Title: The smell of moulting: N-acetylglucosamino-1,5-lactone is a premoult biomarker and candidate component of the courtship pheromone in the urine of the blue crab, *Callinectes sapidus*

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Running headline: Moulting biomarker in urine of blue crabs: Kamio et al.

Key words: biofluids, chemoreception, courtship, metabolite profiling, moulting, pheromone, quantitative NMR, urine
Word count for the text: 8598 words excluding reference list including figure legends and tables
SUMMARY

Female blue crabs (*Callinectes sapidus*) in their pubertal moult stage release unidentified sex pheromone molecules in their urine, causing males to respond with courtship behaviours including a display called courtship stationary paddling and a form of precopulatory guarding called cradle carry. We hypothesized that pheromones are mixtures of molecules and are more concentrated in urine of pubertal premoult females compared to other mouling stages and thus that these molecules are biomarkers (i.e. metabolites that can be used as an indicator of some biological state or condition) of pubertal premoult females. We tested this hypothesis by combining bioassay-guided fractionation and biomarker targeting. To evaluate the molecular mass of the putative pheromone by bioassay-guided fractionation, we separated urine from pubertal premoult females and intermoult males by ultrafiltration into three molecular mass fractions. The <500 Da fraction and the 500–1000 Da fraction but not the >1000 Da fraction of female urine induced male courtship stationary paddling, but none of the fractions of male urine did. Thus, female urine contains molecules <1000 Da that stimulate courtship behaviours in males. Biomarker targeting using nuclear magnetic resonance (NMR) spectral analysis of the 500–1000 Da fraction of urine from premoult and postmoult males and females revealed a premoult biomarker. Purification, NMR, mass spectrometry, and HPLC analysis of this premoult biomarker identified it as *N*-acetylglucosamino-1,5-lactone (NAGL) and showed that it is more abundant in urine of premoult females and males than in urine of either postmoult or juvenile females and males. NAGL has not been reported before as a natural product or as a molecule of the chitin metabolic pathway. Physiological and behavioural experiments demonstrated that blue crabs can detect NAGL through their olfactory pathway. Thus, we hypothesize that
NAGL is a component of the sex pheromone and that it acts in conjunction with other yet unidentified components.
INTRODUCTION

In many animal species, individuals that are ready to mate release chemical signals, called sex pheromones, that are detected and used by other individuals to identify potential mates (Wyatt, 2003, 2010; Johansson and Jones, 2007; Gomez-Diaz and Benton, 2013; Petrulis, 2013). Pheromones can be the basis for the identification of many features of these potential mates, including species (Billeter et al., 2009; Levesque et al., 2011; Leary et al., 2012), physiological condition related to readiness to mate (Kobayashi et al., 2002; Ferrero et al., 2013), and quality of the mate (Thornhill and Gangestad, 1999). In this context, mate recognition using multicomponent chemicals is one way to establish the specificity of a chemical signal.

For many insects and some vertebrates, pheromones are mixtures of molecules acting in synergy when combined in particular ratios (Wyatt, 2003, 2010). For example, in lepidopteran insects, sex pheromones are species specific by virtue of their blend combinations and ratios, thus ensuring reproductive isolation (Ando et al., 2004; Lassance et al., 2010). In male mice, two volatile constituents of urine, dehydro-exo-brevicomin (DB) and 2-(sec-butyl)-dihydrothiazole (BT), act synergistically to evoke inter-male aggression when added to urine of castrated males but not when added to water (Novotny et al., 1985). The required presence of urine indicates that other compounds in urine of males work in synergy with these two compounds to stimulate aggressive behaviour in other males. Major urinary proteins are also known to promote inter-male aggression and thus may be part of the pheromone (Chamero et al., 2007). DB and BT also have additional functions besides mediating aggression, such as attracting females (Osada et al., 2008).
To identify pheromone molecules, one approach is bioassay-guided fractionation. This involves a progressive fractionation of natural products based on physicochemical properties such as polarity, molecular mass, or molecular charge (Sarker et al., 2006) coupled with bioassays to identify which fractions are bioactive (Hay et al., 1998; Koehn and Carter, 2005) until bioactive molecules are isolated and identified. However, using this approach to identify components in a synergistic blend of pheromone molecules may be difficult because when tested alone each individual component may lack biological activity, as in the case of the nematode *Caenorhabditis elegans* (Srinivasan et al., 2008).

A second approach in identifying pheromone compounds is biomarker targeting. Biomarker targeting has been used to discover bioactive molecules associated with a variety of specific conditions (Robinette et al., 2012). An example in mice is DB, which was first identified as a testosterone-level dependent urinal biomarker (Novotny et al., 1984) and then later confirmed as a pheromone through behavioural bioassays (Novotny et al., 1985). A family of glycosides, the ascarosides, which differentially regulate *C. elegans* development and behavior, were also revealed by biomarker targeting using two-dimensional nuclear magnetic resonance (2D NMR) (Pungaliya et al., 2009).

Research on sex pheromones of crustaceans has often used as models brachyuran crabs that mate only after the female's pubertal moult. These species have a long courtship period with precopulatory mate guarding (Christy, 1987) in which the pubertal premoult female releases pheromones and the male carries the female until she mouls (Dunham, 1988; Asai et al., 2000; Kamio et al., 2003). In such species with precopulatory mate guarding, males may choose females that are close to mouling in order to maximize their mating success during a limited reproductive season by mating...
more often and with a relatively short mate-guarding time period. For example, males of the hermit crab *Pagurus nigrofascia* prefer females that are closest to their pubertal moult (Suzuki et al., 2012). For these reasons, sex pheromones of mate-guarding crustaceans have sometimes been thought to be moulting-related metabolites. Crustecdysone, a moulting hormone, was reported as a sex pheromone in several brachyuran crab species (Kittredge et al., 1971; Kittredge and Takahashi, 1972). This idea, however, has not been supported by later work on several crustacean species (Atema and Gagosian, 1973; Gleeson et al., 1984; Hayden et al., 2007), and crustecdysone may play a role in other chemosensory-driven behaviors than courtship and reproduction (Hayden et al., 2007; Coglianese et al., 2008).

To date, only a few compounds have been identified in pre- or postmoult female crabs and reported as sex pheromones or potential sex pheromones. These include uridine diphosphate from the urine of premoult females of the shore crab *Carcinus maenas* and possibly other species of decapod crustaceans (Bublitz et al., 2008; Hardege and Terschak, 2011; Hardege et al., 2011) and ceramides from the water surrounding postmoult females of the hair crab *Erimacrus isenbeckii* (Asai et al., 2000). Thus, our knowledge of pheromone molecules of crab species is very limited, and more extensive research in diverse species is necessary to characterize the nature of sex pheromones in crabs, including the possibility of multicomponent pheromones.

Moulting and mating are also closely linked in blue crabs, *Callinectes sapidus* (Jivoff et al., 2007). Female blue crabs are sexually active only once in their life, just after they experience a terminal, or pubertal, moult. Pubertal premoult females release pheromone in their urine, and males detect the pheromone via their olfactory organs, leading to initiation of the courtship (Teytaud, 1971; Gleeson, 1980).
Courtship consists of two behaviours. One is courtship stationary paddling (see Movies 1A and 1B in supplementary material of Kamio et al., 2008). This behaviour involves raising the swimming legs vertically over the carapace, turning them so that the flat side of the paddle blades face anteriorly to push water forward, and then moving them from side to side. In addition to the side-to-side movement, each blade moves from outside to inside and backward to forward to make a circular motion, with the left and right blades 180 degrees out of phase (Wood and Derby, 1995; Kamio et al., 2008).

When a crab performs this behaviour, it does not walk or move backward or forward, and it always performs chelae spread and high on legs behaviours (see Table 1 for descriptions of these behaviours). A second behavior in courtship is precopulatory mate guarding, or cradle carry, in which a male crab holds the female underneath him by his first and second walking legs, such that both crabs face in the same direction (Kamio et al., 2008). Precopulatory guarding lasts for several days until the female moults and copulation begins (Jivoff and Hines, 1998). When a male can easily access a female, he initiates precopulatory mate guarding; but if the male cannot reach the female, the male initiates courtship stationary paddling to produce a water current toward the female which may deliver his sex pheromone to her (Kamio et al., 2008). Thus, male and female blue crabs use bidirectional chemical communication via sex pheromones.

Characterization of the molecular nature of the female sex pheromone of *C. sapidus* by Richard Gleeson showed that its molecular mass is in the range of 300 to 600 Da and it is stable at 95°C (Gleeson et al., 1984; Gleeson, 1991).

Towards our goal of identifying the molecular nature of sex pheromones in blue crabs, we hypothesized that the female’s sex pheromone is a multicomponent mixture contained in urine that includes small polar molecules that in combination code the
species, sex, moulting, and reproductive status of the female (Kamio, 2009; Kamio and Derby, 2011). This hypothesis can be tested by identifying biomarkers of pubertal premoult female contained in urine and measuring the ability of these compounds, alone and in combinations, to produce courtship behaviours. Towards identify such molecules, we combined bioassay-guided fractionation and biomarker targeting. Because male blue crabs mate only with pubertal premoult females, we hypothesized that the concentration of pheromone molecules is higher in urine of pubertal premoult females compared to other moulting stages. In other words, the pheromone is a biomarker of pubertal premoult females that is contained in its urine and detected by males using their olfactory system. To test this hypothesis, we first assayed ultrafiltrate fractions of urine of pubertal premoult females and postmoult males and females for their courtship-inducing activity using a behavioural bioassay with male crabs, to identify a fraction containing the pheromone. Second, we compared the molecular profiles of urine of male and female crabs of various moult stages using $^1$H NMR spectroscopy, to identify a biomarker of pubertal premoult females. Third, we elucidated the structure and quantified the concentration of a biomarker of pubertal premoult females. Finally, we tested the bioactivity of this biomarker in physiological and behavioural assays with male crabs. Our results identify N-acetylglucosamino-1,5-lactone as a moulting biomarker and candidate courtship signal in the urine of the blue crab, *C. sapidus*. Some generalities of the findings of this paper were summarized in reviews (Kamio, 2009; Kamio and Derby, 2011).

RESULTS
Bioassay of ultrafiltrate fractions of urines

Bioassay-guided fractionation was used to determine the approximate molecular mass of pheromone molecules in the urine of pubertal premoult females that elicits courtship behaviour in adult male crabs. Urine from intermoult males, which does not contain the pheromone, was used as a control. Four behaviours, defined in Table 1, were recorded in this assay: courtship stationary paddling, grasping, high on legs, and chelae spread.

Courtship stationary paddling is the only behaviour of the four that is specific to courtship; the others can occur in either courtship or agonistic encounters. Three ultrafiltrate fractions of urine of pubertal premoult females and adult males were tested: small (<500 Da), medium (500-1000 Da), and large (>1000 Da).

All four behaviours – courtship stationary paddling, grasping, high on legs, and chelae spread – showed a significant difference in degree of activation in males across the 10 stimuli tested (Cochran's Q test, $P < 0.01$) (Fig. 1). The small and medium ultrafiltrate fractions and a mixture of the small, medium, and large ultrafiltrate fractions of urine from pubertal premoult females induced adult intermoult males to perform courtship stationary paddling and chelae spread at significantly higher levels than the seawater control (McNemar test, $P < 0.05$) (Fig. 1A, D). The small fraction of female urine but not the medium fraction also evoked two other behaviours at higher levels than the seawater control: standing high on legs and grasping (McNemar test, $P < 0.05$) (Fig. 1B, C). The large ultrafiltrate fraction of urine from pubertal premoult females did not induce any behaviour (Fig. 1A-D). The small fraction of male urine induced standing high on legs and chelae spread (McNemar test, $P < 0.05$) (Fig. 1C, D). Thus, the small fraction of pubertal premoult female urine induced all of the behaviours and
the medium fraction of female urine induced courtship stationary paddling and chelae spread, indicating that these two fractions contain the pheromone molecules. For our subsequent biomarker targeting experiments, we used the medium fraction of female urine because compounds in it were easier to separate from inorganic salts in urine and we were able to purify and identify a pubertal premoult female biomarker molecule from it.

NMR spectral detection of a premoult biomarker in urine of pubertal premoult females

NMR-based biomarker targeting was used to identify molecules that uniquely identify premoult animals. We examined the medium fraction of urine from the four types of crabs that were available to us at that time: pubertal premoult females, sexually mature postmoult females, adult premoult males, and adult postmoult males. $^1$H NMR spectra for the medium fraction of urine appeared different for pubertal premoult females compared to either adult premoult males, sexually mature postmoult females, or adult postmoult males collected in North Carolina (Fig. 2). Urine from pubertal premoult females had a prominent singlet signal at 2.07 ppm, a doublet of doublet signals at 4.19 ppm, and a doublet signal at 4.36 ppm, all of which were absent from the urine of postmoult adult males and females. In the urine of premoult adult males, these signals existed, albeit with smaller intensity. Similar differences occurred with urine samples from premoult females and males and from postmoult females collected in Florida. The molecule associated with this set of signals is thus a premoult biomarker preferentially contained in pubertal premoult female urine, and we therefore sought to purify and identify this molecule.
Purification and structure determination of the premoult biomarker

A 0.8 mg sample of this biomarker compound was purified from 300 ml of urine from pubertal premoult females using HPLC (Fig. 3). The molecular formula of this compound was determined by HR ESI TOF-MS to be C_{8}H_{13}NO_{6} (m/z 242.0596 [M+Na]^+, Δ-4.5 mDa). ^1H NMR and Heteronuclear Single Quantum Coherence Spectroscopy (HSQC) 2D NMR experiments identified one acetyl methyl group, four N- or O-linked methane groups, and one O-linked methylene group (Table 2). H-H Correlation Spectroscopy (COSY) 2D NMR experiment and Heteronuclear Multiple Bond Correlation Spectroscopy (HMBC) 2D NMR experiments connected these groups to elucidate a N-acetyl amino sugar-like structure missing an anomeric proton at 5.0 to 5.2 ppm but with a carbonyl group. The Nuclear Overhauser Effect Spectroscopy (NOESY) 2D NMR experiments revealed a correlation between H2 and H4 and between H3 and H5, indicating that this compound is a derivative of D-glucose, N-acetylglucosamino-1,5-lactone (NAGL) (Fig. 4). NMR spectral data for this compound and for an NAGL standard were identical (Fig. 5), and co-injection of the two using HPLC resulted in only one peak, thus confirming that this compound is NAGL.

Quantification of NAGL in urine

To quantify NAGL in urine of male and female crabs at different stages of moulting, the concentration of NAGL in urine from intermoult juveniles and from premoult and postmoult adult males and pubertal premoult females was measured using quantitative ^1H NMR spectroscopy. NAGL was detected in juveniles of both sexes and in both
The concentrations of NAGL in urine from males and females were not significantly different from each other, while the concentrations were different across moulting stages (two-way ANOVA, see Fig. 6 legend). NAGL levels ranged from 7 to 50 μmol l⁻¹ in female urine and from 0 to 46 μmol l⁻¹ in male urine, with the highest levels in the premoult stage of males and females. Significant differences were observed between urine from pubertal premoult females vs. urine from either postmoult males or juvenile females (Tukey test, \( P < 0.05 \)).

**Behavioural responses of males to NAGL**

We assessed the activity of NAGL in behavioural assays using adult male crabs. In this experiment, males did not respond to urine from pubertal premoult females with courtship behaviour. Thus, these males were sexually inactive, and consequently this assay could not be used to ascertain whether NAGL elicits sexual behaviour. Nonetheless, we were able to use this assay to determine if males can detect NAGL at 1 mmol l⁻¹ and respond to it with either epipod waving, a behavior that produces a water current directed at its antennules and thus is associated with chemosensory detection (Brock, 1926; Denissenko et al., 2007), or cheliped extension forward, an appetitive behaviour associated with attempts to acquire the source of the chemicals (Fig. 7). Cheliped extension forward was evoked in 17 out of 24 male crabs by shrimp juice, but not by the other stimuli: premoult female urine, NAGL, and the negative control, seawater. Epipod waving was elicited by all stimuli except the seawater control, 21 crabs responded to shrimp juice, 10 responded to pubertal premoult female urine, and 13 responded to NAGL. All of these responses were significantly different compared to
the seawater control ($P < 0.01$, McNemar test). This experiment demonstrates that male blue crabs can detect 1 mmol l$^{-1}$ NAGL.

**Responses of olfactory receptor neurons to NAGL**

We assessed whether NAGL is detected by the olfactory pathway of crabs by using Ca$^{2+}$ imaging to examine NAGL’s ability to excite olfactory receptor neurons (ORNs). We recorded responses from 17 ORNs in three preparations. Representative responses from three cells in one preparation are shown in Figure 8. In total, 15 of these 17 ORNs showed robust (i.e. maximum $\Delta F/F > 0.02$) and transient increases in intracellular Ca$^{2+}$ in response to one or more of the tested stimuli. Of the three ORNs tested only with 0.1 mmol l$^{-1}$ NAGL, two showed Ca$^{2+}$ responses. Of the 14 ORNs tested with 1% TM and 0.1 mmol l$^{-1}$ NAGL, six responded to both stimuli (Figs. 8A, B), seven responded only to 1% TM and not to 0.1 mmol l$^{-1}$ NAGL, and one did not respond to either stimulus (Fig. 8). None of the ORNs in our sample responded to 0.1 mmol l$^{-1}$ NAGL but not to 1% TM. Thus, our experiments show that some ORNs in blue crabs can detect NAGL and that ORNs have different response specificities to this set of odorants.

**DISCUSSION**

Our study used a combination of biomarker targeting and bioassay-guided fractionation towards identifying components of the sex pheromone of pubertal premoult female blue crabs, *Callinectes sapidus*, that elicit courtship behaviour in males. Bioassay-guided fractionation using a behavioural assay with adult male crabs showed that the sex pheromone of pubertal premoult females, which is the sexual stage, is one
or more molecules that are small and polar. Both the <500 Da fraction and the 500-1000 Da fraction of urine of pubertal premoult females evoked males to perform courtship stationary paddling, a distinctive behaviour evoked by the sex pheromone of females (Gleeson, 1980, 1982, 1991; Kamio et al., 2008). Fractions of urine of males did not evoke courtship stationary paddling. Interestingly, the <500 Da fraction of urine of pubertal premoult females, but not the 500-1000 Da fraction, also evoked behaviors that are a part of courtship but not specific to it, including behaviours evoked by male urine, such as grasping, and standing high on legs Chelae spread was evoked in males by the <500 Da and 500-1000 Da fractions of urine of pubertal premoult females and the <500 Da fraction of urine of adult males. Thus, our results indicate that urine of pubertal premoult females contains one or more sex-specific pheromone molecules that are less than 1000 Da and other molecules <500 Da that might be pheromone molecules but also might play a role in agonistic interactions.

Our results agree with Gleeson that the female sex pheromone is 300 to 600 Da (Gleeson, 1991) and non-volatile (Gleeson et al., 1984). Our results thus show that the sex pheromone in urine of female blue crabs consists of small (<1000 Da) molecules. This also appears to be the case for water-borne sex pheromones (including those released in the urine) of other brachyuran decapod crustaceans, including the shore crab Carcinus maenas (Bublitz et al., 2008; Hardege and Terschak, 2011; Hardege et al., 2011), hair crab Erimacrus isenbeckii (Asai et al., 2000), helmet crab Telmessus cheiragonus (Kamio et al., 2002), and other species (Kittredge et al., 1971; Kittredge and Takahashi, 1972; Bublitz et al., 2008; Breithaupt and Thiel, 2011). Unlike the above cases, water-borne sex pheromones of some other crustaceans, such as the parasitic sea louse Lepeophtheirus salmonis, may be small volatile molecules.
Unlike water-borne sex pheromones (including those released with the urine), contact pheromones are located on the body surface of animals and are detected by the mating partner with contact chemoreceptors. These contact pheromones may be large (>1000 Da) and non-volatile molecules such as glycoproteins (copepods: Ting and Snell, 2003; caridean shrimp: Caskey et al., 2009) or possibly small (<1000 Da) hydrocarbons (caridean shrimp: Zhang et al., 2011).

We identified \(N\)-acetylglucosamino-1,5-lactone (NAGL) in the 500-1000 Da fraction of urine as a biomarker of premoult crabs and a potential component of the sex pheromone from pubertal premoult females. This molecule may act in conjunction with other unidentified components to stimulate courtship behaviour of males. Although NAGL has a molecular mass of 219 Da, it was retained on a YC-05 membrane during fractionation of urine, thus demonstrating that estimates of molecular mass by ultrafiltration are not strict and that the 500-1000 Da fraction of urine may contain other molecules outside of this range. The concentration of NAGL is highest in urine of the premoult stage of both males and females (Figs. 2 and 6), thus demonstrating that NAGL is a biomarker for premoult crabs, regardless of sex. Urine of postmoult females has NAGL in Figure 6 but not in Figure 2. This might result from a difference in when urine was collected over the 1- to 3-day postmoult period for the samples used in these two experiments. For example, urine used for Figure 6 might have been collected before the decline of NAGL after moultng, and urine for Figure 2 might have been collected after the decline. NAGL has never before been identified as a natural product or metabolite from any organism. NAGL is probably produced from \(N\)-acetylglucosamine by an oxidase, as shown in Figure 9. Although such an enzyme has never been found, a functionally similar enzyme for glucose, which has the identical sugar structure as
N-acetylglucosamine, is well known to produce gluconolactone from glucose (Wong et al., 2008). NAGL is known as an inhibitor of the enzyme N-acetylglucosaminidase in several species including *E. coli*, limpets, mice, and bovines using artificially synthesized compounds (Findlay et al., 1958; Yem and Wu, 1976; Legler et al., 1991), although NAGL was not previously known as a natural product. This inhibition might be a natural biological function of NAGL in the molting process of blue crabs. Since N-acetylglucosaminidase is involved in the degradation of chitin (Muzzarelli, 1996; Merzendorfer and Zimoch, 2003), we propose that NAGL plays a role in chitin metabolism in the premolt stage of blue crabs by inhibiting N-acetylglucosaminidase (Fig. 9). Preparation for molting during the premolt stage involves degrading old cuticle and generating new cuticle (Smith and Chang, 2007). NAGL may control chitin degradation in the old cuticle or protect chitin in the new cuticle by inhibiting N-acetylglucosaminidase, as shown in Figure 9.

Our physiological and behavioural experiments showed that NAGL is detected by the olfactory pathway and evokes behavioural responses of male blue crabs. ORNs in aesthetasc sensilla on the lateral flagellum of the antennules of crustaceans are known to uniquely detect conspecific chemicals associated with sex, social interactions, and alarm (Aggio and Derby, 2011; Schmidt and Mellon, 2011; Breithaupt and Thiel, 2011), including courtship pheromones of blue crabs (Gleeson, 1982, 1991). Our observation from Ca$^{2+}$ imaging that NAGL activates these ORNs is consistent with NAGL being a candidate intraspecific signal, either as an indicator of molting or as a sex pheromone. The fact that these ORNs have different chemosensitivities, some sensitive to both NAGL and food-related chemicals and others sensitive to only one of these, supports the notion that male crabs can discriminate between these odorants. Our observation...
that a single aesthetasc sensillum contains ORNs that are sensitive to both food-related
chemicals and NAGL is also consistent with previous activity labeling of ORNs in blue
crabs (Cate et al., 1999). Not only does NAGL activate ORNs but it also produces
behavioural responses from males, including cheliped extension forward and epipod
waving. Unfortunately, we could not evaluate if NAGL evokes courtship behaviour
from males, since the males used in this experiment were not responsive to female urine.
The moulting hormone 20-hydroxyecdysone, or crustecdysone, suppresses feeding
behaviour of male shore crabs, Carcinus maenas, during the mating season, possibly to
suppress cannibalism by males on the vulnerable, recently moulted females (Hayden et
al., 2007). Whether NAGL has such an effect during mating in blue crabs remains to be
determined.
Together, our results show that NAGL is a biomarker of the premoult stage of
blue crabs and that it is detected by adult male crabs through their chemical senses, thus
making NAGL a candidate component of the sex pheromone of female crabs. We
hypothesize that the NAGL is one member of a multicomponent blend in the sex
pheromone of female blue crabs (Kamio, 2009; Kamio and Derby, 2011). According to
this model, males also may use other compounds in urine of pubertal premoult females
to recognize them as mating partners, similar to the case for the pheromones mediating
intrasexual aggression in male mice (Novotny et al., 1985). This hypothesis can be
tested by identifying more biomarkers of specific conditions and measuring the ability
of these compounds, alone and in combinations, to produce courtship behaviours.

MATERIALS AND METHODS
Collection, maintenance, and moult staging of animals

For collection of urine and for behavioural experiments, juvenile and adult male and female blue crabs (*Callinectes sapidus* Rathbun) were caught using cage traps or hand nets from waters near St. Augustine, FL, and Swan Quarter and Beaufort, NC, USA. Animals were held in aquaria equipped with flow-through natural seawater at an ambient temperature of 28°C in St. Augustine, 18-23°C in Beaufort for behavioural experiments, and 28°C in Swan Quarter for urine collection. Behavioural experiments were performed at the Whitney Laboratory for Marine Bioscience, University of Florida, St. Augustine, FL, or the Duke University Marine Laboratory, Beaufort, NC. For calcium-imaging experiments, male and female crabs were purchased from local seafood markets and brought to Georgia State University where they were held in 80-liter aquaria with re-circulated, filtered, and aerated artificial seawater (Instant Ocean™, Aquarium Systems Inc., Mentor, OH, USA) at 20–25°C and a 12 h:12 h light:dark cycle. Animals were fed shrimp, though late premoult crabs of either sex did not eat. Moult stage was determined by viewing the generation of new cuticle in the edge of the paddles of the swimming legs, according to Smith and Chang (2007): premoult animals had new cuticle beginning to separate from the old cuticle (moult stages D₀ to D₄), postmoult animals were 1–3 days after the moulting, and intermoult animals lacked any moulting indicators on their paddles (i.e. moult stage C) and did not moult during experiments. Dark blue or purple coloration of the abdomen is another indicator of pubertal premoult females (Jivoff et al., 2007)

Analytical chemistry equipment
Beckman HPLC system (Beckman Coulter, Inc., Brea, CA, USA) equipped with a 168 photodiode array was used for purification and identification of compounds. For NMR, Bruker Avance 400 and 500 MHz (Bruker Co., Billerica, MA, USA) and Varian Inova 500, 600 MHz instruments equipped with room temperature probe, and 800 MHz instruments equipped with cold probe (Agilent Technologies, Inc., Santa Clara, CA, USA). NMR data were processed and analyzed using MestReNova (Mestrelab Research, Santiago de Compostela, Spain), X-WinNMR (Bruker Co.), or TopSpin software (Bruker Co.). Mass spectroscopy data were obtained by Waters ESI-QTOF micro instrument (Milford, MA, USA) using carrier solvent, methanol:water = 1:1 containing 0.5% formic acid.

**Bioassay of ultrafiltrate fractions of urines**

**Collection of urine**

We collected urine from the nephropores of the antennal glands (Fig. S1), as was the case in previous studies on blue crabs (Gleeson, 1980), green crabs *Carcinus maenas* (Bamber and Naylor, 1997), and helmet crabs *Telmessus cheiragonus* (Kamio et al., 2000). To do this, crabs were cuffed with silicone tubes that fit over their major chelae and then shaken to remove the water in the gill chambers that might leak out and contaminate the urine. The cuticular lid covering the nephropore was lifted with the unsharpened tip of a micro-knife (plastic handle, 15° cutting angle: Fine Science Tools Inc., Foster City, CA, USA). This allowed urine to leak from the nephropore, which was then collected into 5 or 50 ml plastic tubes by aspirating with a plastic-tipped micropipette connected to a vacuum pump. These urine samples were immediately
frozen and stored temporarily at -20°C, then transported to Georgia State University where they were stored at -80°C.

Preparation of ultrafiltration fractions

Ultrafiltration was used to prepare fractions of urine based on molecular mass. Ultrafiltration membranes are given a nominal molecular mass to describe their pore size; however, these membranes do not separate strictly by molecular mass because the shape of a molecule in aqueous solution affects its movement through membranes during ultrafiltration. Nonetheless, this method is useful in estimating the molecular mass of the pheromone components. Ten ml of urine were collected from pubertal premoult females and intermoult males and pooled respectively, and each of these samples was separated into three molecular mass fractions by ultrafiltration using Amicon YM-1 and YC-05 (EMD Millipore, Billerica, Massachusetts, USA) under nitrogen gas atmosphere at 4°C. The fractions are <500 Da (S, small), 500–1000 Da (M, medium), and >1000 Da (L, large). These fractions were lyophilized and stored at -20°C and solubilized with deionized water to their original, full-strength concentration just before behavioural bioassay.

Behavioural bioassay

Behavioural bioassays were performed on animals held in a 60 x 30 x 25 cm transparent plastic aquarium equipped with flow-through natural seawater at 6 l min⁻¹ with a water depth of 20 cm and salinity of 35 ppt. An intermoult female and intermoult male were introduced into the aquarium and acclimated for 1 h until they stopped moving and interacting. Then, chemical stimuli were introduced into the aquarium through an inlet,
and the behaviour of males was recorded. Stimuli were 20 μl of each of the following:

- the three molecular mass fractions (S, M, L) of urine from pubertal premoult females
- and from intermoult males, as described above; a mixture of these three molecular mass fractions (Mix); female urine (from pubertal premoult females, as a positive control);
- and seawater (as a negative control). Stimuli were presented in a blind fashion such that the observer was not aware of the identity of each, and the order of presentation of stimuli was randomized. Several behaviours, as defined below and as shown in supplemental videos in Kamio et al. (2008), were studied in assays of 55 male crabs, and used for data analysis.

Courtship in blue crabs involves courtship display prior to forming pairs, and then after pairing, precopulatory mate guarding occurs in which a male carries a female in a posture called cradle carry. Only a subset of the male’s behaviour during this long courtship is useful as a measure of response to courtship pheromone or its components. The behaviours recorded in these experiments are courtship stationary paddling, grasping, high on legs, and chelae spread (Table 1). Of these behaviours, only courtship stationary paddling is specific to courtship, as the others occur in both courtship and agonistic encounters.

Each male crab was scored for production of each of the above behaviours. For each behaviour, a Cochran's Q test was used to determine if there was a difference in the responsiveness to the 10 stimuli tested. If so, then McNemar tests (one-tailed, $P < 0.05$) were performed using web-based statistical program R (R Foundation for Statistical Computing) version 2.14.1 to determine which of the stimuli evoked a greater response compared to seawater, the negative control.
NMR detection of a premoult biomarker in urine of pubertal premoult females

Urine was collected from 10 pubertal premoult females, 10 sexually mature postmoult females, 10 adult premoult males, and 10 adult postmoult males. 0.2 ml aliquots of urine from each individual for a given condition were pooled to make 2 ml samples for each of the four conditions. To compare metabolites in the four pooled samples, 2 ml of each was separated using ultrafiltration as described above, and the 500–1000 Da fraction of each was lyophilized and solubilized in 500 μl of deuterium oxide mixed with 5 nmol of trimethylsilyl propanoic acid (TSP), 70 mmol l⁻¹ phosphate buffer at pH 7.6, and 0.1% of sodium azide. ¹H NMR spectra were obtained for these samples using a Varian Inova 600 MHz instrument and a NOESY pulse sequence with water pre-saturation. A total of 512 scans were accumulated for a single increment.

Purification and determination of the molecular structure of the premoult biomarker

To isolate the premoult biomarker molecules, urine was collected from pubertal premoult females and pooled, yielding 1550 ml of urine. A 300-ml aliquot of this pooled sample was separated by ultrafiltration to produce a 500–1000 Da fraction, which was separated into 10 sub-fractions using reversed phase HPLC with a Developsil RP-AQUEOUS C30 column (Nomura Chemical, Seto, Aichi, Japan) with 100% water as the mobile phase. These fractions were screened by ¹H NMR to find the premoult biomarker molecules. Fractions containing the compounds were separated by hydrophilic interaction HPLC using a TSKgel Amide 80 column (Tosoh Bioscience, Minato-ku, Tokyo, Japan) with 60% acetonitrile as the mobile phase. Ultraviolet light
(wavelength 210 nm) was used to detect chromatographic peaks. The molecular formula of the purified compound was determined using high resolution electrospray ionization time of flight mass spectrometry (HR ESI TOF-MS). Detailed partial structure of the compound was analyzed using 1D and 2D NMR, H-H Correlation Spectroscopy (COSY), Nuclear Overhauser Effect Spectroscopy (NOESY), Heteronuclear Single Quantum Coherence Spectroscopy (HSQC), and Heteronuclear Multiple Bond Correlation Spectroscopy (HMBC) by observing $^1$H to estimate the distance to other $^1$H and $^{13}$C atoms connected to the $^1$H through and not through covalent bonds. To determine the structure of the compound from candidate structures, NMR spectra of the compound and a standard sample of N-acetylglucosamino-1,5-lactone (NAGL) (CAS Number: 19026-22-3) (Toronto Research Chemicals, North York, Ontario, Canada) were compared in phosphate buffered deuterium oxide at pH 7.6 with deuterated methanol (CD$_3$OD) as an internal reference.

**Quantification of NAGL in urine**

To elucidate changes in the concentration of NAGL in urine of male and female crabs at different stages of moult ing, NAGL levels were measured using quantitative $^1$H NMR spectroscopy. Urine samples were collected from individual animals of different maturity, sex, and moult stage: intermoult juveniles, pubertal premoult females, sexually mature postmoult females, adult premoult males, and adult postmoult males. Samples were stored individually at -80°C. A 500-μl aliquot of each individual urine sample was lyophilized and solubilized in 500 μl of deuterium oxide mixed with 5 nmol of TSP, 70 mmol l$^{-1}$ phosphate buffer at pH 7.6, and 0.1% of sodium azide. 1D $^1$H NMR spectra were obtained for these samples using a Varian Inova 800 MHz instrument and a
The concentration of NAGL was calculated based on the methylene doublet signal at 4.32 ppm by standardizing with TSP as an internal reference. Statistical evaluation of differences in NAGL concentrations for the variables 'sex' and 'moult stage' were performed using two-way ANOVA, and then differences between each moult stage were analyzed using post-hoc Tukey’s tests, using R version 2.14.1.

**Behavioural responses of male crabs to NAGL**

Responses of male crabs to NAGL were measured using behavioural assays as described above. For this, we recorded three behaviours: courtship stationary paddling, cheliped extension forward, and epipod waving (Table 1). Chemical stimuli were presented to the animals by micropipetting 100-μl aliquots to the antennular region of the crabs, and behavioural responses were recorded for 10 min. Stimuli tested included: SW (seawater, a negative control); Shrimp (shrimp juice, which is a supernatant of a homogenate of 9 g of shrimp abdominal muscle in 40 ml of seawater); PFU (pubertal premoult female urine at 10% full strength in seawater); NAGL (1 mmol l⁻¹ NAGL in seawater, which is 20 times higher than the concentration in urine of pubertal premoult females). All stimuli were tested on 24 males in randomized order. Overall differences among the stimuli were tested by Cochran’s Q test, and data were compared between SW and each of the other stimuli by one-tailed McNemar tests using program R version 2.14.1.
Calcium imaging of responses of olfactory receptor neurons of blue crabs to NAGL

Female pheromones are detected by olfactory receptor neurons (ORNs) in the aesthetasc sensilla of the lateral flagellum of the antennules of male crabs (Gleeson, 1982). In blue crabs, each aesthetasc has 40–160 ORNs whose somata are arranged as a cluster near the aesthetasc’s base and whose dendrites ascend into the lumen of the sensilla (Gleeson et al., 1996). To determine if blue crabs can detect NAGL using their olfactory pathway, which is a prerequisite for any substance to act as distance pheromone, we examined the responses of these ORNs using calcium imaging. Calcium imaging has been reliably used to study odor-activated responses in spiny lobsters (Ukhanov et al., 2011; Tadesse et al., 2014) and many other animals (Grienberger and Konnerth, 2012). We used methods modified from those used for spiny lobsters, which are described in detail elsewhere (Tadesse et al., 2014) and here more briefly.

Antennular lateral flagella (length: 1.8–2.0 mm; diameter: ~ 0.6 mm at base tapering to ~ 0.3 mm at tip) were collected from adult intermoult blue crabs of either sex and immersed in saline (pH 7.4) composed of, in mmol l⁻¹, 459 NaCl, 13.4 KCl, 13.6 CaCl₂, 3 MgCl₂, 14.1 Na₂SO₄, and 9.8 HEPES. Antennular lateral flagella were split sagitally using microscissors to expose ORN somata. These ‘antennule slices’ were enzymatically digested for 7 min (2 ml saline + 500 µl of 2.5 mg ml⁻¹ papain + 8 mg of L-cysteine to activate papain), rinsed, and then loaded with the calcium indicator dye, Oregon Green 488 BAPTA-1 AM (OGB) (Molecular Probes, Invitrogen, Carlsbad, CA, USA). OGB was prepared as 50 µg in 50 µl Pluronic ® F127 with DMSO (Molecular Probes, Invitrogen) and diluted to a final concentration of 50–60 µmol l⁻¹ in saline.
Antennule slices were incubated in this solution for 1 h at room temperature, rinsed in saline for 20 min, and transferred into another dish containing saline until imaging.

Antennule slices were secured in a flow-through chamber (polycarbonate ring with an inner diameter of 12 mm and a thickness of 5 mm glued on a 60-mm plastic Petri dish) mounted on an upright epifluorescence microscope (Zeiss Axioplan 2, Jena, Germany) equipped with a 40 x water immersion objective with long working distance.

Antennule slices were continuously perfused with saline at a rate of 3.0–3.6 ml min\(^{-1}\) for the duration of the experiment. Excitation light at 488 nm was provided by a monochromator (Polychrome V, Till Photonics, Munich, Germany), and fluorescent images (688 x 520 pixels and 2 x 2 binning) were captured with a fast CCD camera (Imago QE, Till Photonics). An experimental protocol was customized in Vision software (Till Photonics) that synchronized image acquisition with an external processor (Imaging System Controller, Till Photonics), allowing control of the stimulus onset and offset via solenoids. For quantification of Ca\(^{2+}\) signals, a region of interest was outlined for each selected OBG-loaded ORN soma, and the average pixel value of mean fluorescence intensity was calculated using Vision software. These raw data were transferred to Excel (Microsoft, Redmond, WA, USA) and from there to PSI-Plot 8.5 (Poly Software International, Pearl River, NY, USA) for further data analysis.

We tested two substances as chemical stimuli. One was 0.1 mmol l\(^{-1}\) NAGL as a candidate pheromone. It was prepared as 10 mmol l\(^{-1}\) stock solution by dilution in saline, and aliquots of stock solution were kept at -20°C. Before each experiment, an aliquot was thawed and diluted 1:100 with saline to yield the working stimulus solution. A second stimulus was an extract of the fish food TetraMarine (TM) (Tetra Werke, Melle, Germany) that contains numerous chemicals in unknown concentration and is highly...
stimulatory for many ORNs of other crustacean species (Ukhanov et al., 2011; Tadesse et al., 2014). It was prepared as 2 g of TM mixed in 60 ml saline for ~3 h, centrifuged at 1400 x g for 20 min, filtered, aliquoted, and stored at -20°C until used for each experiment, at which time an aliquot was thawed and diluted 1:100 with saline. Negative controls for preparations tested with NAGL and TM were trials in which no stimulus was presented, because stimulation with a saline control in other preparations reliably did not elicit responses. Stimuli were presented as 1-s pulses using a six-barrel stimulation pipette driven by a Picospritzer III (Parker, Pine Brook, NJ, USA) as described in Tadesse et al. (2014). For each stimulation, 500 images with an exposure time of 4 ms were acquired at 150 ms frame$^{-1}$ (for a total recording time of 75 s) and the stimulus was applied at 15 s. Consecutive stimuli were presented with a 2-min inter-trial interval.

Raw data were converted to relative fluorescence change expressed as $\Delta F/F = (F-F_0)/F_0$ where $F$ is the fluorescence intensity measured at a given time point and $F_0$ is the baseline fluorescence] in PSI-Plot 8.5 (Poly Software International, Pearl River, NY, USA). $F_0$ was calculated as the arithmetic mean of the first 100 data points. The amplitude of odor-activated Ca$^{2+}$ transients was determined as the most positive value of $\Delta F/F$ in a time series.

ACKNOWLEDGMENTS

We thank Dr. Richard Gleeson for inspiration, assistance, and advice throughout the project, Dr. Peter Anderson and Dr. Barry Ache of the Whitney Laboratory for Marine Bioscience of the University of Florida and Dr. Dan Rittschof of Duke University Marine Laboratory for allowing us to work in their facilities, Dell Newman (Swan...
Quarter, NC) and Norman Byrd (Hastings, FL) for use of their crab shedding facilities in the collection of urine, Dr. Sekar Chandrasekaran of Georgia State University and Dr. John Glushka of University of Georgia for assistance with NMR spectroscopy, Dr. Siming Wang of Georgia State University for assistance with mass spectrometry, Dr. Yusai Ito of National Institute of Health Sciences for preliminary NMR experiment and Dr. Toshiyuki Wakimoto of University of Tokyo and Dr. Hiroshi Nagai of Tokyo University of Marine Science and Technology for valuable discussions on chitin metabolism and chemistry of NAGL.

AUTHOR CONTRIBUTIONS
M.K., C.D.D., and J.K. contributed to the initial planning and design of the project; M.K. designed experiments and collected and analyzed data for all aspects of the project; J.K. contributed to the chemical identification experiments and the structural determination of NAGL; M.W.G. contributed to the NMR projects; M.S. designed experiments and collected and analyzed data in the calcium imaging study; and M.K. and C.D.D. synthesized all aspects of the work and wrote the manuscript.

COMPETING INTERESTS
No competing interests declared.

FUNDING
M.K. was supported by seed grants from the Brains & Behavior Program and the Center for Behavioral Neuroscience through the Science and Technology Centers Program of
the National Science Foundation (NSF) under Agreement No. IBN-9876754, the Japan
Society for the Promotion of Science (JSPS) Postdoctoral Fellowship for Research
Abroad, Narishige Zoological Science Award, The Sumitomo Foundation for Basic
Science Research Grant Number 120594, and JSPS KAKENHI Grant Number
23780212. M.S. was supported by seed grants from the Brains & Behavior Program.
Research at Georgia Tech was supported by NSF grant OCE-0134843 to J.K.
REFERENCES


Fig. 1. Behavioural responses of male blue crabs to ultrafiltration fractions of urine.

Four behavioural responses were: A. Courtship stationary paddling. B. Grasping. C. High on legs. D. Chelae spread. Stimuli tested were: Female urine = urine from pubertal premoult females; Male urine = urine from adult intermoult males; S (Small) = <500 Da fraction; M (Medium) = 500-1000 Da fraction; L (Large) = >1000 Da fraction; Mix = combination of S+M+L; Pos = urine from pubertal premoult females (Positive control); Neg = seawater (Negative control). Data are from 55 males that were tested with all 10 stimuli and responded to one or more of them with one or more of the four behaviours.

There was a significant effect of Stimulus for all four behaviours, based on Cochran’s Q tests: (A) $\chi^2 = 47, df = 9, P = 4.3 \times 10^{-7}$; (B) $\chi^2 = 46.7, df = 9, P = 4.29 \times 10^{-7}$; (C) $\chi^2 = 32, df = 9, P = 1.9 \times 10^{-5}$; (D) $\chi^2 = 21, df = 9, P = 1.1 \times 10^{-5}$. An asterisk indicates a stimulus producing responses significantly greater than did seawater (Neg) by one-tailed McNemar test ($P < 0.05$).

Fig. 2. $^1$H NMR spectra of 500-1000 Da fraction of urine from A. Pubertal premoult females (purple). B. Adult Premoult males (light blue). C. Sexually mature postmoult female (light green). D. Adult postmoult male (brown). Red rectangles indicate NMR signals that are specific for urine of premoult crabs. Peak sizes were standardized to the internal standard.

Fig. 3. Purification of the female-specific compound by HPLC. The 500-1000 Da fraction of urine from pubertal premoult females was separated using HPLC. A.
Separation by C30 column with 100% water as the mobile phase. The fraction between the dotted lines (blue) was used for subsequent chromatography using Amide 80, shown in B. B. Separation by Amide80 column with 60% MeCN as the mobile phase. Elution of compounds was monitored at 210 nm. The fraction between the dotted lines contains pure NAGL. The amount of purified compound was 0.8 mg from 300 ml of urine from 300 pubertal premoult females.

Fig. 4. Structure of NAGL. Arrows indicate 2D NMR NOESY correlations to determine relative stereochemistry of this molecule. Numbers for carbon atoms correspond to those in Table 2.

Fig. 5. $^1$H NMR spectra of the urine-derived compound (A) and standard NAGL (B). Spectra were obtained in 70 mmol l$^{-1}$ sodium phosphate in deuterium oxide, pH 7.6. TSP was used as reference for 0 ppm. N-Acetyl methyl signals at 2.07 ppm (not shown) were observed in the same position in (A) and (B).

Fig. 6. Concentration of NAGL over moult cycle for male and female blue crabs. Urine from juvenile intermoult males and females, pubertal premoult females, adult premoult males, sexually mature postmoult females (adult female), and adult postmoult males are compared. Values are mean ± s.e.m. from 8 to 10 individual crabs in each condition. Two-factor ANOVA results: for moult stage, $F = 5.50$, $P = 0.007$; for sex, $F = 0.35$, $P = 0.55$; for moult stage x sex interaction, $F = 4.00$, $P=0.025$. An asterisk indicates values significantly different from pubertal premoult females by post-hoc Tukey tests, $P < 0.05$. 

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Parametric ANOVA was performed because population variances were not significantly different for either moult stage or sex (Bartlett test, $P > 0.05$).

Fig. 7. Behavioural responses of adult male crabs to NAGL. Stimuli tested: NAGL = 1 mmol l$^{-1}$ NAGL in seawater; Shrimp = supernatant of homogenate of shrimp abdominal muscle (0.225 g ml$^{-1}$ seawater); PFU = Pubertal premoult female urine, 10% full strength diluted in seawater; SW = seawater. A 100-μl aliquot of each stimulus was micropipetted toward the antennules of males. Twenty-four males were tested. There was a significant effect of Stimulus for A. Cheliped extension forward (Cochran's Q test. $\chi^2 = 51$, $df = 3$, $P = 4.9 \times 10^{-11}$) and B. Epipod waving ($\chi^2 = 28$, $df = 3$, $P = 3.3 \times 10^{-6}$). An asterisk indicates a stimulus producing a response significantly greater than seawater (one-tailed McNemar test, $P < 0.05$).

Fig. 8. Responses of olfactory receptor neurons of blue crabs to NAGL. Shown are odorant-evoked responses of three olfactory receptor neurons (ORNs) in the antennular lateral flagellum of an adult crab based on calcium imaging. Stimuli are 0.1 mmol l$^{-1}$ NAGL and 1% TetraMarine (TM), a food extract in saline (see Methods). Vertical dashed line indicates the onset of the ~2 s chemical stimulus.

Fig. 9. Metabolic pathway for chitin and a proposed role and position of NAGL in the pathway. Shown is the pathway for the biosynthesis and degradation of chitin (Horst et. al., 1993; Muzzarelli, 1996; Merzendorfer and Zimoch, 2003), and a proposed role for NAGL in this pathway. According to this proposed scheme, NAGL is synthesized from NAG by NAG oxidase and controls the degradation of chitin by inhibiting
Supplementary Materials

Fig. S1: Nephropore of blue crabs and the collection of urine. The arrows indicate the cuticular lid of the nephropore. Urine was released by lifting this lid and collected using a micropipette tip connected to a vacuum pump.
Table 1. Behaviours observed in this study

<table>
<thead>
<tr>
<th>Name of behaviour</th>
<th>Context in which it occurs</th>
<th>Description of behaviour</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Courtship</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>stationary</td>
<td>Only in courtship</td>
<td>Crab holds its swimming legs (5th pereiopods) high above its carapace and waves them side to side. The flat surface of each paddle faces forward. In addition to the side-to-side movement, the paddles move from outside to inside as they move from backward to forward to make a circular motion, with the left and right paddles 180° out-of-phase (Wood and Derby, 1995). When a crab performs this behaviour, it does not walk or move backward or forward, and it always performs chelae spread and high on legs behaviours (Kamio et al., 2008).</td>
</tr>
<tr>
<td>paddling</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Grasping</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Courtship and other context such as fighting</td>
<td>Male crab tries to hold a female using both his chelae without pinching. In our experiments, females were not sexually receptive and so when the males tried to hold them, the females always resisted so that stable guarding pairs were not established.</td>
</tr>
<tr>
<td><strong>High on legs</strong></td>
<td></td>
<td>Crab extends its legs and stands high.</td>
</tr>
<tr>
<td><strong>Chelae spread</strong></td>
<td></td>
<td>Crab lifts its chelae and fully extends them laterally, with chelae closed.</td>
</tr>
<tr>
<td><strong>Epipod waving</strong></td>
<td></td>
<td>Crab waves its maxillary epipods (Brock, 1926; Denissenko et al., 2007).</td>
</tr>
<tr>
<td><strong>Cheliped extension forward</strong></td>
<td>Appetitive behaviour to grasp food</td>
<td>Crab extends its chelipeds forward and opens and closes its chelae in an attempt to grasp food.</td>
</tr>
</tbody>
</table>
Table 2. NMR chemical shift assignments of NAGL in deuterium oxide

<table>
<thead>
<tr>
<th>Position</th>
<th>$^1$H (ppm)$^b$</th>
<th>$J_{H-H}$ (Hz)$^c$ (multiplicity)$^d$</th>
<th>$^{13}$C (ppm)$^e$</th>
<th>HMBC$^f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>176.7</td>
<td>H-2,3</td>
</tr>
<tr>
<td>2</td>
<td>4.32</td>
<td>5.1 (d)</td>
<td>58.2</td>
<td>H-3</td>
</tr>
<tr>
<td>3</td>
<td>4.14</td>
<td>3.2, 5.1 (dd)</td>
<td>70.1</td>
<td>H-2</td>
</tr>
<tr>
<td>4</td>
<td>3.56</td>
<td>3.2, 7.6 (dd)</td>
<td>71.9</td>
<td>H-5</td>
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<tr>
<td>5</td>
<td>3.68</td>
<td>2.9, 6.9, 7.6 (ddd)</td>
<td>71.4</td>
<td>H-4,6</td>
</tr>
<tr>
<td>6a</td>
<td>3.58</td>
<td>6.9, 11.7 (dd)</td>
<td>62.9</td>
<td>H-4</td>
</tr>
<tr>
<td>6b</td>
<td>3.78</td>
<td>2.9, 11.7 (dd)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>-</td>
<td>173.9</td>
<td>H-2.8</td>
</tr>
<tr>
<td>8</td>
<td>2.01</td>
<td>-</td>
<td>22.2</td>
<td>-</td>
</tr>
</tbody>
</table>

$^a$ Positions of elements are in Fig. 4.

$^b$ Recorded at 500 MHz.

$^c$ $J_{H-H}$ is a coupling constant between proton ($^1$H) and proton.

$^d$ Multiplicity of NMR signals, d means doublets, dd means doublet of doublets, ddd means doublet of doublets of doublets.

$^e$ Recorded at 125 MHz. Carbon chemical shifts were assigned by HMBC and HMQC.

$^f$ HMBC correlations are from H listed in column 5, to C listed in column 1.

Chemical shifts were referenced for deuterated methanol (CD$_3$OD).
Fig 1

A. Courtship stationary paddling

- S: Sample
- M: Male
- L: Low
- Mix: Mixed
- Pos: Positive
- Neg: Negative

B. Grasping

- S: Sample
- M: Male
- L: Low
- Mix: Mixed
- Pos: Positive
- Neg: Negative

C. High on legs

- S: Sample
- M: Male
- L: Low
- Mix: Mixed
- Pos: Positive
- Neg: Negative

D. Chelae spread

- Female urine
- Male urine
- Control

Number of males that responded

n = 55
Fig 4
The graph shows the NAGL concentration (mmol/L) in different conditions for both female and male individuals.

- **Juvenile Intermoult**: Male (30) and Female (10)
- **Adult or Pubertal Premoult**: Male (7) and Female (50)
- **Adult Postmoult**: Male (5) and Female (10)

The asterisks (*) indicate significant differences between groups.
Fig. 9

NAGL is excreted or secreted into urine and detected by olfactory receptor neurons of male.