VARIATION OF LARVAL TRAITS AND COPPER TOLERANCE IN AN INVASIVE CRYPTIC SPECIES COMPLEX (*WATERSIPORA*: BRYOZOA)

By

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ABSTRACT

VARIATION OF LARVAL TRAITS AND COPPER TOLERANCE IN AN INVASIVE CRYPTIC SPECIES COMPLEX (*WATERSIPORA*: BRYOZOA)

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Many factors contribute to the potential of a non-indigenous species to invade an area and become established. For bryozoan colonies of the cryptic species complex *Watersipora* (Neviani, 1896), this may include larval characteristics such as settlement rate, competency of metamorphosis, swimming duration, and the ability to tolerate copper, a common component in marine anti-fouling paints. Two common groups of *Watersipora* that occur along the California coast are *W. subatra* Clade A and an undescribed new species, Clade N. The goal of this research work was to discover what differences, if any, exist in the larval traits and copper tolerances of these two clades. Colonies of Clade A and N were collected around Humboldt Bay and induced to release larvae. Individual larvae were pipetted into petri dishes with either a circle of copper paint or an unpainted control and placed in a common-garden experiment where larval characteristics were measured between species and experimental treatments. Both species had markedly different larval characteristics, with *W. subatra* settling faster and at a higher rate than Clade N in the control treatment. When exposed to copper anti-fouling paint, however, these trends reversed. This study is the first to investigate larval differences between these two species. A number of studies on bryozoans are presumed,

but not verified, to be *Watersipora subatra*. This fact, coupled with the strong observed differences in larval behavior that these results show, suggests that a *Watersipora* species-specific approach needs to be taken in future work with this cryptic species complex.

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No researcher is an island and there were many people that helped to make this research work possible. Reginald Blackwell III taught me how to perform the molecular

genetic work necessary to differentiate the two species of *Watersipora* in my study and allowed me to pass on this information to a number of lab assistants who, in turn, used this experience for their own research work or professional development. I'd like to thank Susan Wright for always taking time from her busy schedule to help out with the numerous chemical and material needs that this project had. Thanks also go to Dave Baston who oversaw the Core Research Facility during this laboratory portion of my research work.

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INTRODUCTION

Non-indigenous species (NIS) are a threat to biodiversity in many ecosystems throughout the world (Ruiz et al. 1997; Molnar et al. 2008). NIS also pose significant economic costs either directly, through management efforts (Lovell et al. 2006), or indirectly through other mechanisms such as loss of ecosystem services, tourism and recreation opportunities, and lowered climate resilience (Katsanevakis et al. 2014). Despite the importance of combatting the spread of NIS in marine environments, there remain numerous challenges to addressing questions of their management. One such challenge is the lack of clarity around what the driving factors of invasion patterns are. Ruiz et al. (2000) grouped hypotheses addressing observed invasion patterns into three main categories: (1) supply and quality of NIS propagules, (2) biases in data, and (3) invasibility of environments and resistance by NIS to environmental conditions.

The supply and quality of propagules involves many factors, both anthropogenic and biological. Anthropogenic factors include aspects such as the frequency of shipping traffic (Ruiz et al. 1997; Seebens et al. 2013), which can allow "hitchhiking" organisms to travel to a new area through either ballast water (Gollasch 2002; Verling et al. 2005) or by settling on a ship's hull and releasing larvae in a new port (Godwin 2003; Piola and Johnston 2008a). Ruiz et al. (2015) estimated that upwards of 82 percent of initial invasion events over the last three decades were due to commercial shipping and shipping traffic continues to be a primary driver of NIS introduction (Iacarella et al. 2020; Costello et al. 2022). Biological factors may include the production of numerous larvae (Clark and

Johnston 2009; Johnston et al. 2009), the size of those larvae (Marshall et al. 2003; Burgess et al. 2009), the duration of larval swimming and thus their ability to passively disperse on currents and find appropriate habitats (Orellana et al. 1996; Burgess et al. 2009).

Biases in data may be the result of organismal size, as a smaller or less conspicuous invader is less likely to be noticed than a large one (Ruiz et al. 2000) and invasions may go unnoticed. Another factor is the amount and quality of research work in an area. For example, an invader in San Francisco is more likely to be noticed and documented than one in a small coastal town (Ruiz et al. 2015). Additionally, species that are not easily distinguishable from each other pose a unique challenge as genetic methods may be required to resolve identities in cryptic invasive species (Geller et al. 2010; Viard et al. 2019).

Invasibility and resistance as a driver of invasion patterns can involve biotic and abiotic elements. Biotic factors effecting habitat resistance are diversity and predation (Noè et al. 2018), resource competition (Comerford et al. 2020), and others. Abiotic factors may be resistance to environmental stressors such as salinity, metal pollution, and temperature (Piola and Johnston 2008b; Crooks et al. 2010; Lenz et al. 2011) or the ability to withstand heavy sedimentation (Houle 2015).

In particular, the ability to withstand metallic pollutants such as copper in an aquatic environment can confer a major advantage to spreading non-indigenous species (Piola and Johnston 2008b; Piola et al. 2009; McKenzie et al. 2011) and may explain why some NIS are more successful at invading than others (McKenzie et al. 2011; Mckenzie

et al. 2012). This can be especially true for invasion into bays and estuaries, long known to be hotspots for NIS (Cohen and Carlton 1995; Carlton 1996; Cohen and Carlton 1998), and which may have elevated levels of dissolved copper in the seawater (Valkirs et al. 2003; Schiff et al. 2004). Much of this copper comes from antifouling paint on boat hulls, where it is used to ward off settlement of "fouling" marine invertebrates and algae (Valkirs et al. 2003; Schiff et al. 2004; Turner 2010).

The high copper concentrations of marinas and harbors within bays are associated with reduced native diversity and change in fouling community structure (Piola and Johnston 2008b; Piola and Johnston 2009; Canning-Clode et al. 2011; Susick et al. 2020). While copper tolerance is a frequently observed trait across many NIS, it is less often found in native species (Dafforn et al. 2009; Piola and Johnston 2009; Crooks et al. 2010). Moreover, increased copper levels in the water may actually enhance the success of marine invaders (Mckenzie et al. 2012). One particular group of marine invertebrates that displays a high degree of invasiveness as well as copper tolerance are Cheliostome bryozoans in the genus *Watersipora* (Ryland et al. 2009; McKenzie et al. 2011; Mckenzie et al. 2012).

Watersipora is a good study group for investigating marine invasions as it typifies aspects of all three groups of Ruiz et al.'s explanatory hypotheses: high propagule pressure through abundant production of larvae and multiple invasion events (Carlton and Geller 1993, Ryland et al. 2009), survivability in new habitats/invasiveness (Mckenzie et al. 2012; Houle 2015; Korcheck 2015; Lauer 2016), and it is a cryptic species complex

that requires genetic tools to differentiate to species (Mackie et al. 2006; Láruson et al. 2012; Mackie et al. 2012).

The lecithotrophic larvae of *Watersipora* generally settle within 24 hours of release (Lynch 1947; Marshall and Keough 2003; Ng and Keough 2003), although they have been observed settling after as long as 72 hours of release in the lab (personal observation). Larvae can be introduced to a new area through ballast water (Carlton and Geller 1993) or through hull fouling, as adults are tolerant of copper anti-fouling paint (Piola et al. 2009). Small levels of copper may even induce settlement (Ng and Keough 2003; Piola and Johnston 2006; McKenzie et al. 2011). Hence, exposure of *Watersipora* larvae to copper could increase ability of the genus to invade (Mckenzie et al. 2012) by increasing the chances of a larva settling on a ship hull, despite the presence of anti-fouling paint.

Once Watersipora larvae are on/in a ship, greater rates of boat traffic allow for an increased chance of multiple introductions into a new site, and may also help these populations overcome an "Allee" effect, by introducing many potentially different genotypes which can interbreed (Leung et al. 2004). If larvae have evolved increased resistance to the toxic effects of copper and are able to use the presence of copper as a settlement cue, this may offer a mechanism for overcoming the Allee effect and explain why some *Watersipora* species are such successful invaders.

Bryozoan larval traits can have profound effects on settlement (Marshall and Keough 2003; Gribben et al. 2006; Burgess et al. 2009; Marshall and Steinberg 2014), post-metamorphic success (Marshall and Keough 2003; Marshall and Keough 2004;

Marshall and Keough 2008), and geographic range (Watts et al. 1998). In another broadly invasive bryozoan, *Bugula neritina*, (Marshall et al. 2003) found that colonies formed from larger larvae have been shown to have numerous life history advantages when compared to colonies from smaller larvae. The colonies from larger larvae showed greater survivorship, higher growth rates, produced more offspring, and did so sooner than colonies grown from smaller larvae (Marshall et al. 2003). A similar study with *Watersipora subatra* (as *subtorquata)* showed that larger larvae had higher growth rates, but overall survivorship varied with the environment they were reared in (Marshall and Keough 2004). Thus, larval size may offer another explanation for variable success amongst invasive species such as those in the genus *Watersipora*.

The taxonomy of the genus *Watersipora* is uncertain and has been described by Gordon (1989) as a "can of worms" due to the lack of distinguishable taxonomic features and a history of uncertainty with type specimens. This uncertainty continues today with the genus *Watersipora* continually being revised (see Vieira et al. 2014 for the latest revision, though this has also been contested, see Fofonoff et al. 2018). Although the taxonomy may be uncertain, the fact that numerous species of *Watersipora* have recently been introduced to many areas necessitates careful species identification, as this genus is considered to be one of the most invasive groups of bryozoans in the world (Mackie et al. 2006; Ryland et al. 2009).

Clouding matters even further is the fact that one of the most well studied species of *Watersipora* (*W. subatra*, formerly *W. subtorquata*), has been found to be a cryptic species complex containing three different clades in two separate species. Nucleotide

sequence divergence in the COI gene of morphologically similar *Watersipora* species suggested *W. subatra* (Ortmann, 1890) consists of two putative cryptic species. Initially, one colony of *W.* 'new species' was identifed as distinct from other *W. subatra* ("*subtorquata*") colonies in California, differing by 17% (Mackie et al. 2006). Anderson and Haygood (2007) found as much as 14.8% difference over 622 nucleotides between samples of the Californian "*W. subtorquata*" COI cluster (presumably two species). The internal portion of COI sequences of five *Watersipora* species differed by an average divergence of 18.5% (Kimura-2 parameter model); further, the Bayesian phylogeny did not support clade N / new species as the most closely related sister taxon to *W. subatra* A and B clades (Mackie et al. 2012). Additionally, a microsatellite study performed by Wostenberg (2015) found that Clade A and Clade B lineages are interbreeding, whereas Clades AB and Clade N were supported as genetically distinct. Clade A was the most widely dispersed (introduced) lineage in recent surveys (Ryland et al. 2009; Mackie et al. 2012). The known distribution of clade N was from Oxnard, California to Humboldt Bay California as well as in Bremerton, WA and South Korea.

These two species have often been lumped together in previously published work, treating them all as *W. subtorquata.* This represents a major shortcoming in research done on *Watersipora*. The nature of cryptic species often obscures the frequency of introduction events due to morphological similarities amongst species (Bastrop et al. 1998; Holland et al. 2004; Geller et al. 2010). This is a serious problem because conflation of sister taxa can muddy our understanding of the evolution and ecology of the species concerned and how they are able to invade new habitats, hampering research

advancements as well as management efforts. Understanding the role of species-specific larval characteristics on the settlement and growth rate of organisms will improve the understanding of the ecology of a poorly understood, yet highly invasive, cryptic species complex of bryozoans in the genus *Watersipora*.

Pilot Studies

During 2016, I conducted a series of pilot lab studies on the larvae of colonies of *Watersipora* from two different locations in San Francisco Bay: Richmond Marina and Treasure Island Marina. Colonies from these two locations were haplotyped to COI clade using the same methodology as in Láruson et al. (2012). The first study looked at larval size, settlement rate, and mortality in these two species. Following the methodology listed below, settlement rate and successful metamorphosis of newly released larvae (Fig.1) were recorded at 6 hours, 24 hours, and 1 week after larval release.

At release, Clade A larvae were smaller (mean: 0.09mm^2 95% C.I.: 0.09 - 0.10mm^2) than Clade N larvae (mean: 0.13mm^2 95% C.I.: 0.11 - 0.16mm^2; t (35.44) = 3.17, N= 60, p= .0016)). Additionally, Clade A settled and began metamorphosis at each interval of time (6 hours, 24 hours, and 1 week) sooner than Clade N (χ^2= 3.44, p= 0.067; χ^2= 5.49, p= 0.020; and χ^2= 8.65, p= 0.006, respectively). After 2 weeks, Clade A showed a much higher rate of successful metamorphosis, as indicated by a live, fully formed ancestrula, relative to Clade N (χ^2= 13.89, N= 24, p= 0.0003).

A separate study was done exposing larvae to copper via anti-fouling paint applied as 5 small 30mm^2 circles evenly distributed to the bottom of a 60mm diameter plastic petri dish. Of the 12 larvae of each species released into each dish, *Watersipora* Clade N preferentially (5 of 11 larvae) settled on the anti-fouling paint compared to Clade A, which avoided the paint (0 of 11 larvae settled on copper; χ^2= 5.60, *p*= .018). There was no statistical difference in the overall number of individuals settling between these two clades, however (N=11/12 larvae settled for each species, χ^2= 0, *p*= 1.0).

Research Focus

Based upon these pilot study results, I focused my research on assessing if there is a difference in the invasion potential between these two species of *Watersipora* (*W. subatra* and *W.* "new species" / Clade N) with a specific lens towards their larval characteristics and copper tolerance. This was done through exposing larvae to either a control treatment or copper antifouling paint treatment and measuring: (1) larval size, (2) mortality, (3) swimming duration, (4) settling time and rate, (5) metamorphic competency, and (6) growth rates of metamorphosed colonies for both *W. subatra* and *W.* "new species" / Clade N.

Looking at these characters and correlations across these traits may provide evidence of different evolutionary strategies between these two species which may, at least in part, account for the different geographic distributions that have already been observed (Mackie et al. 2012). For instance, having a life history with fast settlement and

growth and with early onset of reproduction may favor invasion in warmer temperatures, in contrast to other bryozoans whose growth rates have been shown to increase relative to cold water (Amui-Vedel et al. 2007). Conversely, producing larger "choosier" larvae that grow more slowly, but ultimately form larger colonies, may be more advantageous when invading colder waters, where higher levels of nutrients and dissolved oxygen may facilitate the growth of larger zooids and colonies (Amui-Vedel et al., 2007; Hunter Hughes, R. N., 1994; Lombardi et al., 2006; O'dea et al., 2007). These larger colonies may have a greater level of fitness as colony size has been shown to be a good proxy for fitness in several studies (D. Marshall & Keough, 2003; D. Marshall et al., 2006; D. Marshall & Keough, 2006). Having a different suite of larval characteristics, and potentially different invasion styles, may help explain observed patterns of latitudinal separation of these two *Watersipora* species.

During the Fall of 2017, I tested the hypotheses that: Clade A larvae would have a (1) smaller cross-sectional area, (2) higher survivorship, (3) shorter swimming duration/faster settlement, (4) higher settlement rates, (5) higher metamorphosis rates, and (6) higher rates of colony growth as adults relative to Clade N. Additionally, given the preference Clade N displayed for copper antifouling paint in the pilot studies, I tested the hypotheses that the addition of copper antifouling paint would cause a reversal with respect to hypotheses (2), (3), (4), (5), and (6). There was no reason to suspect a reversal in (1) since it is unlikely the effect of copper exposure would be immediate.

Since pilot studies suggested a possible difference in larval size between the two species of *Watersipora*, it is possible that a difference in the rate of larvae that settled in

the copper treatment could be skewed towards the larger larvae. As larger larvae have a lower surface area to volume ratio, they may be receiving lower relative copper exposure.

Since little is known about the differences between these two species of *Watersipora*, I also performed a series of correlation analyses to investigate what aspects of the larval life histories of these two species may be correlated with one another, as well as how these relationships may change with the addition of antifouling paint.

METHODS

Collection

Specimens of *Watersipora* were collected from locations in Humboldt Bay where past research work has shown the presence of both Clade A or Clade N. For Clade A, the primary collection site was the Eureka Public Marina across the entrance channel to the bay, whereas for Clade N, the primary collection sites were docks and outcroppings along the southern bank of the Eureka Channel (Fig. 1). After collection, these bryozoans were placed in bubbler-aerated coolers and transported back to the Telonicher Marine Laboratory (TML) in Trinidad, CA where individual colonies from each locale were placed into separate, closed, and labeled, and aerated aquaria bathed in a recirculating seawater table maintained at 12° C. These colonies were maintained separately from each other so that I could individually identify the colonies within them genetically using PCR-based haplotyping.

Each aquarium had half of its water drained out and replaced with fresh seawater daily. All colonies were fed a mixture of phytoplankton (*T. isochrysis lutea* and *Tetraselmis* sp.) daily after these seawater changes. The holding tables surrounding these aquaria had large black plastic tarps covering them to prevent light-induced larval release as well as the growth of unwanted invertebrates and algae.

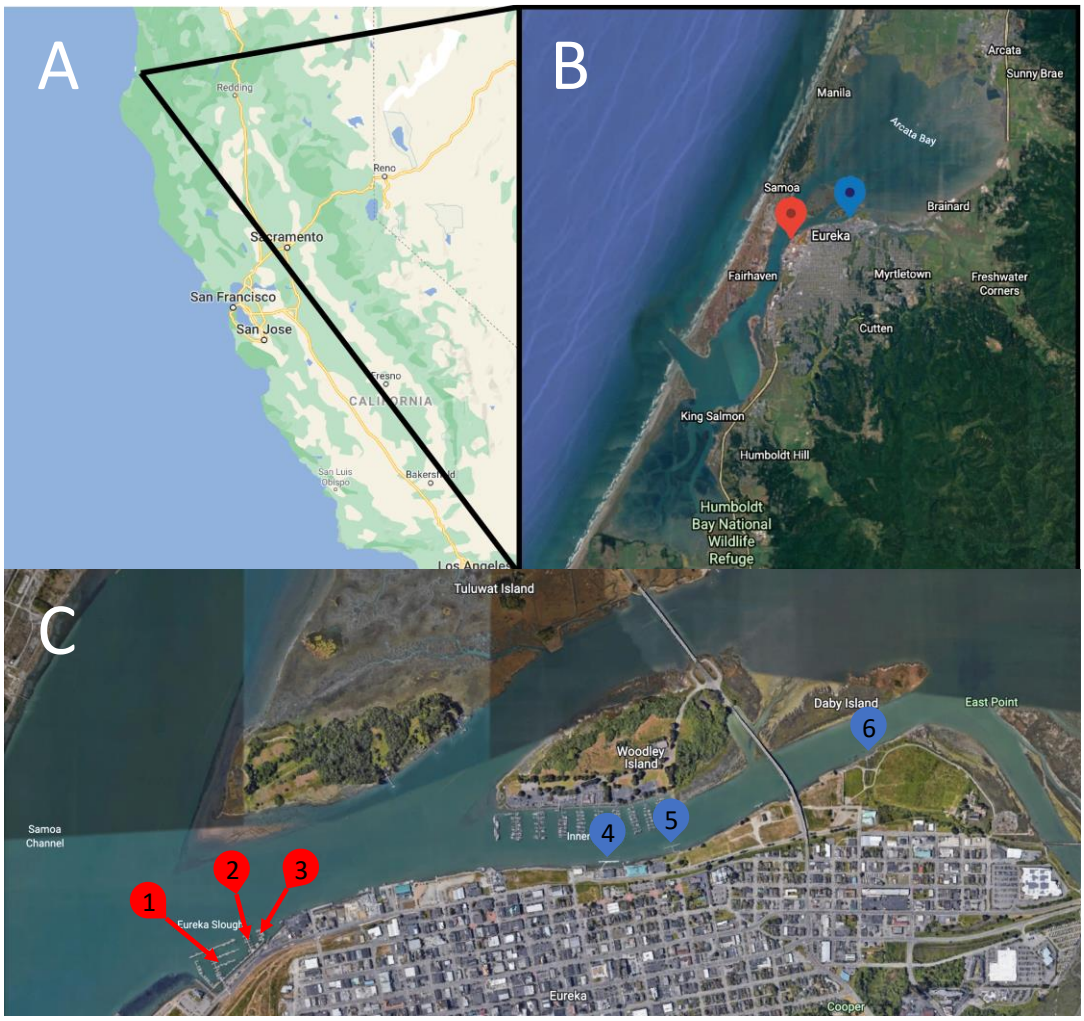

Figure 1: Location of Humboldt Bay (above right) with the location sites used for collecting *Watersiopra* Clade A (blue) and Clade N (red) with specific locations shown for each species (below). Collection sites listed from Left to Right: (1) Eureka Public Marina Dock J (Clade N), (2) Eureka Public Marina Dock C (Clade N), (3) Eureka Public Marina Dock D (Clade N), (4) Humboldt Bay Aquatic Center Dock (Clade A), (5) Bonnie Gool Guest Dock (Clade A), and (6) the southern bank of the Eureka Channel (Clade A).

Species Identification Using Molecular Genetic Typing

To determine the genetic identity to A, B or N COI lineage of each *Watersipora* colony collected in the field, a small piece of each colony was taken and placed in individually marked microcentrifuge tubes filled with 75% ethyl alcohol. Samples underwent a cetyl trimethyl ammonium bromide (CTAB) DNA extraction protocol. Isolated DNA was then amplified with PCR using a multiplex primer cocktail of five different primer sets that amplify polymorphic segments of cytochrome oxidase I that allow differentiation of the A, B, and N clades of *Watersipora* (Láruson et al. 2012). Of the 6 Clade N candidate colonies sampled, 5 were confirmed as being Clade N as evidenced by an amplified DNA band of 460 base pairs (see Fig. 2). One Clade A candidate colony failed to amplify the COI sequence of interest and was removed from the study. Of the 8 Clade A candidate colonies, all 8 were confirmed as being Clade A as evidenced by a single DNA band of 177 base pairs (Láruson et al., 2012; Fig. 2).

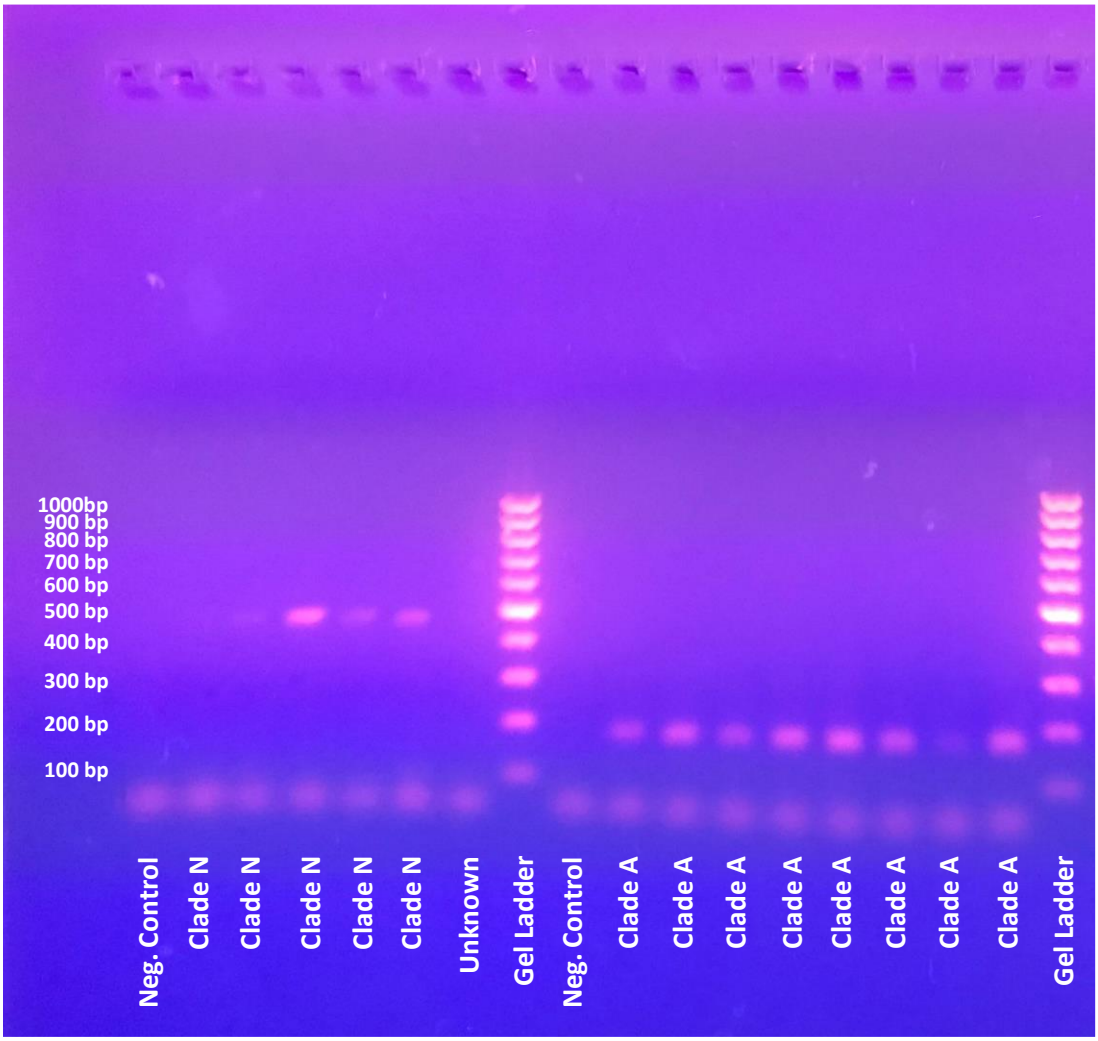

Figure 2: Electrophoresis gel of 1.5% agarose (weight to volume) run at 100 V for 45 minutes and stained with ethidium bromide. Hazy bands below the 100bp increment ladder are likely primer dimers. Gel wells are labeled with the species that corresponds to the band fragment length of the sample, “Unknown” in the case of a sample that failed to amplify, “Gel Ladder”, and “Neg. Control” for negative control PCR samples run with no DNA present.

Larval release

Induction of larval release from colonies was done by exposing these colonies to light from 120-Watt incandescent flood light bulbs mounted above the aquaria. Individual larvae from each aquarium were then pipetted into uniquely labeled petri dishes that had been conditioned with seawater for the previous 24 hours to develop a biofilm then emptied and filled with 20mL of filtered sterile seawater. Petri dishes had either no modifications (control treatment) or a small circle (approximately 125 mm^2 in area, or the size of a standard paper hole punch) of Pettit Paint (Unepoxy Standard Anti-fouling Paint 1628) which contained 33% cuprous oxide (copper treatment). There were more Clade N larvae released by colonies than Clade A larvae during the 1.5-hour larval release process, though there was more mass of Clade N parent colonies.

Due to the low number of larvae initially released from Clade A colonies, (20 larvae) these larvae were used for an initial control treatment along with 20 larvae released from Clade N. A second round of larval release was done two weeks later for *Watersipora* colonies of both clades. This second larval release period produced the following combinations: 20 Clade A larvae to the copper treatment group an additional 8 Clade A larvae to the control group plus 30 Clade N larvae to the copper treatment group and an additional 30 Clade N larvae to the control group.

The larvae from different trials were grouped together for statistical analyses but records of the trial and timing of larval release were kept to standardize the amount of

time passed since larval release between the two trials. This yielded a total of 48 *Watersipora* Clade A larvae and 80 Clade N larvae in these experiments (see Table 1).

Table 1: Number of individual larvae of each *Watersipora* clade collected and separated into individual petri dishes in both the control and copper treatment.

Clade	Control	Copper Treatment	Total Larvae
Clade A	28	20	48
Clade N	50	30	80

Measurements of Larval and Colony Sizes

To measure the size (area) of larvae from each *Watersipora* clade, several photographs of each larva were taken with an Infinity 2 digital camera and Infinity Capture software through a dissecting microscope with discrete magnification factors allowing for repeated measurements to be taken. Photos where larvae displayed the largest and clearest projected area were used to measure their cross-sectional area. This was done by photographing a 0.01mm calibration slide and calculating a pixel to mm conversion factor. Adobe Photoshop CS5 was used to outline the perimeter of the *Watersipora* larvae, set the conversion factor, and then measure the actual cross-sectional area of the inside bounds of the perimeter. Similar methodology was used for photos of

individual *Watersipora* colonies at 3 different time points during their development: (1) during settlement but before metamorphosis, (2) after metamorphosis, and (3) once every week for 14 weeks for adult *Watersipora* colonies.

Differences in larval size between the two *Watersipora* clades were analyzed with a t-test. Larvae were not separated according to control and copper treatment, since there was no expectation that copper has an instantaneous effect on larval size.

Time Until Settlement

After single larvae were released, separated, pipetted into individual petri dishes, and photographed, petri dishes were monitored every 4 hours for signs of settlement, determined by a change in morphology **(**Fig. 3) as well as the ability of a larva to remain attached when subjected to a gentle squirt of water from a pipette (Marshall and Keough 2003). Larvae not settled after 72 hours were removed from the experiment and assumed to be non-competent. A 2-way ANOVA was used with time until settlement as the response variable and copper/control treatment and species as the 2 fixed factors.

Figure 3: Morphological changes used to define stages of settlement and metamorphosis in *Watersipora* larvae. In the "Larval" stage, the larvae are actively moving and have a band of cilia still present and an apical furrow present (above left). In the "Settled" category, the larvae have adhered down to the surface and lost their cilia (above center). The individual larva's shape changes to one akin to either a pair of lips or a volcano at this stage. The "Metamorphosed" category (above right) is characterized by a fully formed ancestrula with a complete operculum (black) clearly present. The lophophore may or may not be extended but the edges of the zooecium and operculum appear darkened.

Rate of Larval Settlement and Metamorphosis

At every time interval each larva was scored with a discrete "yes/no" for each category (settlement, metamorphosis) and these data were analyzed with a Fisher's Exact Test to examine the difference between species and the effect of the copper treatment.

Individuals that had not successfully settled were excluded from the metamorphosis analyses.

Time Until Settlement and its Effect on Size Increase Post-Metamorphosis

To measure the effect of timing of larval settlement on post-metamorphic size change in these larvae, the ratio of the size of the metamorphosed ancestrula relative to the pre-metamorphosed, settled larva's cross-sectional area was taken for each larva that successfully metamorphosed, and these data were compared to the time it took for settlement of each larva (from release to settlement) using a linear regression analysis.

Effect of Size, Treatment, and Clade on Settlement Probability

To see if the size of the larva, treatment, and clade had an effect on the probability of settlement as well as to control for differing surface area to volume ratio differences in the copper control treatment, I performed a quasibinomial logistic regression using a logit link function. The model looked at the probability of settlement as a function of size (as cross-sectional area), clade, treatment, and the interaction of clade and treatment.

Colony Growth

For the 14 weeks following larval release, *Watersipora* colonies were maintained in a common garden setting in their individual petri dishes at 12° C, maintained by a temperature-controlled chiller, with daily exchanges of ultraviolet-sterilized seawater and approximately 5 mL of microalgal mix of *T isochrysis lutea* and *Tetraselmis* sp. added daily after the seawater exchange. Colonies also had twice-weekly cleanings to remove built-up feces in their dishes as well as any motile ciliates that had made their way into their petri dishes. This was done by using a fine-haired paint brush to dislodge any accumulated material around the colony and the periphery of the petri dish while viewing the colony under a dissecting scope. Cleaning was followed by a squirt of sterile seawater from a pipette, allowing the water and detritus to flow out of the petri dish. Petri dishes were then filled with sterile seawater and microalgae (as above). Photographs were taken of colonies (exactly as described for Larval and Colony Measurements above) every other week.

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RESULTS

Larval Size

Mean larval size of *Watersipora* Clade N larvae (0.107mm^2; 95% C.I.: 0.103-0.112 mm^2) was approximately 33% bigger, significantly larger than that of Clade A (0.080 mm^2, 95% C.I.: 0.076-0.085 mm^2; T=-8.40, DF=114.23, $p < 0.0001$). There was no difference in the size of larvae between the control and experimental copper treatment for either clade of *Watersipora*, however (Clade N: p=0.859, Clade A: p=0.762; Fig. 4).

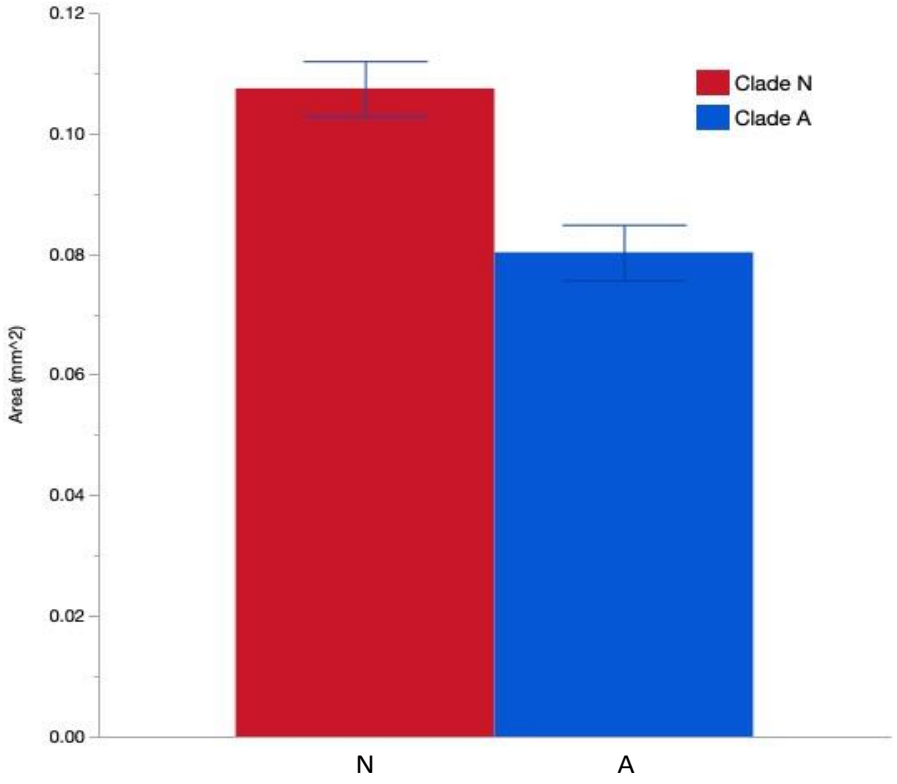

Figure 4: Cross-sectional area of *Watersipora* Clade N and Clade A larvae. Error bars represent a 95% confidence interval of the mean (T=-8.40, DF=114.23, p<0.0001).

Larval Size Between Larval Release Groups

There was a slight but significant difference in larval size between the two batches of larvae released for *Watersipora* Clade N (T=-2.89, DF=55.84, p=0.0055) with those from Trial 1 being roughly 11% larger than larvae from Trial 2 (0.116 mm^2; 95% C.I.: 0.110-0.112 mm^2 and 0.104 mm^2; C.I.: 0.099-0.110 mm^2, respectively). Although

there was a difference in larval size between the two larval release batches of Clade N, the difference in average size between the two Clade N trials was less than half the degree of difference between the average size of each species. There was no difference in the size of larvae between the two trials of *Watersipora* Clade A larvae, however (T=-0.097, DF=31.4, p=0.923; Fig. 5).

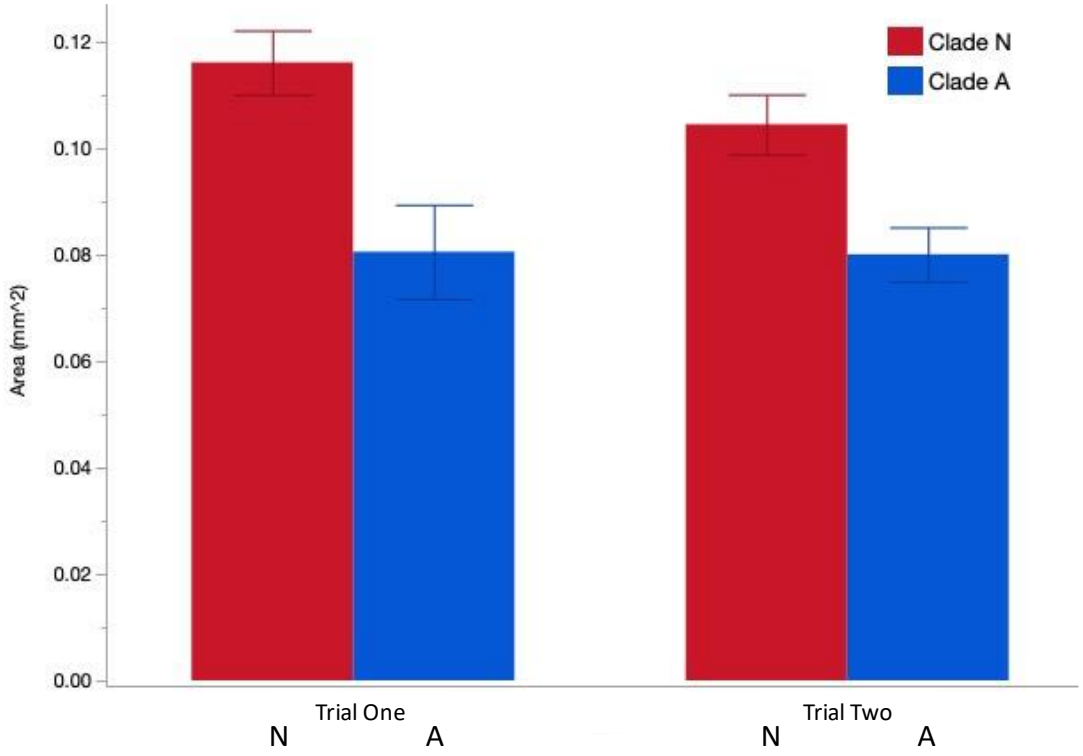

Figure 5: Comparison of cross-sectional area of larvae of the two *Watersipora* clades in each of the two trials. There was a small difference in the size of Clade N between the two trials (T=-2.89, DF=55.84, p=.0055) but no difference in the size of Clade A (T=-0.097, DF=31.4, p=.923). Error bars represent a 95% confidence interval from the mean

Settlement and Metamorphosis Rates

The rate of larval settlement for Clade N was much lower than Clade A in the control treatment (42% versus 81%, respectively; N=76, DF=1, p=0.0011), but with the addition of copper, the pattern reversed and considerably more Clade N settled than Clade A (83% versus 55%, respectively; N=49, DF=1, p=0.0370; Fig. 6).

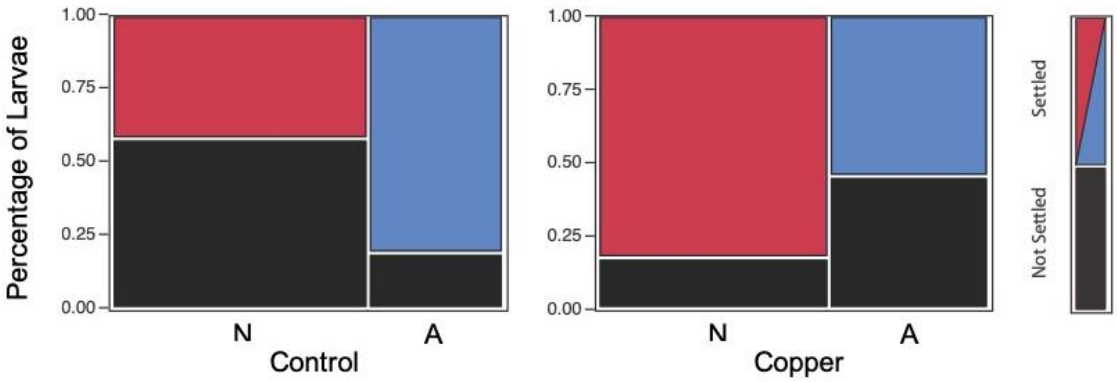

Figure 6 Contingency table of the percentage of each Clade in either the control (N=76, DF=1, p=0.0059) or copper treatment (N=49, DF=1, p<0.0001) that settled during the first 72 hrs. of the experiment. The colored bars represent the percentage of settled larvae from each clade with Clade N in red and Clade A in blue. The black bar represents the percentage of each clade that failed to settle. The width of each bar corresponds to the percentage of total individuals in each category.

Of the larvae that settled in the control treatment, only 40% of *Watersipora* Clade N successfully metamorphosed compared to 73% of Clade A (N=76, DF=1, p=0.0059). Of the larvae exposed to copper, 76% of settled Clade N metamorphosed compared to a mere 15% of Clade A (N=49, DF=1, p<0.0001; Fig. 7).

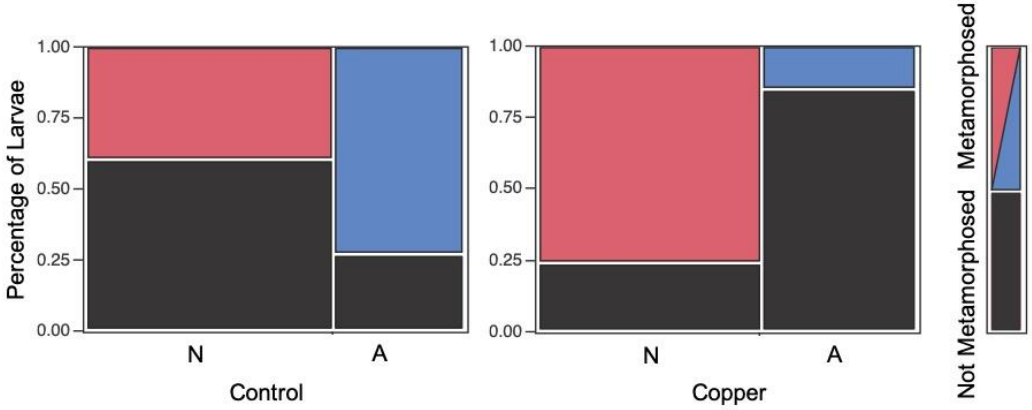

Figure 7: Contingency table of the percentage of larvae that settled and went on to successfully metamorphose between *Watersipora* clades in the control (N=76, DF=1, p=0.0059) and copper (N=49, DF=1, p<0.0001) treatments. The colored bars represent the percentage of metamorphosed larvae from each clade with Clade N in red and Clade A in blue. The black bar represents the percentage of each clade that failed to metamorphose. The width of each bar corresponds to the percentage of total individuals in each category.

Time Until Settlement

There was a marked effect of species and treatment on settlement time. Clade A larvae settled over 135% faster than Clade N in the control treatment with Clade A settling an average of 12.1 hours after larval release (95% C.I.: 7.0-17.2 hrs) versus 28.5 hours for Clade N (95% C.I.: 18.3-38.7 hrs). There was no significant difference in the time until settlement between Clades A and N for the copper treatment, with Clade A exposed to copper settling at an average time of 33.8 hours after larval release versus 29.5 hours after larval release for Clade N (95% C.I.: 26.3-41.4 hrs. and 23.9-35.0 hrs., respectively). However, for Clade A larvae exposed to copper, time to settlement increased over 100% compared to Clade A larvae not exposed to copper (Table 2., Fig. 8). However, there was no interaction effect between species and treatment in their settlement time (p= 0.12; see Table 2).

Table 2: Two-way ANOVA of the effect of *Watersipora* clades and experimental treatment on the time (in hrs.) until settlement ($F(3, 74) = 7.68$, $p= 0.0002$).

Term	**Estimate**	**Std Error**	**t Ratio**	**Prob>|t|**
Clade	5.1916017	1.895112	2.74	0.0077*
Treatment	-5.680779	1.895112	-3.00	0.0037*
Clade*Treatment	2.9988745	1.895112	1.58	0.1178

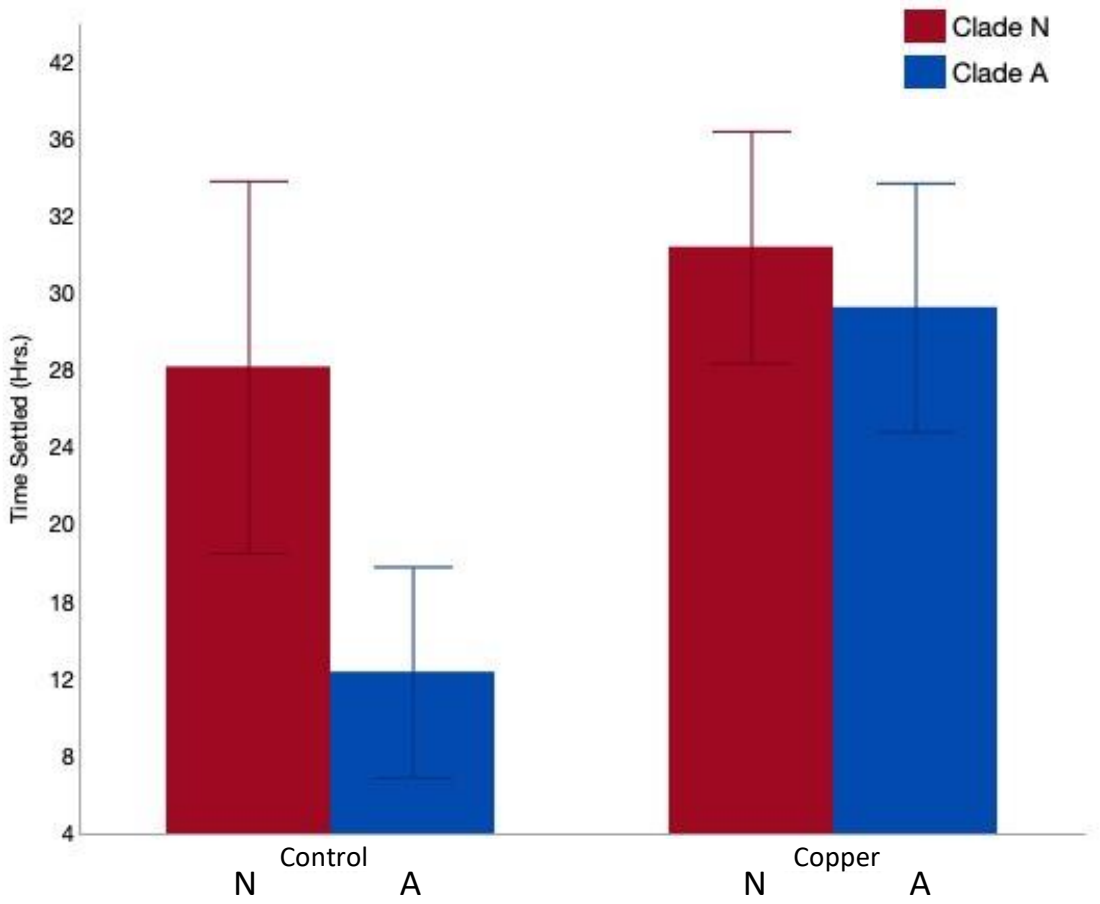

Figure 8: Comparison of cross-sectional area of larvae of the two *Watersipora* clades in each of the two trials. There was a small difference in the size of Clade N between the two trials (T=-2.89, DF=55.84, p=.0055) but no difference in the size of Clade A (T=-0.097, DF=31.4, p=.923). Error bars represent a 95% confidence interval from the mean

Time Until Settlement and its Effect on Size Increase Post-Metamorphosis

In the control treatment, the overall gain in size (measured as cross-sectional area) from settled larvae to metamorphosed adult zooids was dependent on the time it took for the larvae to settle in Clade N but not in Clade A ($p<0.0095$, and $p=0.91$, respectively). There was no difference in the rate of increase in cost to the ancestrula between treatments (Table 3), but ancestrula exposed to copper had a lower initial size-increase during metamorphosis (Fig. 9).

Table 3: ANCOVA for the reduction in post-metamorphosis size increase of *Watersipora* "new species" / Clade N as a function of time taken until settlement, the treatment, and the interaction of time taken until settlement and treatment ($F(2,50) = 12.76$, $p < 0.0001$).

Term	Estimate	Std. Error	t-Ratio	P
Intercept	219.04	28.7	7.65	<0.0001
Time Until Settlement	-2.42	0.90	-2.70	0.0095
Treatment	58.62	16.1	3.66	0.0006
Time Until Settlement * Treatment	-0.52	0.90	-0.58	0.564

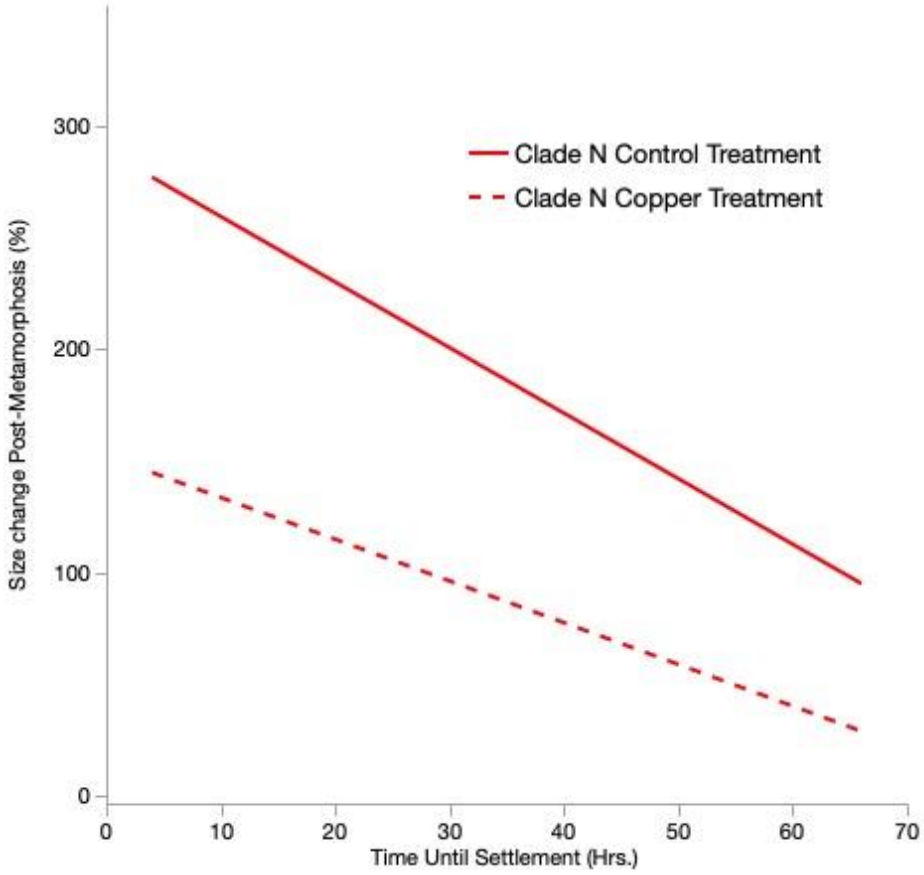

Figure 9: ANCOVA of the size increase from settled larvae to metamorphosed ancestrula exposed to copper and the control treatment for *Watersipora* Clade N ($F(2,50) = 12.76$, $p = <0.0001$, $R^2 = .43$). Size change post-metamorphosis was dependent upon time settled ($p = 0.0095$) and clade ($p = 0.0006$), but there was no interaction effect ($p = .56$).

Unfortunately, there were too few successfully metamorphosed Clade A larvae in the copper treatment to analyze the effect of time until settlement on the increase in size post-metamorphosis (N=3).

Effect of Size, Treatment, and Clade on Settlement Probability

The size of the larvae had no effect on the probability of settlement in *Watersipora* when controlling for other aspects ($p = 0.10$). There was a small increase in the log odds ratio of settlement for the copper treatment and a larger increase in the log odds ratio of settlement for Clade A, but a considerable decrease in the log odds ratio of settlement for Clade A larvae exposed to copper (see Table 4).

Table 4: Quasibinomial model of larval traits' effect on settlement probability. The dispersion parameter of this model is 1.05. The treatment, clade, and interaction of treatment and clade all had an effect on a larva's probability of settlement ($p = 0.001$, 0.001, and < 0.0001, respectively). There was no effect of size on settlement probability ($p = 0.10$).

Coefficients	Intercept	Std. Error	T-value	p
Intercept	-2.41	1.29	-1.86	0.06
Larval size	19.27	11.48	1.68	0.10
Copper Treatment	.1972	.60	3.30	0.001
Clade A	2.35	.71	3.3	0.001
Copper*Clade A	-3.26	.92	-3.54	< 0.001

Colony Growth

Control colonies of both Clade A and Clade N showed similar rates of growth through the 14 weeks of the experiment. However, the Clade N colonies grown in the presence of copper exhibited reduced growth (Fig. 10). Clade N colonies not exposed to copper were 230% larger (3.07 mm^2, 95% C.I : 1.39-4.75 mm^2) than colonies in the copper treatment (0.93 mm^2, 95% C.I : 0.31-1.56 mm^2) at the conclusion of the experiment . Too few Clade A colonies that were exposed to copper survived (n=3) to include in this analysis.

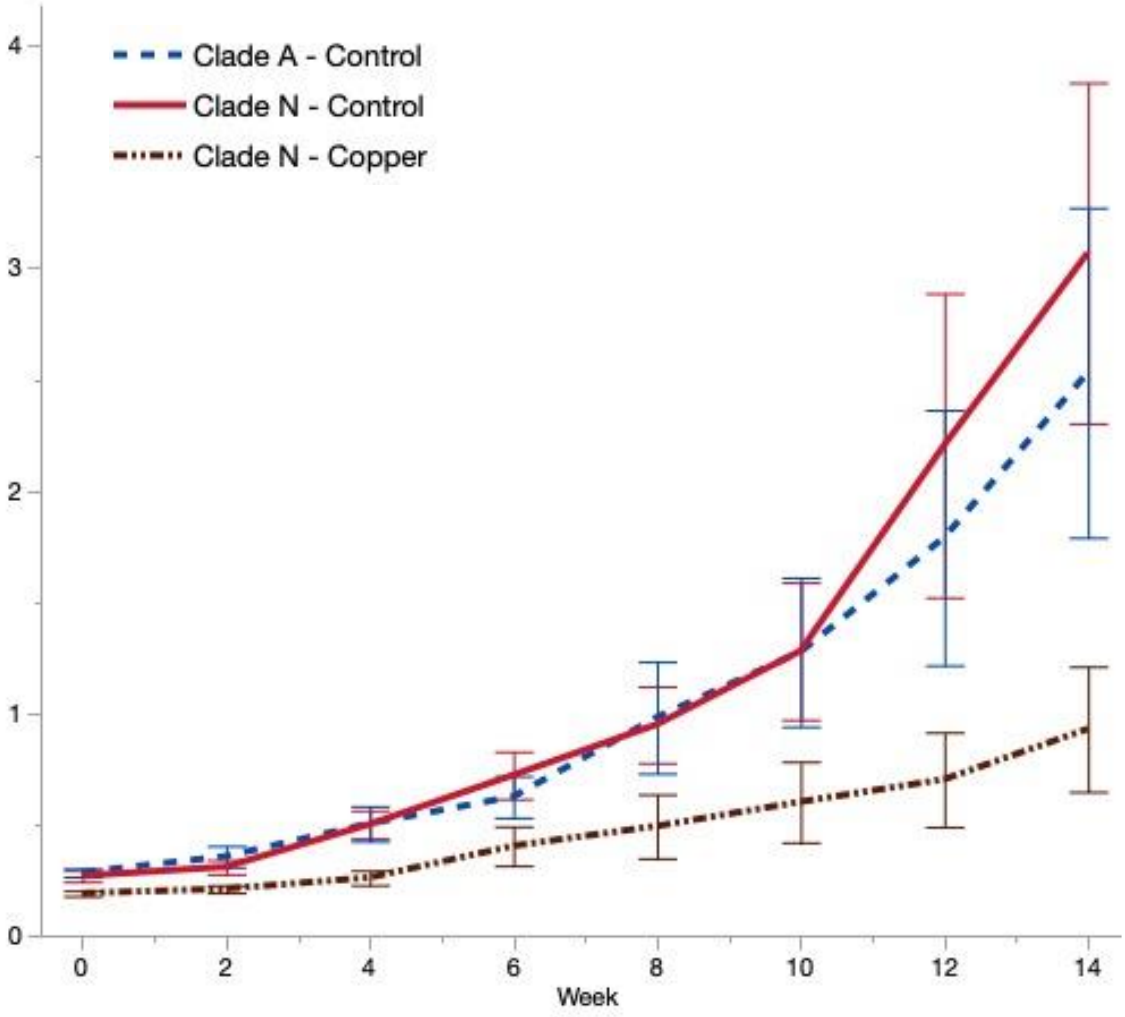

Figure 10: Mean surface area (in mm) of *Watersipora* colonies during the course of 14 weeks. Clade A grown in the copper treatment were excluded from the analysis since there were too few samples that successfully metamorphosed. Error bars represent +/- 1 standard error from the mean.

DISCUSSION

This study's intent was to determine what, if any, differences exist in a suite of larval traits between two cryptic species of *Watersipora*: (1) *W. subatra* Clade A and (2) *W.* "new species" Clade N. The results of this study show that these two species differ drastically in their larval size, rate and speed of settlement, survivorship, and behavioral responses to copper anti-fouling paint.

Clade A larvae were generally smaller (75% the size of *W.* "new species") and settled faster (135% faster) than Clade N larvae. Clade A larvae also successfully settled at a rate 92% greater and metamorphosed at a rate 83% greater than Clade N in the control treatment. However, a reversal occurred in the copper treatment: *Watersipora* Clade N larvae either outperformed or performed at the same rate as their congeners in the presence of copper. The following summarizes the results of the different larval traits studied herein and discusses the implications for these life history differences between larvae of these two *Watersipora* species.

Control Treatment

The larvae of *Watersipora* Clade N were roughly 33% larger than those of *Clade A*, having an average cross-dimensional area of 0.107mm^2 versus 0.080mm^2, respectively (Fig. 7). According to the "desperate larva hypothesis" (Toonen and Pawlik 1994), lecithotrophic larvae become less choosy about substratum as their size and energetic

reserves dwindle. Smaller larvae that have been less well provisioned therefore ought to be less picky about where they settle, especially after having spent some time swimming.

The *Watersipora* Clade N larvae, with larger energetic reserves, as evidenced by their larger size, certainly took longer to settle, taking on average 28.5 hours after larval release compared to only 12.1 hours with Clade A (Fig. 8). At face value, this result supports the "desperate larva hypothesis" (Toonen and Pawlik 1994; Gribben et al. 2006; Burgess et al. 2009) However, a considerably lower percentage of Clade N larvae actually settled: Clade A larvae settled nearly twice as frequently as Clade N larvae in the control treatment (81% compared to 41% settling; Fig. 6). This contradicts the idea that the Clade N can "afford" to be choosy in their settlement search, perhaps despite having the energetic reserves to do so, since 58% of Clade N larvae failed to settle at all, even after 72 hours.

Even the *Watersipora* Clade N larvae that did settle showed relatively low rates of successful metamorphosis, with only 40% of their settled larvae going on to competently metamorphose into adults. Of the settled individuals that failed to metamorphose successfully, some simply never fully developed into an ancestrula while others metamorphosed into deformed adults with non-functioning lophophores.

Despite being smaller, *Watersipora* Clade A larvae out-performed Clade N larvae in the control treatment for most metrics investigated in this study in the absence of copper (control treatment). In fact, while 59.1% of newly released Clade A larvae went on to become functioning adult ancestrulae (ancestrulae that survived metamorphosis and

developed a feeding lophophore), only 16.3% of Clade N larvae successfully metamorphosed into functioning, feeding adults.

Copper Treatment

Behavior of the larvae tested changed drastically with the addition of copper anti-fouling paint to the experiment. While copper had no effect on time to settlement for *Watersipora* Clade N larvae, Clade A took substantially longer to settle than they did in the control treatment and were no different in settlement timing than Clade N in the copper treatment (Fig. 8).

Other patterns reversed entirely from that in the control treatment, such as *W. subtorquata* settling much less than Clade N, with 55% versus 83% of their larvae settling, respectively, in the presence of copper (Fig. 7). Of the larvae that settled in the copper treatment, a higher proportion of Clade N also successfully metamorphosed (76%) while barely any of the already paltry few Clade A larvae that settled metamorphosed into adult zooids (15%).

Hence in the presence of copper, the larger *Watersipora* Clade N larvae fared much better with 63.1% of the released larvae successfully metamorphosing into adults whereas a mere 8.2% of Clade A larvae made it through metamorphosis. Despite the larger surface area to volume ratio Clade N larvae compared to Clade A larvae and commensurate copper exposure (though this study did not measure actual copper exposure), the size of larvae had no effect on the probability of settlement of *Watersipora* larvae (Table 4).

Differences Between the Two Clades

The presence of copper seems to dramatically change the larval response of these two species, swapping many of their respective outcomes. General awareness of this phenomenon in *Watersipora* goes back as far as the 1950s (Wisley 1958). There have been a number of studies showing increased settlement in *Watersipora subatra* (as *subtorquata*) larvae that were exposed to copper, either by exposure to anti-fouling paint (Piola and Johnston 2009; Mckenzie et al. 2012) or dissolved copper in the water (McKenzie et al. 2011; McKenzie et al. 2012), although oftentimes settlement resulted in non-competent adults (Ng and Keough 2003; Piola and Johnston 2006; Mckenzie et al. 2012). While this study observed similar results, there were important Clade-specific differences which were not examined previously. Clade N in the presence of copper seemed to have a higher number of competent settlers compared to the Clade A control treatment, though this came at the cost of a decreased growth rate in adult colonies (Fig. 10).

There was a markedly different response of *Watersipora subatra* Clade A to copper, resulting in numerous mortalities and greater percentages of failed settlement and metamorphosis in contrast to the previously reported increases in settlement of *Watersipora subatra* found in other studies (Piola and Johnston 2009; McKenzie et al. 2011; Mckenzie et al. 2012). A possible reason for this could be the lack of differentiation between these two cryptic clades of *Watersipora*.

A possible reason for the different response of *W. subatra* Clade A to copper found in this study could be due to regional differences between colonies found in Humboldt Bay and those found in Australia. Previous research has shown that there are both genetic (Wostenberg 2015) and life history trait (Korcheck 2015) differences between *W. subatra* colonies from different geographically separated populations. Still, the results of this study show a clear difference in the response to copper between *Watersipora* Clade A and Clade N within Humboldt Bay. This coupled with the results of pilot research work on these two species in San Francisco Bay suggest the pattern may be more broad spread.

Watersipora Clade N could be using the presence of copper as a settlement cue, eschewing otherwise suitable copper-free surfaces. But what benefit might settling on or near a copper antifouling paint-rich surface confer? It may be that the presence of copper is a good indicator of available space, since few other organisms are able to successfully settle on it. It also seems likely that settling on copper hull paint confers an advantage in spreading to new environments. Since copper antifouling paint is so ubiquitous on ship hulls, and has been for many years with the earliest reported usage of copper as an antifouling agent in 1625 (Woods Hole Oceanographic Institution 1952) settling and surviving on it may facilitate transport and spread to new harbors where it could further proliferate.

Watersipora Clade N in the control treatment showed substantially larger gains in size after metamorphosis compared to the copper treatment. It seems that a similar pattern might be found with Clade A, but too few larvae of this species survived the copper

treatment to allow meaningful insights about changes in post-metamorphic size. Nevertheless, in addition to taking longer to settle, *Watersipora* Clade N also incurred a greater "cost" to its lengthy settling behavior versus Clade A. The longer Clade N larvae took to settle, the smaller the post-metamorphic ancestrulae became. There was no difference in the rate of increase in cost to the ancestrula between treatments (Table 3), but ancestrula exposed to copper had a lower initial size-increase during metamorphosis (Fig. 9). A smaller ancestrula may reflect the energetic and physiological costs of dealing with copper, both during metamorphosis and as a larva. These sub-lethal effects of copper exposure are found within bryozoans (Ng and Keough 2003; Piola and Johnston 2006) and other marine invertebrates (Pease et al. 2010; Rouchon and Phillips 2017).

Different Life Histories Resemble Traits of *r*- and K- selected species

Many invasive organisms exhibit traits of *r*-selected or "weedy" organisms (Williamson and Fitter 1996; McMahon 2002; Davis 2005; Lagos et al. 2017). These include: fast growth, short-lives, early reproduction, less parental investment (either in rearing effort or larval provisioning), etc. (Eric R . Pianka 1970; Gadgil and Solbrig 1972) whereas K-selected species are often more long-lived, reproduce later and repeatedly, and have greater parental investment in their offspring (Grosberg, 1988; Grosberg, 1982). While this binary model is an overly simplistic view and, in actuality, life history characteristics vary along a continuous spectrum, the two Clades of *Watersipora* investigated herein seem to represent two different points along this

spectrum. Clade A is a species that has smaller, presumably less well-provisioned larvae (Marshall and Keough 2004; Marshall and Keough 2007; Marshall and Keough 2008), that settle and metamorphose quickly. There is some evidence that *W. subatra* may begin to brood larvae sooner (Korcheck 2015) and anecdotal evidence that there is some seasonal die-off of *Watersipora subatra* colonies during the winter (Mackie, Personal Communication 2017).

Watersipora Clade N colonies, however, grow to larger sizes, forming much larger, three-dimensional lettuce-like "heads" in Humboldt Bay (personal observation). Though *W. subatra* can cover extensive areas of substrate, they generally occur in a predominately encrusting two-dimensional form with limited vertical growth (personal observation). Whether the larger three-dimensional form of a Clade N colony is a character trait of this species or an inherent consequence of continued growth of the colony long past what we normally see in *W. subatra*, is unknown. Since larval production in bryozoans is directly related to the number of zooids in a colony which, in turn, is proportional to their surface area (Hayward and Ryland 1975), the larger, more complex, "fractal-like" heads of Clade N may be able to produce considerably more larvae per colony, though larval output was not looked at in this study. It is also possible, however, that Clade N larvae settle in aggregate more often and that large, 3-D growth "heads" of this species represent cases of intense competition between multiple genets.

The modus operandi of *Watersipora subatra* seems to be: settle fast, settle in large numbers, do not provision your lecithotrophic larvae very well, and do not settle on copper. The strategy for *Watersipora* Clade N appears to be: release plump larvae into

the water that swim around but largely don't settle unless there is a copper cue, then settle on or near it, grow to a larger size while continuing to produce numerous larvae at a much later point in time (Korcheck 2015).

This may help *Watersipora* Clade N proliferate in polluted bays and harbors where it is often found. If this strategy is indeed reflective of a more "K-selected" species, we can expect that over long stretches of time, *Watersipora* Clade N may eventually outcompete its congener. In fact, despite *W. subatra* being found fairly ubiquitously as recently as 2015 in Humboldt Bay, especially on the Eureka Marina near the Wharfinger building, very few locations were found to have any *W. subatra* during sampling and subsequent genotyping in either Humboldt Bay, Santa Cruz Harbor, Monterey Harbor, or San Francisco Bay back in 2017-2018. Potential ramifications of this are that Clade N may become a more established NIS in areas where it overlaps with *W. subatra*, due to its increased ability to settle and grow on copper antifouling paint. This may also confer an advantage to further introduction events if these traits also allow it to better "hitchhike" to new locations on boat hulls.

One factor that may help to control the spread of *Waterispora* Clade N is that Korcheck's work (2015) on *Watersipora subatra* (as *subtorquata*) Clades A, B, and *W.* "new species" Clade N showed that *W. subatra* clades had higher growth and survivorship in warmer waters than Clade N. With the heat of the ocean continuing to increase (United States Environmental Protection Agency 2021), Clade N's range may be limited by higher sea surface temperatures. Mackie et al. (2012) also found a latitudinal

separation amongst the two species, suggesting a possible temperature hurdle that Clade N would need to overcome to expand its range.

Issues with Cryptic Species

The results of this study conflict with some of the body of work that has been done on the effect of copper and copper tolerance in *Watersipora subatra*. Ng and Keough (2003) found that exposure to copper in *W. subatra* (as *subtorquata*) larvae sped up and increased their percentage of attachment, although there were some seasonal differences and copper exposure did slow their metamorphosis. McKenzie et al. (2012) saw increased settlement on panels that had copper antifouling paint painted around the border, and although they also found greater mortality in what was presumed (there was no molecular genotyping performed) to be *W. subatra* on copper panels, overall recruitment was still higher in the copper treatments.

These discrepancies and inconsistencies may be caused by the cryptic nature of these two species, *Watersipora subatra* and *Watersipora* "new species" / Clade N. Because few, if any, of these other studies differentiate between these two species, it may be that they are commonly and incorrectly assumed to all be *W. subatra* and traits of one species' larvae are being attributed to the other (or vice-versa). This research study is the first of its kind, to my knowledge, that directly looks at differences in the larval traits and behaviors of these two species and it has shown that there are significant differences between *W. subatra* and *W.* "new species" / Clade N larvae.

There is significant overlap between the ranges of these two species (Mackie et al. 2012) and a paucity of work done on genotyping populations of *Watersipora* to determine the occurrence and relative abundance of these different cryptic species in most bays. Much of the work that has been done to determine the makeup of *Watersipora* species in invaded bays has been done on the California coastline (Blackwell III and Craig 2012; Mackie et al. 2012; Wostenberg 2015). There is a serious risk of conflating species that appear to have markedly different traits here. In fact, there is little mention of *W.* "new species" / Clade N in the literature despite numerous studies being performed on what is purported to be *W. subatra*, and the majority of studies do not mention genotyping specimens in areas where both species may be found, or even where *W.* "new species" / Clade N is the dominant invader (Sellheim et al. 2010; Edwards and Stachowicz 2011; Simons et al. 2016; Page et al. 2019; Scott and terHorst 2019; Scott and terHorst 2020).

Although there are numerous biodiversity concerns with misidentifying cryptic species (Bickford et al. 2007), the main issues with failing to account for a cryptic species within NIS are those of management and control, including identifying the frequency and spread of invasions (Geller et al. 2010; de Barro and Ahmed 2011; Rius et al. 2015; Viard et al. 2019). This is especially true if a cryptic species complex contains species that differ considerably in their life histories, as I have shown to be the case here in *Watersipora subatra* and *W.* "new species" / Clade N larvae. Without knowing the identity and life history traits of invaders the potential means of control and management efforts risk inefficiencies or even failing in their objectives. For example, copper-based

anti-fouling paints may not be the best preventative measure for stopping the spread of *W.* "new species" / Clade N, due to its resistance and settlement response to copper. Perhaps other anti-fouling paints are needed for this species, because the results of this study suggest that Clade N may have already adapted to take advantage of the presence of copper. Without properly identifying the species of note and/or species composition of invaders, management decisions may be shots in the dark.

This is of particular concern with *Watersipora* species given the extent to which they have already invaded all around the world. While *Watersipora spp.* are generally limited to bays and harbors, (Mackie et al. 2012; Wostenberg 2015) areas which are already subject to intense NIS establishment (Ruiz et al. 1997; Crooks et al. 2010), *Watersipora spp.* are being found with increased frequency in the rocky intertidal on the outer coast of California (Zabin et al. 2018; Myron et al. 2019; Page et al. 2019). Given the propensity for *Watersipora* species to hold space and over-grow competing benthic organisms (Wilson 2011; Liu et al. 2017), this poses a threat to recently established Marine Protected Areas (MPAs) in intertidal areas near heavily invaded bays including Bodega Bay, San Francisco Bay, Humboldt Bay, Crescent City, and Monterey Bay. The rocky intertidal zone is an invaluable ecosystem heavily used in both biodiversity studies and ecological research and many important ecological theories have been discovered and tested in these habitats (Connell 1961; Paine 1966; Dayton 1971). The spread of *Watersipora* "new species" may also jeopardize restoration and recovery efforts in estuaries (DeRivera et al. 2005; Lonhart et al. 2019) and kelp beds (Lonhart 2012) already hampered by anthropogenic disturbances and climate impacts. To combat the

slow creep of *Watersipora* spp. outward from bays and harbors, we must first know more about the two most common species of *Watersipora* in these areas, given their very different life histories and larval characteristics. Genetic analysis is an important tool for differentiating *Watersipora subatra* and *Watersipora* "new sp." to determine which species are actually spreading outwards to the open coast of California.

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