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Effect of Adult Chemical Cues on Molting of Fiddler Crab Megalopae in Low Salinity Seawater

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ABSTRACT

Three species of fiddler crabs, *Uca minax*, *U. pugnax*, and *U. pugilator*, are commonly found in estuaries along the Atlantic coast, each with distinct adult habitats differing in salinity and sediment grain size. Prior research has found evidence for larvae exhibiting selective settlement; however, the degree to which and the method by which they choose their species-appropriate habitat to settle in is still unknown. Additionally, a recent study determined that chemical cues from adult crabs stimulate molting in field-caught fiddler crab megalopae, as previously determined in lab-reared megalopae; however, in 35 ppt seawater, few *U. minax* molted. This study tested the hypotheses that 1) chemical cues from adult fiddler crabs would stimulate molting of conspecific megalopae in 10 ppt seawater, and 2) that at 10 ppt, more *U. minax* would molt than at 35 ppt. Adult chemical cues accelerated molting in field-caught megalopae of *U. pugilator* and *U. pugnax*, which both molted significantly earlier in all odor water treatments than in the control water, and fastest in conspecific water. *U. pugilator* megalopae were stimulated to molt in all treatments, whereas *U. pugnax* molted mostly in conspecific odor. However, even at 10 ppt, few *U. minax* molted and there were no differences in molting frequency or time among treatments. The few *U. minax* molts suggest that these experimental conditions may still not be ideal, and other factors may be responsible for stimulating and accelerating their molting and settlement site selection.

INTRODUCTION

In North Carolina and estuaries along the Atlantic coast of the United States, three species of fiddler crabs (genus *Uca*) are commonly found. These three species (*Uca minax*, *Uca pugilator* and *Uca pugnax*) share a common larval life history, in which near high tide at night, female adults release zoeae that then swim to the surface and are carried out of the estuary on ebb tides (Epifanio 1988, De Vries *et al.* 1994). Once in offshore waters, zoeae develop into megalopae before returning to the estuary using flood tide transport where they settle and further develop into juvenile crabs (Epifanio 1988, De Vries *et al.* 1994). Although morphologically similar as larvae, the adults differ in appearance and by the estuarine habitats in which they live. *U. minax* inhabit low salinity areas of salt marshes, *U. pugnax* inhabit moderate to high salinity salt marshes with muddy sediments, and *U. pugilator* inhabit moderate to high salinity sandflats and sandy regions of salt marshes (Teal 1958, Miller & Maurer 1973, Crane 1975).

Fiddler crabs serve a vital role to salt marsh ecosystems, as they feed on decaying plant material in the sediment and are an important link in the food web, preyed upon by herons, egrets, ibises, and blue crabs (Crane 1975, Petit & Blidstein 1987, Zhong 2006). When they consume algae and other organic matter in the sediment by sifting through the surface sediment, fiddler crabs aid in redistributing nutrients in the salt marsh (Zhong 2006, Smith & Green 2015). In addition, fiddler crabs are bioturbators that improve salt marsh quality by redistributing oxygen in the sediment, enhancing plant growth, and allowing for the retention of meiofauna when they construct burrows for protection and mating (Christy 1983, Frix *et al.* 1991, Qureshi & Saher 2012, Bolton *et al.* 2013).

Prior research has provided evidence that suggests that larvae exhibit selective settlement (O'Connor 1993, Brodie *et al.* 2005, Welch *et al.* 2015), however, it is still uncertain how larvae

choose their species-appropriate habitat to settle in. Determining the mechanisms used in fiddler crab species-specific habitat selection, as well as their ideal molting conditions, can aid in understanding community composition and predicting the species of settlers in habitats with varying salinity, sediment grain size and adult species present. Additionally, since fiddler crabs can be used as environmental indicators for contaminants, such as insecticides, understanding their habitat selection mechanisms can also aid in assessing the health of salt marsh ecosystems (Zhong 2006).

Previous studies suggest that chemical cues given off by settlement sites stimulate molting of megalopae by possibly indicating a proper habitat (Wolcott & De Vries 1994). For example, sediment from adult habitats (Christy 1989) and water containing adult crabs have both individually and jointly stimulated molting of *U. pugilator* (O'Connor 1991). Similarly, for *U. pugnax*, seawater conditioned by adult crabs (O'Connor & Gregg 1998), extracts of adult crabs (O'Connor 2005), and sediment conditioned by adult crabs (O'Connor & Van 2006) have also stimulated metamorphosis. Additionally, field experiments have determined that molting of either *U. pugnax* or *U. minax* megalopae is accelerated when placed in adult salt marsh habitats and experience a rapidly diminishing effect the further away they are from the adult habitat (O'Connor & Judge 1997, 1999, 2004). Although the results from these studies have provided much support for habitat cues stimulating the molting of fiddler crab megalopae, they were all conducted using laboratory-reared megalopae from known-species adults. Once molecular techniques were discovered and implemented, species identification of field-caught fiddler crab megalopae became possible (Behum *et al.* 2005, Welch *et al.* 2015), and research has thus been capable of executing additional field experiments in studying fiddler crab larval development.

A recent study used field-caught *Uca* megalopae to determine whether cues from adult fiddler crabs stimulate molting, as they do for laboratory-reared megalopae, and determined that field-caught *Uca* megalopae are stimulated to molt by conspecific odors (Welch *et al.* 2016). This study was repeated in the summer of 2016 at the Duke University Marine Lab at 20 ppt to determine whether *U. minax* would molt more frequently in reduced salinity water, as compared to the initial experiment done at 35 ppt. However, only a few more *U. minax* molted at 20 ppt, and very late in the 10-day experimental period. This may imply that the conditions were not ideal for them, as *U. minax* tend to inhabit the lower salinity regions of salt marshes (Teal 1958).

Although research has been carried out on molting and larval development of fiddler crabs, research focused on the impact of salinity on molting has not occurred. Nevertheless, research has been conducted on effects of salinity on larval development of other crabs. For instance, a study on the larval development of blue crab *Callinectes sapidus* Rathbun, reared in the laboratory, determined that *C. sapidus* molts to the first crab stage earlier in 26.7 ppt than in 20.1 ppt and 31.1 ppt at 25°C (Costlow & Bookhout 1959). Larvae maintained at 20.1 ppt and 26.7 ppt had similar durations of the megalopa stage (6-9 days), compared to higher salinities at 31.1 ppt with longer time to molt periods of 10-20 days (Costlow & Bookhout 1959). Megalopae of the mud crab *Panopeus herbstii,* reared in the laboratory, molted similarly in 20.1 ppt, 26.5 ppt, and 31.1 ppt waters, while none survived to molt in 12.5 ppt water (Costlow *et al.* 1962). Additionally, both zoeae and megalopae experienced delays in molting at 20.1 ppt water (Costlow *et al.* 1962). Similar research conducted with lab-reared *Rhithropanopeus harrisii* crab larvae discovered the highest percentages of survival and molting of megalopae to the first crab stage at 20°C in 15 ppt and 25 ppt and at 25°C in 15 ppt (Costlow *et al.* 1966). However, these salinity-temperature combinations required the greatest time for molting, as compared to other

combinations with shorter molting times and lower survival percentages (Costlow *et al.* 1966). These studies involving different crab genera bring to the forefront the need and importance to conduct similar research on fiddler crabs and determine if they are similarly affected by varied salinity.

This study repeats the experiment conducted in Welch *et al.* (2016), but at 10 ppt estuarine water, and aims to determine whether *U. minax* megalopae molt more frequently in the reduced salinity estuarine water than in the experiments conducted at 35 ppt and 20 ppt. The study also seeks to determine how salinity affects the molting stimulation of the other two species, *U. pugilator* and *U. pugnax*. Another goal of conducting the Welch *et al.* (2016) study at 10 ppt is to determine the importance of salinity and chemical cues produced by adult fiddler crabs on settlement site selection by fiddler crab megalopae. It is hypothesized that *U. minax* would molt more frequently and earlier in the 10-day experimental period than in the previous experiments carried out in 35 ppt and 20 ppt.

MATERIALS AND METHODS

Field collection of megalopae and adult fiddler crabs

Fiddler crab megalopae were collected with a 0.75 m diameter 333 μm mesh plankton net, deployed from a platform under the Pivers Island Bridge in Beaufort, North Carolina, USA (34° 43.20' N, 76° 40.40' W), for each of five experiments conducted from June to August 2017. Plankton nets were deployed for 45 minutes surrounding the time of maximum nocturnal flood current, since fiddler crab megalopae utilize flood tide transport to travel upstream into estuarine habitats (DeVries *et al.* 1994, Forward & Tankersley 2001). *Uca* megalopae were separated from the other organisms collected in the net sample and held in 20 ppt estuarine water for ~8-12

hours, to transition them from 35 ppt estuarine water to 10 ppt estuarine water used in this study. Estuarine water (salinity ranging from 33-35 ppt) was collected by bucket off a dock at Duke University Marine Lab and filtered through a 5 μm bag filter before being diluted with deionized water to 20 ppt or 10 ppt.

Adult *U. pugilator* were collected from a sandflat in the Rachel Carson Estuarine Research Reserve (34° 42.71' N, 76° 40.47' W) and adult *U. minax* and *U. pugnax* were collected from Bell Creek Salt Marsh (34° 47.39' N, 76° 40.14' W). Collected fiddler crabs were maintained in large tanks in Lab 4 at the Duke University Marine Lab. They were given running seawater (salinity = 34 ppt) and sediment from their collection site, which was periodically added to the tanks, serving as fresh food for the crabs. *U. pugilator* were held separately in a rectangular tank (122 cm x 70 cm x 30 cm), while *U. minax* and *U. pugnax* were held together in a large circular tank (122 cm diameter, 60 cm depth). Groups of each of the species of fiddler crabs were used in lab each day and returned to their holding tanks following usage in odor water preparation. More crabs were collected and held than needed to ensure that different groups of crabs were used to prepare the odor waters each day. Adult fiddler crabs were released back to their appropriate habitat at the conclusion of the experiment.

Odor water preparation

Species-specific odor waters were prepared each day during the experiments. Estuarine water was collected off the dock at Duke University Marine Lab, filtered through a 5 μm bag filter, and diluted to 10 ppt with deionized water. The prepared 10 ppt estuarine water served as the control water treatment for the experiments. Approximately 25 grams of each species of adult fiddler crabs were soaked in 500 ml of the prepared 10 ppt seawater for 1 hour in species-specific 2800 ml Erlenmeyer flasks. The estuarine water was aerated during the hour of soaking.

After one hour, each of the odor waters was filtered through a 500 μm sieve into species-specific beakers to remove any particles left by the crabs during the incubation period. The sieve was rinsed with warm tap water then deionized water between uses.

Experimental set-up

For each of the five 10-day experiments, individual megalopae were placed into separate 20 ml scintillation vials containing 10 ml of one of the four water treatments. Vials were arranged in an 8 x 8 array, in two groups of 64 for a total of 128 vials. The water treatments were systematically varied in each row. The vials were kept at 25°C with ambient 14 hour light:10 hour dark cycle for a period of 10 days. Each day, megalopae were transferred, using a glass wide bore pipet, to clean vials with newly prepared water to prevent degradation of the chemical cues. Megalopae were fed recently hatched *Artemia* nauplii daily, after receiving a water change, and monitored four times per day, at 06:30, 11:00, 17:30 and 23:00 hours. Megalopae found molted or dead at each observation period were preserved in 95% ethanol for later species identification and date and time were recorded. During the last observation period on day 10, the remaining megalopae were preserved for species identification.

***Uca* species identification**

Each of the preserved *Uca* megalopae and juveniles species was identified using the method of Welch *et al*. (2015). DNA was extracted from each individual (Estoup *et al.* 1996), followed by multiplex PCR. Gel electrophoresis was used to identify the species of each individual based on the number of base pairs displayed in the amplified ITS157 gene (Welch *et al.* 2015).

Data analysis

The average time to molt in each odor water treatment for *U. pugilator* and *U. pugnax* species was compared with separate 1-way ANOVAs (Sokal & Rohlf 1981), and individual treatments were compared with Tukey post-hoc tests using SPSS 13. Since *U. minax* megalopae only had average time to molt data in two odor water treatments, the times to molt were compared using a *t*-test (Sokal & Rohlf 1981). A *z*-test for proportions (Walpole 1974) was used to compare the proportions of the megalopae that molted in each water treatment with the proportions that molted in the control treatment, as well as in the other water treatments, for each species.

RESULTS

Megalopae of the three fiddler crab species were evenly distributed across the four water treatments (Table 1). A total of 623 megalopae, consisting of 98 *U. pugilator*, 340 *U. pugnax*, and 185 *U. minax*, were tested and identified over five experimental periods (Table 2). There were 9 megalopae omitted from the results and data analysis, as they could not be identified due to failed amplification of DNA in PCR. Of the unidentified larvae, four had died, two remained megalopae, and three had molted. Of the identified larvae, 33.1% molted, 62.3% remained megalopae, and 4.6% died (Table 2).

A majority of the megalopae molted during days 3-6 of the 10-day experimental period. The shortest average time to molt (91.79 hours, ranging from 65 to 227 hours) for *U. pugilator* megalopae occurred in their own species-specific odor water, which was significantly earlier than in the control water (*U. pugilator*: ANOVA, $F_{3,75} = 5.522$, $p < 0.01$; Fig. 1a). Similarly, for *U. pugnax*, the shortest average time to molt (103.94 hours, ranging from 43 to 227 hours) was

observed in their conspecific odor water, which was also significantly earlier than in the control water (*U. pugnax*: ANOVA, $F_{3,106} = 4.722$, $p < 0.01$; Fig. 1b). Few *U. minax* molted in this study, and those that did molted in the conspecific water and *U. pugnax* water, except for one *U. minax* that molted in the control water treatment (Fig. 1c). The shortest time to molt for *U. minax* (71.88 hours) was in the control water treatment; however, it was of no statistical significance and thus not possible to determine if there was a significant difference between the average time to molt in the control water versus the other two water treatments (Fig. 1c). The majority of *U. minax* that molted did so in their own species-specific odor water treatment (12 individuals), with an average time to molt of 126.56 hours, ranging from 60 to 235 hours (Fig. 1c). There was not a significant difference between the average time to molt in the *U. pugnax* and *U. minax* odor waters ($t_3 = 0.21$; $p = 0.85$). No *U. minax* megalopae molted in *U. pugilator* odor water (Fig. 1c).

Molting was observed in all water treatments for *U. pugilator*, and significantly more with crab odor than without, with 95.0% in *U. pugilator,* 92.6% in *U. pugnax*, and 85.71% in *U. minax* (Fig. 2a). However, there was no significant difference in the percent of molting between the three odor water treatments. Additionally, all but one *U. pugilator* that were placed in the *U. pugilator* water treatment molted (Fig. 2a). *U. pugnax* also molted in each of the water treatments, with a significantly largest percent of molting in their conspecific odor water treatment (89%, Fig. 2b). Unlike *U. pugilator*, *U. pugnax* had significantly lower percent molting in the other two odor water treatments, with no significant difference between the *U. minax* and control treatments (Fig. 2b). Few (16 out of 185, 8.65%) *U. minax* megalopae molted in any water treatment during the experiments, with no molting observed in the *U. pugilator* odor water (Fig. 2c). Of the water treatments *U. minax* molted in, the highest percent of molting (25.5%)

occurred in their conspecific odor water, which was statistically more than in the control or *U. pugilator* treatments but not statistically different from *U. pugnax* odor water (Fig. 2c).

DISCUSSION

The results of this study indicate that chemical cues accelerate molting in conspecific odor waters for *U. pugilator* and *U. pugnax*. Both *U. pugilator* and *U. pugnax* molted significantly earlier in their conspecific odor water treatment, which was significantly different from the control water but not from the other two odor water treatments. Due to insufficient data, acceleration conclusions are unclear for *U. minax*, as not enough molted in the control water treatment to determine if there was a significant difference in molting time in the *U. minax* and *U. pugnax* waters.

The results of this study also support the hypothesis that chemical cues stimulate molting of field-caught megalopae, but differently for each species. *U. pugilator* molted significantly more in all three odor water treatments than in the control water, but not significantly more in its own species-specific odor water than the other two odor treatments. Since molting was observed in all water treatments for *U. pugilator*, this indicates that they may be relatively unselective on where they settle. In contrast, *U. pugnax* was highly selective in the water they molted in. They molted significantly more in their own conspecific water than in any other water treatment, indicating that they prefer to molt in areas that already have *U. pugnax* adults present. Finally, *U. minax* were not significantly stimulated to molt by an odor water treatment, as still few *U. minax* molted in 10 ppt water. However, more total *U. minax* molted in their conspecific water in this study at 10 ppt seawater than in the 35 ppt experiment (Welch *et al.* 2016).

Since *U. minax* did not molt more frequently in this study as hypothesized, it indicates that these experimental conditions may still not be ideal and other factors besides salinity may stimulate and accelerate their molting. One reason *U. minax* may not have had a high percentage of molting is because they may require more time to molt than the 10-day experimental period under these conditions. This may be a probable reason because *U. minax* inhabit the upper portion of the marsh, meaning that it may take longer for them to reach their species appropriate habitat than the other two species, and thus require a longer time to molt (Teal 1958). Another reason could be that *U. minax* respond better to habitat cues, such as from the sediment or *Spartina* cord grass, than adult crab odor chemical cues. Sediment from adult habitats has stimulated molting in *U. pugilator* and *U. pugnax* species in previous studies, however, have not been explicitly tested with *U. minax* species in a lab setting (Christy 1989, O'Connor & Van 2006). Another alternative is that *U. minax* may need a stronger chemical cue concentration in order to be stimulated to molt. For instance, the odor water prepared by soaking ~25 g of adult crabs may not be an ideal concentration for molting stimulation in *U. minax* as it is for the other two species. Instead, they may require a larger number of crabs soaking in the same amount of seawater to receive the same effect. Future research should thus focus on the mentioned factors not accounted for in this study to determine the most ideal molting and habitat selection conditions for *U. minax*. Once determined, this information could aid in assessing salt marsh ecosystem health by predicting community composition under various environmental conditions that impact fiddler crab molting, and thus their presence, in the marsh.

TABLES

Table 1. Number of megalopae of each *Uca* species tested in each odor treatment.

Treatment	*U. pugilator*	*U. pugnax*	*U. minax*
Control	20	87	49
U. pugilator odor	20	86	50
U. pugnax odor	29	87	39
U. minax odor	29	80	47

Table 2. Number and percentages of megalopae, of each *Uca* species, that molted, remained megalopae or died during the experiment. Unidentified megalopae omitted.

Species	Total Tested	Number molted	Number remained megalopae	Number died
U. pugilator	98	80 (81.6%)	15 (15.3%)	3 (3.1%)
U. pugnax	340	110 (32.3%)	211 (62.1%)	19 (5.6%)
U. minax	185	16 (8.6%)	162 (87.6%)	7 (3.8%)
Total	623	206 (33.1%)	388 (62.3%)	29 (4.6%)

FIGURES

Figure 1. Mean time to molt (±SE) in hours for megalopae of (a) *Uca pugilator*, (b) *U. pugnax*, and (c) *U. minax* exposed to estuarine seawater (control) or seawater treatments with different adult chemical cues. Numbers in bars represent the total number of megalopae that molted in each treatment. Lowercase letters above error bars indicate treatments that did not differ by Tukey post-hoc tests.

Figure 2. Percent of megalopae of (a) *Uca pugilator*, (b) *U. pugnax*, and (c) *U. minax* that molted (dark bars) and did not molt (light bars) during 10 days of exposure to estuarine seawater (control) or seawater treatments with adult chemical cues. Different lowercase letters indicate statistically significant differences in percent molting by z-test for proportions. Megalopae that died during the experiments were excluded from analysis.
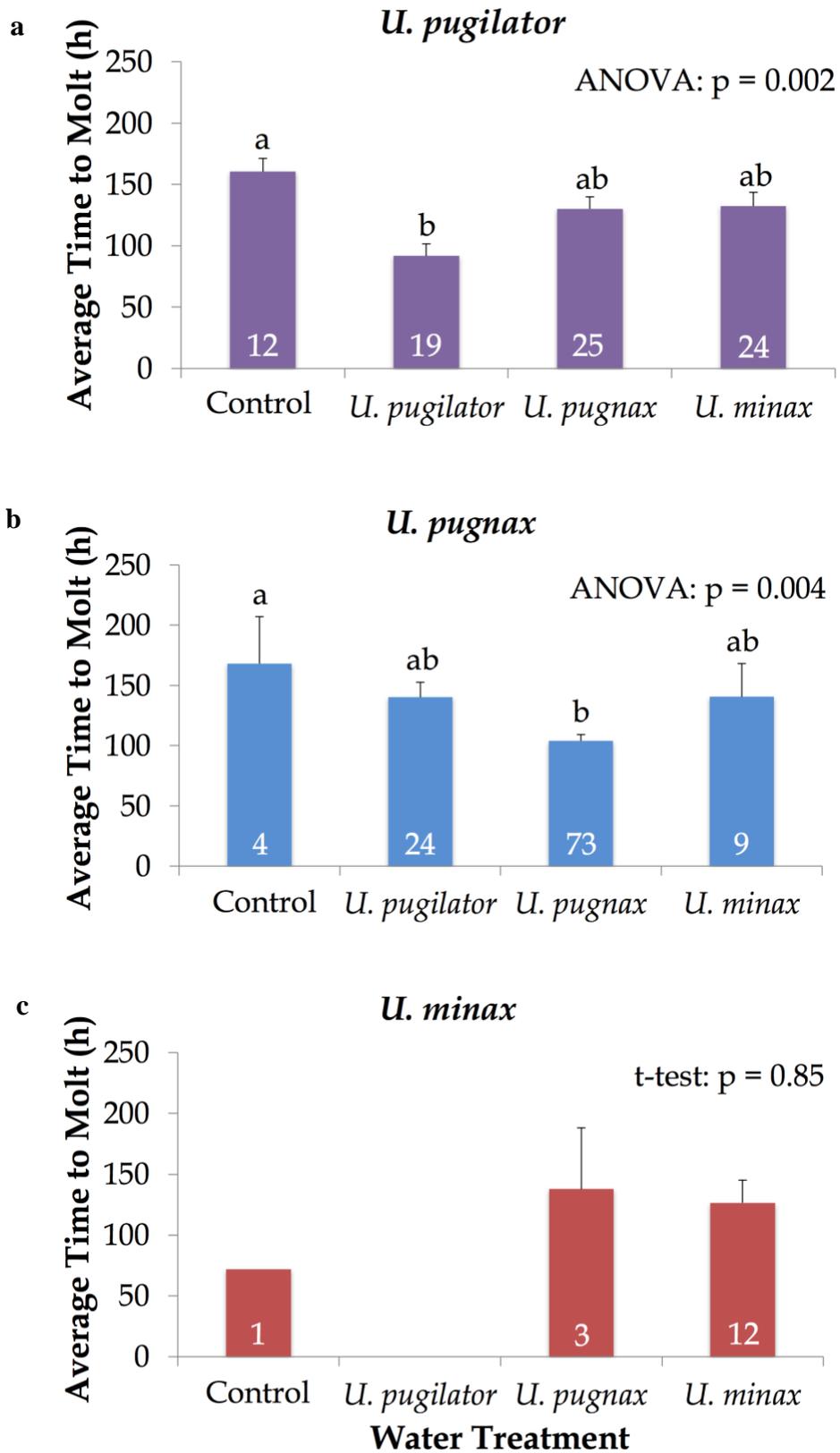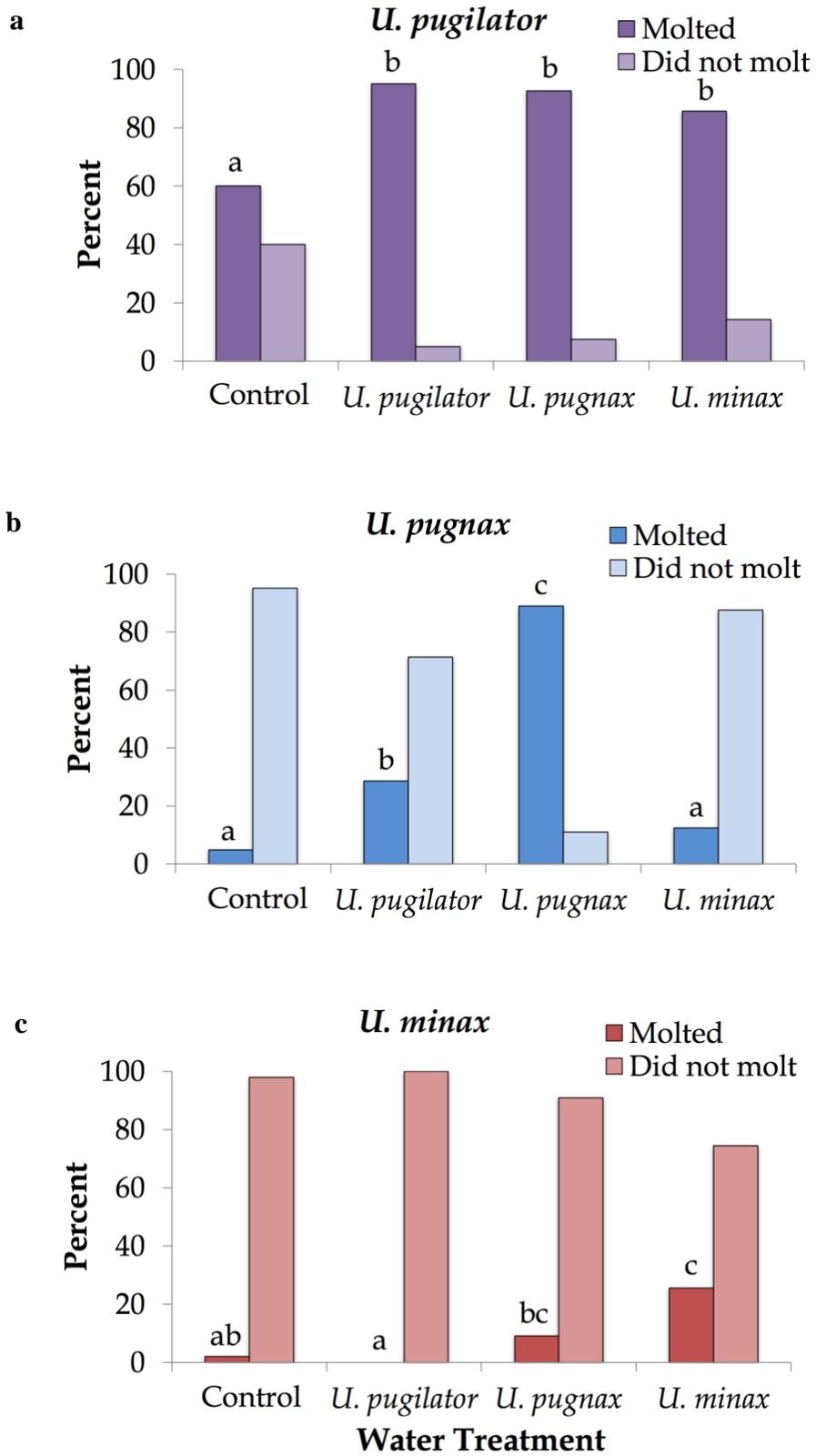
Figure 1.

Figure 2.

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