griffenis* that has displaced or replaced *U. pugettensis* in the San Francisco Bay area since its introduction around 2007 (Chapman et al. 2021). Comparisons of *O. griffenis* and *N. rugifera* infestations between co-occurring *U. pugettensis* and *U. major* populations are needed to assess their impacts on the native and introduced hosts. *U. major* is less vulnerable to *O. griffenis* than *U. pugettensis* but possibly more vulnerable to *N. rugifera* (Chapman et al. 2021). The presence of *U. major* assures *O. griffenis* persistence even where *U. pugettensis* has been extirpated. *U. major* has the same distribution in Asia

as *O. griffenis* and thus can potentially invade all areas in North America that are presently invaded by *O. griffenis* (Punta Banda, Mexico, to Sitka, Alaska) (Chapman et al. 2021). *U. pugettensis* and its associated native symbionts are thus threatened first by *O. griffenis* and now by *U. major*. Greater understanding of *U. pugettensis* ecology is needed to aid in its conservation but also, more hopefully, to assess whether *U. pugettensis* can evolve or adapt to *O. griffenis*.

5. CONCLUSIONS

Our results show that differential symbiont settlement and survival are important drivers of symbiont aggregation on *Upogebia pugettensis*. Larger mud shrimp hosts attract and accumulate more symbionts. In addition, *Orthione griffenis* are likely to impact host survival, growth, and resource allocation and thereby alter probabilities of co-infestation. Further experimental manipulations are needed to distinguish among these potential mechanisms and their consequences for co-infestation, host fitness, and conservation. Tests of how host body energetics, water flow, or host-microbial interactions impact symbiont larval settlement and growth using common garden experiments, for example, would be particularly valuable. Future studies should also focus on understanding how the parasite and symbiont species differentially impact host growth, given their drastically different host energy needs.

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Appendix. Additional results

Table A1. Results of generalized linear mixed models assessing overall *Orthione griffenis* and *Neaeromya rugifera* infestation in response to host size (carapace length, which was \log_{10} -transformed [+1] and scaled for inclusion in the models), sex, and symbiont presence

Response variable	Predictor	Coefficient \pm SE	z	p
<i>O. griffenis</i> infestation	Carapace length	0.973 \pm 0.163	5.980	<0.001
	Clam infestation	1.103 \pm 0.357	3.086	0.002
	Sex [male]	-0.618 \pm 0.242	-2.558	0.011
<i>N. rugifera</i> infestation	Carapace length	1.693 \pm 0.359	4.722	<0.001
	Isopod infestation	1.209 \pm 0.365	3.318	0.001
	Sex [male]	0.235 \pm 0.360	0.652	0.514

Table A2. Results of generalized linear mixed models assessing small (first-year) and larger (older) *Orthione griffenis* and *Neaeromya rugifera* infestation in response to host size (carapace length, which was \log_{10} -transformed [+1] and scaled for inclusion in the models), sex, and symbiont presence

Response variable	Predictor	Coefficient \pm SE	z	p
Small <i>N. rugifera</i> infestation	Carapace length	1.069 \pm 0.351	3.05	0.0023
	Isopod infestation	0.611 \pm 0.469	1.302	0.193
	Sex [male]	0.899 \pm 0.466	1.93	0.054
Large <i>N. rugifera</i> infestation	Carapace length	2.914 \pm 0.735	3.965	<0.001
	Isopod infestation	1.732 \pm 0.551	3.147	0.0017
	Sex [male]	-0.677 \pm 0.560	-1.207	0.227
Small <i>O. griffenis</i> infestation	Carapace length	0.664 \pm 0.179	3.704	0.0002
	Clam infestation	0.704 \pm 0.423	1.663	0.096
	Sex [male]	-0.373 \pm 0.296	-1.258	0.208
Large <i>O. griffenis</i> infestation	Carapace length	1.025 \pm 0.233	4.407	0.00001
	Clam infestation	0.747 \pm 0.372	2.009	0.045
	Sex [male]	-0.631 \pm 0.308	-2.05	0.040

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