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# The Molting Biomarker Metabolite *N*-acetylglucosamino-1,5-lactone in Female Urine of the Helmet Crab *Telmessus cheiragonus*

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**Abstract.** *N*-acetylglucosamino-1,5-lactone (NAGL) is a molting biomarker in the blue crab *Callinectes sapidus*. The concentration of this compound in urine is highest at the premolt stage. Since sexually mature premolt females release sex pheromone in their urine, NAGL is a candidate sex pheromone molecule in *C. sapidus*. This compound has not been reported in other species. In the present study, we quantified the concentration of NAGL in the urine of the helmet crab *Telmessus cheiragonus*, using nuclear magnetic resonance spectroscopy, and found that the concentration increases toward the day of molting and decreases after molting. However, the total amount of NAGL collected from individual animals was greatest two to five days after molting, because the amount of urine collected was the lowest at the premolt stage, and it increased after molting. The highest median concentration of NAGL in *T. cheiragonas* was 29 $\mu$mol $l^{-1}$, which is 75% of the highest concentration reported in *C. sapidus*. This is the first report of NAGL as a molting biomarker in a species other than *C. sapidus*. We assume that NAGL is part of a pheromone bouquet in these two species.

## Introduction

Many and diverse species of decapod crustaceans mate after the female molts (Salmon, 1983). Females preparing to molt generate new exoskeletons under their existing ones (Smith and Chang, 2007). In some crab species, these females release sex pheromones in their urine. Four such species include the blue crab *Callinectes sapidus* (Gleeson, 1980); the shore crab *Carcinus maenas* (Eales, 1973; Bamber and Naylor, 1997); the three spot swimming crab *Portunus sanguinolentus* (Ryan, 1966); and the helmet crab *Telmessus cheiragonus* (Kamio *et al.*, 2000). Since the release of pheromones is linked to molting in these species, metabolites that increase in concentration in the urine of—or in the water surrounding—premolt females have been reported as the potential sex pheromone, or one of the components of a pheromone bouquet (Kittredge *et al.*, 1971; Asai *et al.*, 2000; Hardege *et al.*, 2011; Kamio *et al.*, 2014).

*N*-acetylglucosamino-1,5-lactone (NAGL) recently was reported to be a premolt biomarker metabolite in the urine of *C. sapidus* (Kamio *et al.*, 2014). NAGL is a candidate component of the sex pheromone in this species. The concentration of NAGL in urine is highest at the premolt stage, when sexually mature premolt females release a sex pheromone bouquet in their urine; males detect this compound as a chemical stimulus. Olfactory detection of this compound was confirmed (Kamio *et al.*, 2014), using calcium imaging of olfactory receptor cells (Tadesse *et al.*, 2014). The structure of the NAGL molecule indicates that it is an oxidized form of *N*-acetyl glucosamine, a monomer of chitin, which is a main component of the exoskeleton. Although chitin is common and abundant in crustacean biomass, and degradation and generation of chitin take place at each molt, the NAGL molting biomarker had never been reported in any crustacean, in any other species, or in any synthetic and/or degradation pathway of chitin before its discovery in *C. sapidus* (Kamio *et al.*, 2014).

The helmet crab *Telmessus cheiragonus* is a model crustacean, used to investigate sex pheromones. This species has

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*Abbreviations:* NAGL, *N*-acetylglucosamino-1,5-lactone; $^{1}$H NMR, nuclear magnetic resonance spectra used for observation and quantification of all hydrogen atoms of all metabolites in a biological fluid without chromatographic separation; NMR, nuclear magnetic resonance.



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precopulatory guarding as courtship, and it mates just after the female molts (Kamio *et al.*, 2003) once a year (Nagao and Munehara, 2001). Premolt and immediate postmolt females release a sex pheromone in urine that induces courtship, grasping, and precopulatory guarding behavior in males (Kamio *et al.*, 2000). Following the molt, females release a second sex pheromone, which stimulates males to copulate, insert their sexual appendages into the gonopores of females, and ejaculate (Kamio *et al.*, 2002). The responses of males to the two kinds of sex pheromones are detectable and distinguishable, using a sponge assay (Kamio *et al.*, 2000, 2002). The pheromones are detected by males through their olfactory organ, the outer flagella of their antennules (Kamio *et al.*, 2005). Thus, the mating of *T. cheiragonus* is linked to molting in sexually mature females, as in *Callinectes sapidus*, and the sex pheromones may have molting-related metabolites as a component.

In the present study, we hypothesized that sexually mature females of *T. cheiragonus* release NAGL in their urine, and that the NAGL concentration becomes highest at the premolt stage, as in *C. sapidus*. To test these hypotheses, we collected urine from females before and after molting, and measured the concentration of NAGL using nuclear magnetic resonance spectroscopy (NMR). We also measured the volume of the collected urine to calculate the total amount of NAGL released from one individual. The concentration of NAGL at the premolt stage of *T. cheiragonus* was compared with the concentration detected in *C. sapidus*. Urine from premolt and postmolt males was not tested in this study, because premolt males were not available.

## Materials and Methods

### *Collection of female crabs*

Individual and precopulatory pairs of *Telmessus cheiragonus* were collected from pier walls in Usujiri, Hokkaido, Japan, during the mating season in May. In early May, individual crabs were found on the pier walls; by the middle of May, many precopulatory guarding pairs were observed on these walls. Females were separated from their partner-males, and housed in a flow-through seawater system at ambient temperature under natural photoperiod. Each female was tagged with a number plate, and the molting date was recorded. Collection of urine samples and morphological observations were conducted at the Usujiri Marine Biological Station of Hokkaido University. Females were not fed during the urine collection period, because premolt females and newly postmolt females do not eat.

"Days from molting" were recorded on data sheets using minus (–) and plus (+) signs. One day before the molting day was indicated as "–1 day," one day after the molting day was "+1 day," and the day of molting was "0 day." However, in this paper, signs are not used with numerals denoting a day or days after molting; for example, two days after a molt is shown as "2."

### *Collection of urine samples and measurement of urine volume*

Urine samples were collected from the nephropores (openings of the antennal gland), using a vacuum pump, as described previously (Kamio *et al.*, 2000, 2014). In early May, females were collected as freely walking individuals, without precopulatory guarding by males (Kamio *et al.*, 2003). The molt stage of these females was determined based on morphological characteristics. Individuals with a dark red exoskeleton on the dorsal side and a dark yellowish exoskeleton on the ventral side were identified as early premolt females, and referred to as "pre-solo." Individuals with a light red exoskeleton on the dorsal side, a light yellowish exoskeleton on the ventral side, and a copulatory plug (Kamio *et al.*, 2002) were postmolt and post-mating females, and termed "post-solo." Urine samples from 64 pre-solo and 17 post-solo females were collected only once from each individual, on the day after their capture.

In the middle of May, females guarded by males were captured from the harbor pier. Urine samples were collected from each individual repeatedly every two days, from the premolt to the postmolt stage. Seventy premolt females were used for urine collection. Of these crabs, all molted during the urine collection period; 26 animals died while molting. Urine samples from each individual, collected on different days, were stored in 15-ml plastic centrifuge tubes at −20 °C and used for NMR experiments. Scales on the tubes were calibrated by adding 50 $\mu$l of water, from 50 to 2000 $\mu$l, using a syringe (1700 Series Gastight Syringe; Hamilton Co., Reno, NV). These scales were used to measure urine sample volume. Differences in the volume of urine collected from each individual per day over the molting period were analyzed by Kruskal-Wallis test ($P < 0.05$), followed by the Steel-Dwass test for multiple comparisons ($P < 0.05$).

### *Quantification of NAGL using nuclear magnetic resonance*

A 0.5 ml volume of each urine sample was lyophilized and solubilized in 500 $\mu$l of deuterium oxide mixed with 5 nmol of trimethylsilyl propionic acid (TSP) and 35 mmol $l^{-1}$ of phosphate buffer at pH 7.6. $^1$H NMR spectra, which are used for observation and quantification of all hydrogen atoms of all metabolites in a biological fluid without chromatographic separation, were obtained for these samples, using a Bruker AVANCE III 600 MHz spectrometer (Bruker Corp., Billerica, MA) and nuclear Overhauser effect spectroscopy (NOESY) PR1D pulse sequence. A total of 1024 scans were accumulated for each sample. A set of NMR signals of NAGL in the urine was confirmed by

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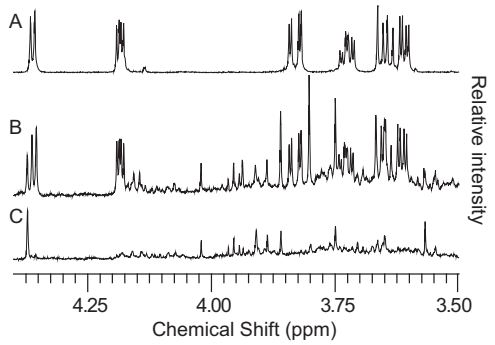


**Figure 1.** Nuclear magnetic resonance (NMR) spectra of urine with and without *N*-acetylglucosamino-1,5-lactone (NAGL). (A) Standard NAGL. (B) Urine from premolt female. NAGL was observed in this spectrum. (C) Urine from postmolt female. NAGL was not detected in this spectrum. ppm, parts per million.

comparison with a standard sample (Fig. 1). Since many of the premolt individuals within 17 to 7 days and 6 to 1 days of molting released less than 0.5 ml of urine per day, multiple urine samples from these same crabs during this period were pooled to make 0.5 ml, then used for NMR analysis. Three groups of premolt urine (pre-solo, −17 to −8 days, and −6 to −1 days) and four groups of postmolt urine (2 to 5 days, 6 to 8 days, 9 to 12 days, and post-solo) were prepared. Days before molting, or premolt, are indicated with a minus (–) sign. Numerals without a sign mean that the molt already occurred, or postmolt. 0 day denotes the day of molting. Between five and ten urine samples were pooled from each of the seven groups. The total amount of NAGL collected in each sampling from each individual crab was calculated by multiplying the concentration, as determined using NMR data, by volume of urine collected from each individual. The difference in concentration and total amount of NAGL in urine over the molting period was analyzed by Kruskal-Wallis test ($P < 0.05$), followed by the Steel-Dwass test for multiple comparisons ($P < 0.05$).

### *Comparison of NAGL concentration between* Telmessus cheiragonus *and* Callinectes sapidus

The concentrations of NAGL in the urine of late premolt-stage females of two brachyuran species, *Telmessus cheiragonus* and *Callinectes sapidus*, were compared by Mann-Whitney U test. The data set of NAGL concentration in the urine from stage −6 to −1 days animals in the present study was used as the concentration of urine samples of *T. cheiragonus* ($n = 10$). The data set for *C. sapidus* ($n = 8$) was from the data set used for pubertal premolt female urine in figure 6 of Kamio *et al.* (2014).

### *Statistics*

Statistical analyses were performed using R version 3.02 (R Core Team, 2015) and two-tailed tests.

## Results

### *Change in amount of urine*

In addition to the 64 pre-solo urine and 17 post-solo urine samples, a total of 288 samples of pre- and postmolt urine were collected from 70 females maintained in the laboratory. These 288 samples were used to determine the change in amount of urine from the premolt to the postmolt period. Urine from females at –17 days premolt and from females 12 days postmolt was obtained. The data were pooled every 2 days in all but 2 groups: in groups "−17 to −8 days" and "7 to 12 days," more days were included in each because fewer urine samples were available. Animals tested for each category numbered between 17 and 64. Pre-solo females produced the most urine, and immediate premolt females produced the least (Fig. 2). The median amounts were 1.2 ml and 0.050 ml, respectively. The amount of collected urine was lowest in the premolt stages within a week of molting, but it increased after molting. Post-solo females produced the second greatest amount of urine, which was 0.80 ml (median). The Kruskal-Wallis test showed a significant difference in urine amount among the molt stages ($\chi^2 = 166$, df = 10, $P < 0.05$). The Steel-Dwass test of multiple comparisons detected significant differences ($P < 0.05$) among molt stages (Table 1). The amount of urine from pre-solo females was significantly greater than for any other stage among guarded females. The amount of urine from the post-solo stage was greater than for urine collected at one and two days postmolt and for all of the premolt stages. The difference between pre-solo and post-solo urine amounts was not significant.

The amount of urine from premolt females that were within a week of molting was significantly less than the amount from postmolt females three and four days after molting. The amount of immediate premolt urine was significantly less than in all postmolt animals and premolt animals at stages earlier than –17 to –8 days. Among the individuals molted in the lab, the amount of urine collected from most premolt animals except for those at the immediate premolt stage, and most postmolt stages, was not statistically different (Table 1). However, postmolt animals that molted in the lab produced more urine than the premolt individuals (Fig. 2).

### *Concentration and total amount of NAGL in urine over the premolt and postmolt stages*

A total of 61 urine samples were used to analyze changes in concentration and total amount of NAGL in the urine from the premolt to the postmolt period. NAGL was observed in the urine samples, as shown in Figure 1. The samples were categorized into the following molt stages: pre-solo, −17 to −8 days, and −6 to −1 days (premolt);

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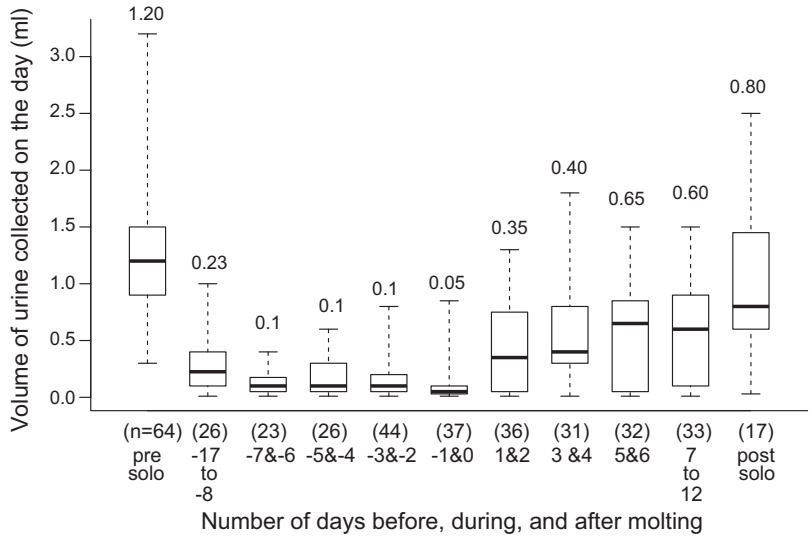


**Figure 2.** Box plot of the amount of urine collected from females before and after molting. The bar graph shows the volume of urine collected from female individuals of *Telmessus cheiragonus* in a single urine collection operation per day. Values are medians, interquartile ranges, minima, and maxima. Urine samples were collected from individuals 17 days before (−17) to 12 days after (12) molting. "Pre-solo," individuals not paired with males when they were collected for the study, and morphologically identified as premolt; "post-solo," individuals not paired with males when they were collected for the study, morphologically identified as postmolt, and having copulated. Differences between the bars, as detected by Steel-Dwass test, are summarized in Table 1. Days before the molting day are indicated by a minus (–) sign for premolt. No sign before the numeral means the animal molted (post-molt). 0 day, the day when the crab molted. The numbers in parentheses below each bar indicate the numbers of samples used. Numbers at the top of each bar show median volumes of urine.

2 to 5 days, 6 to 8 days, and 9 to 12 days (postmolt); and post-solo. NAGL was not detected in urine from the pre-solo or post-solo stages. The median concentration of NAGL ranged from 0 to 28.9 $\mu$mol $l^{-1}$. The concentration of NAGL was zero at pre-solo, and gradually increased toward the molting time. The highest concentration of NAGL was found in the immediate premolt urine, and it gradually decreased after molting (Fig. 3A). The Kruskal-Wallis test showed a significant difference in NAGL concentration among the different days before molting ($\chi^2 = 48.95$, df = 6, $P < 0.05$). Multiple comparisons using the Steel-Dwass test (Table 2) showed that the NAGL concentration at stage −6 to −1 days was significantly higher ($P < 0.05$) than at all the other stages. Interestingly, the test also showed that

**Table 1**

*Results of multiple comparisons by Steel-Dwass test of volumes of urine collected on different days before (Pre) and after (Post) molting*

| | Pre | −17 to −8 | −7 and 6 | −5 and −4 | −3 and −2 | −1 and 0 | 1 and 2 | 3 and 4 | 5 and 6 | 7 to 12 | Post |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Pre | | | | | | | | | | | |
| −17 to −8 | * | | | | | | | | | | |
| −7 and −6 | * | ns | | | | | | | | | |
| −5 and −4 | * | ns | ns | | | | | | | | |
| −3 and −2 | * | ns | ns | ns | | | | | | | |
| −1 and 0 | * | * | ns | ns | ns | | | | | | |
| 1 and 2 | * | ns | ns | ns | ns | * | | | | | |
| 3 and 4 | * | ns | * | * | * | * | ns | | | | |
| 5 and 6 | * | ns | ns | ns | ns | * | ns | ns | | | |
| 7 to 12 | * | ns | ns | ns | ns | * | ns | ns | ns | | |
| Post | ns | * | * | * | * | * | * | ns | ns | ns | |

ns, not significant. Minus (−) sign before numerals denote a premolt day; numerals with no sign are postmolt days. 0 = molt day.
* $P < 0.05$.

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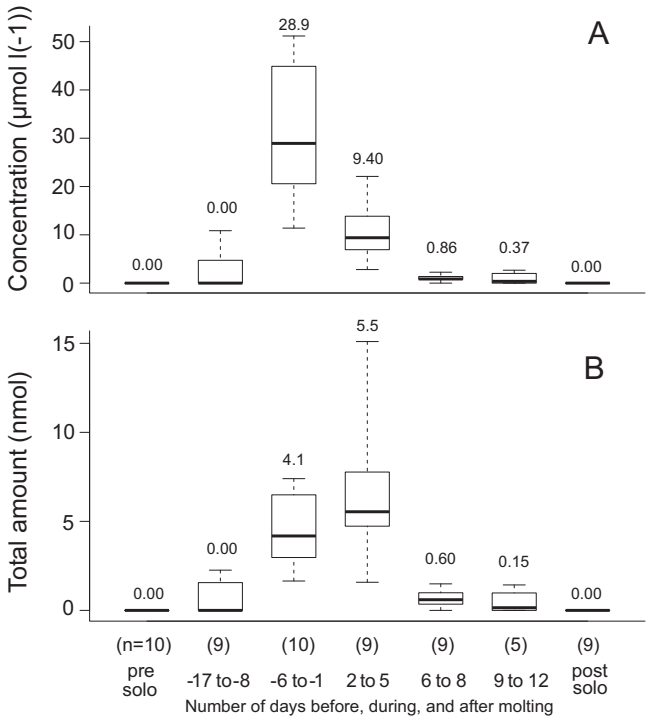


**Figure 3.** Box plots of the total amount and concentration of *N*-acetylglucosamino-1,5-lactone (NAGL) in premolt and postmolt females. (A) Concentration of NAGL in urine samples from premolt and postmolt females. The number at the top of each bar is the median concentration of NAGL. (B) Total amount of NAGL in urine samples from premolt and postmolt females. The number at the top of each bar is the median amount of NAGL. Values are medians, interquartile ranges, minima, and maxima. The number in parentheses (below each bar) is the number of samples used. Numbers with minus signs (–), days before the molt; numbers without minus signs, days after the molt. The differences between the bars, as detected by Steel-Dwass test, are summarized in Tables 2 and 3.

the NAGL concentration at stage 2 to 5 days was significantly higher than at the other stages, except for stages −17 to −8 days and −6 to −1 days. The concentration of NAGL at stage 6 to 8 days was significantly higher than at either the pre-solo or post-solo stage. The total amount of NAGL also increased toward the time of molting, but the highest amount of NAGL was observed at the postmolt stage just after molting (Fig. 3 B).

The median amount of NAGL ranged from 0 to 6.8 nmol. The Kruskal-Wallis test showed a significant difference in the total amount of NAGL among the different days towards molting ($\chi^2 = 48.76$, df = 6, $P < 0.05$). Multiple comparisons using the Steel-Dwass test (Table 3) showed that the total amount of NAGL at stages −6 to −1 days and 2 to 5 days was significantly higher ($P < 0.05$) than at all the other stages. The difference between these two stages was not significant. The amount of NAGL at 6 to 8 days was significantly greater than at either the pre-solo or post-solo stage.

**Table 2**

*Results of multiple comparisons by Steel-Dwass test of NAGL concentration in urine collected on different days before (Pre) and after (Post) molting*

| | Pre | −17 to −8 | −6 to −1 | 2 to 5 | 6 to 8 | 9 to 12 | Post |
|---|---|---|---|---|---|---|---|
| Pre | | | | | | | |
| −17 to −8 | ns | | | | | | |
| −6 to −1 | * | * | | | | | |
| 2 to 5 | * | ns | * | | | | |
| 6 to 8 | * | ns | * | * | | | |
| 9 to 12 | ns | ns | * | * | ns | | |
| Post | ns | ns | * | * | * | ns | |

NAGL, *N*-acetylglucosamino-1,5-lactone, a molting biomarker; ns, not significant. Minus (−) sign before numerals denotes premolt days; no sign before a numeral indicates a postmolt day.

* $P < 0.05$.

### *NAGL concentration at the late premolt stage of* Telmessus cheiragonus *and* Callinectes sapidus

The median highest concentration of NAGL was 28.9 $\mu$mol $l^{-1}$ and 38.5 $\mu$mol $l^{-1}$ in *T. cheiragonus* and *C. sapidus,* respectively (Fig. 4). No statistical difference was found by Mann-Whitney U test ($P = 0.17$, $Z = 1.36$).

## Discussion

NAGL, which had previously been reported only in the urine of *Callinectes sapidus* (Kamio *et al.*, 2014), was found in the urine of sexually mature premolt and postmolt females of *Telmessus cheiragonus* in the present study. The concentration of NAGL in the urine was highest 1−6 days prior to molting. After molting, this concentration gradually decreased. Thus, this report marks the second time that this molecule was found in crab urine as a premolt biomarker in the concentration. In contrast, the total amount of NAGL in

**Table 3**

*Results of multiple comparisons by Steel-Dwass test of the total amount of NAGL in the urine collected on different days before (Pre) and after (Post) molting*

| | Pre | −17 to −8 | −6 to −1 | 2 to 5 | 6 to 8 | 9 to 12 | Post |
|---|---|---|---|---|---|---|---|
| Pre | | | | | | | |
| −17 to −8 | ns | | | | | | |
| −6 to −1 | * | * | | | | | |
| 2 to 5 | * | * | ns | | | | |
| 6 to 8 | * | ns | * | * | | | |
| 9 to 12 | ns | ns | * | * | ns | | |
| Post | ns | ns | * | * | * | ns | |

NAGL, *N*-acetylglucosamino-1,5-lactone, a molting biomarker; ns, not significant. Minus (−) sign before numerals denote premolt days; no sign before a numeral indicates a postmolt day.

* $P < 0.05$.

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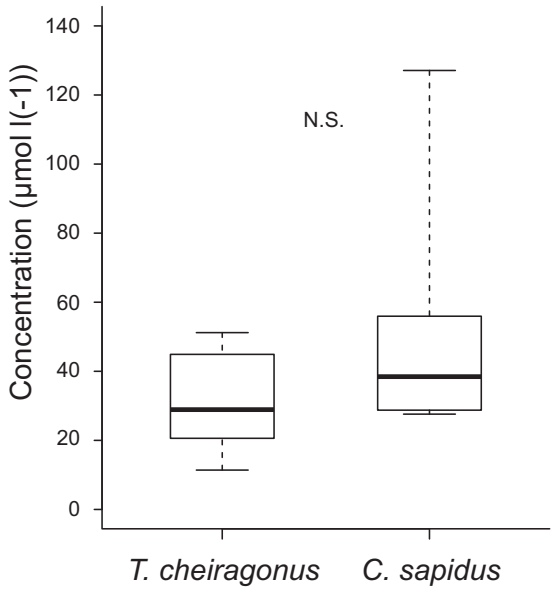


**Figure 4.** Box plot of *N*-acetylglucosamino-1,5-lactone (NAGL) concentrations in late premolt female urine of *Telmessus cheiragonus* and *Callinectes sapidus.* The data set used for *T. cheiragonus* ($n = 10$) is from the urine samples for −6 to −1 day females in Figure 3A. The data set for *C. sapidus* ($n = 8$) is from the data set used for pubertal premolt female urine in figure 6 of Kamio *et al.* (2014). Values are medians, interquartile ranges, minima, and maxima. No statistical differences were noted (Mann-Whitney U test: $P = 0.17$, $Z = 1.36$).

urine was greatest 2−5 days after molting. This is because the volume of urine released decreased toward the time of molting, then recovered after the molting. Therefore, in total amount, NAGL is a postmolt biomarker. As a biomarker for molt stage, the concentration of NAGL is useful only if urine is collected and quantified directly from the nephropores, as was done in the present study. Alternatively, NAGL released in urine could be collected from the aquarium water. In that case, the amount of NAGL would be important, even though there is no method for concentrating the diluted NAGL in seawater to a level high enough to be detected by NMR. When more sensitive detection methods are developed, the total amount of NAGL in tank water will be a more useful measure than NAGL concentration.

In chemical communication in the aquatic environment, both the concentration and total amount of the signal molecules released from senders may be important for receivers to detect by their chemosensory organs, which have some threshold to odorant molecules (Derby and Atema, 1982). In *T. cheiragonus*, the pheromone bouquet is released in urine by premolt and postmolt females (Kamio *et al.*, 2000, 2002). The concentration of signal molecules in the urine as released from nephropores may be diluted by the surrounding water, distributed by water flow generated by the animal's fanning organs (Breithaupt, 2001), and sent to the receiver's aesthetascs, or chemosensory organs (Kamio *et al.*, 2005; Reidenbach *et al.*, 2008; Breithaupt, 2011). The urine released from the females exists as a plume in the surrounding water. The plume has a filamentous structure, and animals detect each filament above some threshold (*e.g.,* detection limit of the receiver animal) (Weissburg, 2011). Using a three-dimensional, laser-induced fluorescence system to collect chemical concentration data, while at the same time observing the behavior of *C. sapidus* in a variety of plume types, Page *et al.* (2011) showed that *C. sapidus* responds to odorant filaments in an all-or-nothing fashion. The total amount of NAGL, which is based on a larger volume of released urine, may enlarge the size of the odor plume, while the concentration of NAGL in the urine may determine if the filaments are above the detection threshold. The highest median concentration of NAGL in *T. cheiragonus* was similar to what was discovered for *C. sapidus*. This finding indicates that NAGL is part of a pheromone bouquet in both species. A comparison of the total amount of urine between the two species would be valuable, but is not possible since the volume of the urine samples was not measured individually in *C. sapidus.* Interestingly, NAGL was detected in the male urine of *C. sapidus* (Kamio *et al*., 2014). In *T. cheiragonus*, it is possible that NAGL is released in the urine of premolt males; however, urine samples from molting males could not be tested because they were not available.

*T. cheiragonus* urine samples, collected from premolt females caught as one of a precopulatory pair from the field and from postmolt females 2−6 days after molting, contained sex pheromone, which stimulates grasping behavior in males (Kamio *et al.*, 2000). This range of pheromone release was similar to the range found in the present study. Thus, NAGL is a possible component of a sex pheromone bouquet in helmet crabs. As a courtship pheromone that evokes guarding behavior in male crabs, the concentration and total amount of NAGL should be at the highest levels during the premolt, not postmolt, stage. However, in the present study the peak in the total amount of NAGL released occurred during postmolt. The postmolt activity is copulation (Kamio *et al.*, 2003), and postmolt urine has the same pheromonally induced activity as premolt urine (Kamio *et al.*, 2002). Thus, releasing the same, or higher, level of NAGL after molting than during premolt is consistent with the pheromone component hypothesis. Another explanation for the greater release of NAGL seen after molting is that females must attract males more when they molt alone. The opportunity to copulate is limited to a short period after molting, and molted females without males must chemically signal to neighboring males.

For many animals, pheromones are mixtures of molecules, or bouquets, acting in synergy when combined in particular proportions (Wyatt, 2003, 2014). For example, in lepidopteran insects, sex pheromones are species-specific by virtue of their blend combinations and proportions (Ando *et al.*, 2004). In male mice, two volatile constituents of

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urine, dehydro-exo-brevicomin (DB) and 2-(secbutyl)-dihydrothiazole (BT), and other compounds in urine act synergistically to evoke inter-male aggression (Novotny *et al.*, 1985). Major urinary proteins are also known to promote inter-male aggression, and thus may be part of the pheromone (Chamero *et al.*, 2007). In sea hares *Aplysia* spp., binary blends of protein pheromones, attractins (Painter *et al.*, 1998), and any one of a number of other proteins (enticin, temptin, seductin) work together to attract mates (Cummins *et al.*, 2006, 2007). A blend of postmolt, female-derived, cuticular hydrocarbons, coated on polyethylene tubes, induced grasping behavior in male decapod shrimp (Zhang *et al.*, 2011); such a blend may work together with a glycoprotein (Bauer, 2011). As with these examples, the urine sex pheromone in premolt females of *T. cheiragonus* may be a blend of molecules. For this reason, the molecular identity of crustacean pheromones is relatively difficult to characterize compared to other systems (Hay, 2011). To identify pheromone blends, in which each component of the blend may not have pheromonal activity by itself, each component must be chemically identified individually in the active fractions and tested as a blend in behavioral bioassay.

Generally, any type of molecule can be a sex pheromone. Molecules from small to large, volatile to nonvolatile, and low to high polarity have been reported as pheromone in animals (Wyatt, 2003, 2014). These molecules include physiologically widely distributed compounds. For example, in polychaete worms, inosine, glutamic acid, glutamate (Zeeck *et al.*, 1992), uric acid (Zeeck *et al.*, 1998a), and cysteine-glutathione disulfide (Zeeck *et al.*, 1998b) are used individually and in a blend as pheromones that stimulate different steps in the reproductive behavior of males and females (Breithaupt and Hardege, 2012). In fish, hormones that are involved in female gonadal function and ovulation are released into water, functioning as sex pheromones that affect the behavior and physiology of males (hormonal pheromones) (Stacey and Sorensen, 2009). A few molting-related compounds have been reported in studies of pheromones of female crabs. Since the mating behavior of many species of crustaceans is tied to molting of females (Salmon, 1983), molecules related to molting have been tested as sex pheromones. Crustecdysone, a molting hormone, was reported as a sex pheromone in several brachyuran crab species (Kittredge *et al.*, 1971; Kittredge and Takahashi, 1972). Uridine diphosphate (UDP), which was identified by bioassay-guided fractionation as a stimulant of male sexual behavior in *Carcinus maenas*, is accumulated from UDP-*N*-acetylglucosamine during chitin biosynthesis in molting (Hardege *et al.*, 2011). These molecules, including NAGL, are supposed to be widely distributed in molting crustaceans, and can be components of the sex pheromone bouquet in these and other crustacean species. However, if these molecules are a part of the pheromone, and the pheromone has species specificity, these molecules must work together with other molecules to form a unique bouquet for each species. To elucidate the components of the pheromone blends, we need to perform simultaneous 1) bioassay-guided fractionation, to find the active molecule and/or physicochemical properties of the active molecules; 2) profiling of molting-related metabolites, to identify candidate pheromone molecules; and 3) evaluation of the synergy between the candidate pheromone molecules.

Having an indicator of molting time will be useful for the study of mating and growth in crustacean species. Morphological evaluation of molt stage without microscopy is possible in *Callinectes sapidus*; it is based on observations of color changes and of a newly generated exoskeleton under the old one (Smith and Chang, 2007). This method is possible because swimming portunid crabs, such as *C. sapidus*, have thin, flat dactyls on their fifth pereiopods. However, many species do not have such macroscopic indicators of molt stage; instead, microscopic observation of surgically removed tissue must be used to determine molt stage (Vigh and Fingerman, 1985). Surgical removal of setae or tissue injures animals and should be avoided if possible, especially when evaluating molt stage in preparation for other experiments using live animals. Measurement of the molting hormone ecdysteroid in hemolymph and tissues is an established method, and has contributed to an understanding of the endocrinology of crustacean molting and development (Chang and Mykles, 2011; Techa *et al.*, 2015). However, collecting hemolymph and tissue injures animals, and thus is unsuitable for repeated sample collecting from the same individual.

Collecting urine for the measurement of molting biomarker metabolites is thus a desirable method to evaluate molt stage, because this procedure does not physically injure the animals. Urine can be collected from the same animals repeatedly over several days. Quantifying NAGL concentration can be a method to evaluate molt stage in other crustacean species, as well as in *C. sapidus* and *T. cheiragonus*. The disadvantage of using NAGL as a biomarker is the low sensitivity of the measurement method (NMR). Although NMR analysis is a useful method for quantifying organic compounds in biological samples (Kamio *et al.*, 2015; Moe *et al.*, 2015), its sensitivity is not as great as the liquid chromatograph mass spectrometer (LC-MS). In the present study, urine volume decreased at the premolt stage, and measurement of NAGL in urine from one individual, using the NMR method, was not possible. More sensitive methods, such as LC-MS or other analytical approaches, are necessary for evaluation of the detailed concentration changes of NAGL in the six days before molting in the case of *T. cheiragonus*. Thus, detection of NAGL may be useful in scientific research on crustaceans. However, it may not be a practical method for fisheries

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industries, because the procedure is complicated and requires expensive equipment. For these industries, more convenient and low-cost methods are needed to detect NAGL.

The concentration of NAGL in hemolymph is unknown in both *Telmessus cheiragonus* and *Callinectes sapidus*. NAGL may be excreted in the urine by a gland such as the nephropore rosette gland (as suggested for *Homarus americanus* by Bushmann and Atema, 1996, and for *Carcinus maenus* by Fontaine *et al.,* 1989), or be excreted from hemolymph into urine, or simply leak out from hemolymph into the urine. Measurement of the NAGL level in hemolymph should be done in *T. cheiragonus* and *C. sapidus* to answer these questions.

Thus, NAGL is a premolt biomarker and candidate component of the courtship sex pheromone in *T. cheiragonus* and *C. sapidus*. Whether this finding holds for other crustaceans requires further comparative testing. Analysis of concentrations of NAGL in urine and in tissues across molt stages, together with behavioral, morphological, endocrinological, and other physiological changes, are necessary to reveal the function and origin of this metabolite.

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