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ARTICLE

# Behavioral assay and chemical characters of female sex pheromones in the hermit crab *Pagurus filholi*

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**Abstract** Males of the hermit crab *Pagurus filholi* perform assessment behavior toward females, as a preliminary step of precopulatory guarding, during the reproductive season. It is known that such behavior is elicited by female sex pheromones, but the compounds involved have never been characterized in this species. Several experiments were conducted to develop a reliable bioassay along with purification procedures to identify potential compounds with pheromonal activity in *Pagurus filholi*. We developed a bioassay protocol to assess pheromonal activity by using an empty shell with cotton containing either artificial seawater (control) or test water. We measured and compared the time duration of male assessment behavior toward each shell if the test water contained female sex pheromones. Ultra-filtering of seawater samples potentially containing pheromones showed that the compound was <1 kDa in molecular weight. Males showed precopulatory assessment behavior toward "female conditioned" water samples treated with open column purification and eluted with MeOH, suggesting that compounds triggering male behavior were low polar molecules. Molecules with pheromonal activity were not volatile after freeze drying, effective even after heating to 90 °C, and remained active in seawater at 12 °C even after 6 days from sample collection, which suggests a rather stable characteristic of the female sex pheromones of this species.

**Keywords** Bioassay · Hermit crab · Low polar molecule · *Pagurus filholi* · Sex pheromone

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## Introduction

Chemical communication is important in many behavioral and ecological interactions. Many aquatic species use chemical signals in attraction and localization of their partners (Salmon 1983; Breithaupt and Thiel 2011). The chemical signals that animals release to communicate with their conspecifics are defined as pheromones, and compounds functioning as sexual attractants are referred to as sex pheromones (Agosta 1992; Wyatt 2010, 2014). Sex pheromones play important roles in reproductive interactions within crustaceans (Dunham 1988; Breithaupt and Thiel 2011). It is known that females release sex pheromones that induce reproductive behavior of mating partners in many crustaceans, such as in the blue crab *Callinectes sapidus* (Gleeson 1980) and the shore crab *Carcinus maenas* (Christofferson 1978; Bamber and Naylor 1996), and many efforts to identify such pheromones have been conducted as well (Kittredge et al. 1971; Gleeson et al. 1984; Asai et al. 2000; Hardege et al. 2002; Bublitz et al. 2008; Kamio 2009; Hardege et al. 2011; Hardege and Teschak 2011; Kamio et al. 2014; Yano et al. 2016). However, there is no complete identification of sex pheromone candidate molecules in decapod crustaceans.

Establishment of a reliable bioassay is required to identify sex pheromones. Such bioassay should be devised independently for each species, because male reactions to pheromones emitted from conspecific females are different among species (Kamio et al. 2000; Hardege et al. 2002; Kamio 2009; Zhang et al. 2010; Kamio and Derby 2011). In many crustaceans, behavior elicited by sex pheromones is usually unclear and difficult to distinguish from other behavior such as feeding and agonistic behavior. In addition, visual and tactile cues may also play an important role in reproductive behavior and pair formation in natural environments (Hazlett and McLay 2000; Bouwma and Hazlett 2001). Therefore, it is difficult to establish reliable bioassay methods to detect sex pheromones in many crustaceans.

Females of the hermit crab *Pagurus filholi* release sex pheromones that induce males to begin assessment and guarding behavior (Imafuku 1986; Goshima et al. 1998; Okamura and Goshima 2010; Kawaminami and Goshima 2015). During guarding, males grasp the shell of a mature female with their left cheliped for up to 5 days before copulation in the reproductive season, and assessment behavior is considered to be a preliminary step toward mate guarding (Goshima et al. 1998; Minouchi and Goshima 1998). Male reactions to sex pheromones are distinctive in this species, and exclusion of other factors such as food cue and odor of predator affecting male behavior is possible; therefore, hermit crabs are a reliable model for developing a bioassay for sex pheromone detection based on a 'releaser reactions' approach (Dunham 1978, 1988) to investigate the chemical character of sex pheromones.

The aim of this study was to develop a reliable bioassay method to detect female sex pheromones in the hermit crab *Pagurus filholi*. Moreover, molecular size, polarity and stability in seawater of potential pheromone compounds are demonstrated in this study.

## Materials and methods

All hermit crabs for the following experiments were collected on a flat rocky intertidal shore at Kattoshi, Hakodate Bay, Hokkaido, Japan (41°44′34″N, 140°36′08″E). The breeding season of *Pagurus filholi* ranges from February to August, but was mainly from March to July at our study site (Goshima et al. 1998). Sampling of guarding pairs was performed during low tides from March to July in 2005–2009.

### Maturity condition of guarded females

Guarding pairs were collected haphazardly 8 times at Kattoshi and were brought to the laboratory. Each pair was separated and the maturity condition of the guarded female was ascertained as non-ovigerous or ovigerous (egg carrying). For the ovigerous females, we determined the egg developmental stages microscopically and classed the females into one of three categories as follows: stage I ovigerous female had newly deposited eggs and more than 70% of original yolk volume; stage II ovigerous female had eggs of less than 70% of original yolk volume and some embryos having incomplete pigmented eyes visible; stage III ovigerous female had pigmented embryo with well-developed oval eyes visible. Percentages of the guarded females were compared among four different maturity conditions using repeated measure ANOVA to clarify whether males in the field frequently guarded any particular maturity stage of the females. As the sphericity assumption did not hold (Mauchly test, $W = 0.00026$; $P < 0.0001$), the $P$ value of the $F$ test was corrected based on the degrees of freedom multiplied by Greenhouse–Geisser epsilon (0.3403). Multiple comparisons were performed by paired $t$-tests with separate error terms depending on the two levels being compared. The type I error rate was corrected by sequential Bonferroni.

### "Female conditioned" water sample collection

Guarding pairs were collected at Kattoshi and brought to the laboratory. Thirty females that had been guarded by males in the field were separated from the males and placed in the same plastic cup (5 cm diameter, 9 cm high) with 100 ml artificial seawater (Perfect Marine, Nisso Corporation) and kept for 24 h at 12 °C. The resulting "female conditioned" water samples were tested to confirm the presence of sex pheromones triggering male assessment response (Imafuku 1986; Goshima et al. 1998; Minouchi and Goshima 1998; Okamura and Goshima 2010). Only females sampled within 2 days from the field were used to collect "female conditioned" water samples.

### Bioassay procedures

Solitary males of the hermit crab often show assessment behavior toward mature females, while no response behavior was observed toward immature females or shells with no females. The assessment behavior is preliminary to guarding behavior, and it is elicited by female sex pheromones (Imafuku 1986; Goshima et al. 1998; Minouchi and Goshima 1998; Okamura and Goshima 2010). The male embraces the female with its ambulatory legs, and rotates the female into a face-to-face position. The male then brings the anterior parts of his cephalothorax close to the shell aperture of the female, and examines it by touch with antennae, walking legs, and chelae on a part of the female's body just protruding from the shell. We

determined whether this assessment behavior occurred or not as well as duration of the interaction when observed in experiments to detect sex pheromonal activity in the samples of "female-conditioned" water as follows.

Guarding males from the field were separated off the females, and each male was placed in a plastic case (10.5 cm diameter, 2 cm high) full of natural seawater. Most guarded females in Kattoshi were in *Batillaria cumingi* shells (Yoshino et al. 2002). Two groups of empty *Batillaria* shells which were similar in size were plugged with cotton containing either artificial seawater ($n = 17$) or "female-conditioned" water ($n = 17$) and then presented, in that order, to the males in the plastic cases. The behavior of the male was observed initially for 30 s. If assessment was initiated during this time period, male behavior was observed for 30 s more. The difference in assessment duration between artificial seawater and "female-conditioned" water shell groups was analyzed with the Wilcoxon signed-rank test.

To verify the assay protocol, the above procedure was repeated, but this time, shells plugged with cotton containing "female conditioned" water ($n = 20$) were presented first, followed by the shells containing artificial seawater ($n = 20$). This was done specifically to test sample presentation order effect on male assessment behavior. The behavior of males presented with plugged shells was observed initially for 30 s. If assessment behavior was initiated during this interval, observation continued for over 30 s. The difference in assessment duration between "female-conditioned" water and artificial seawater shell groups was analyzed with the Wilcoxon signed-rank test.

Only males that performed assessment behavior to the "female-conditioned" water samples (positive control) were used in experiments 2 and 3. In the later experiments, we tested seawater first and then samples because the order of the treatments had no effects on experimental results (see Results Section for details).

### Experiment 1: "pheromone" molecular size

We performed ultrafiltration of the "female-conditioned" water samples using YM-1 filters (Amicon ultra, Millipore) to separate each water sample into two fractions of different molecular size; <1 or >1 kDa. The pheromonal activity of the remaining fraction on the filter (>1 kDa) and filtrate (<1 kDa) was tested for each sample ($n = 30$) by the following procedure.

Guarding males from the field were separated off the females, and each male was placed in a plastic case (10.5 cm diameter, 2 cm high) full of natural seawater to test "female conditioned" water fractions (<1 or >1 kDa) separately, both against seawater. First, one group of *Batillaria* shells was plugged with cotton containing artificial seawater ($n = 30$) while another group of shells was plugged with cotton soaked in unfiltered fraction sample ($n = 30$) or filtrate fraction sample ($n = 30$). The unfiltered fraction (>1 kDa) was obtained by rinsing the YM-1 filter membrane with 100 ml distilled water. Artificial seawater and fraction samples were presented sequentially (one at a time) to the males. The behavior of males was observed initially for 30 s. If assessment was initiated during this period, we observed male behavior for 30 s more. Difference in assessment duration between artificial seawater and fraction shell groups was analyzed with the Wilcoxon signed-rank test.

### Experiment 2: "pheromone" polarity

To reveal the polarity of the compounds with sex pheromone properties, and in order to remove large amounts of salts from "female conditioned" water samples, ODS resin fractioning was employed as follows. "Female conditioned" water was passed through an ODS open column (3.5 cm diameter, 4 cm high, Fuji Silysia Chemical). The column was washed with distilled water and successively eluted with 30 or 50% MeOH. Each extract was evaporated in vacuum and dissolved in 10 ml distilled water and used as a sample. The behavior of males presented sequentially with cotton plugged *Batillaria* shells, containing column samples eluted with 30% ($n = 38$) and 50% ($n = 28$) MeOH, was examined separately, both against plugged shells with artificial seawater for each sample in a bioassay as noted under Bioassay procedures Section.

### Experiment 3: stability

One of the major concerns during handling of natural chemical products including sex pheromones is their stability. Herein, we present handling tests and storing procedures of the pheromone potentially contained within 100 ml of "female conditioned" water and its effect on male assessment behavior.

#### *Freeze-drying*

"Female conditioned" water was freeze-dried and taken up in 10 ml distilled water. The freeze-drying effect on pheromonal activity was evaluated with the behavioral assay for $n = 14$ samples, described under Bioassay procedures Section.

#### *Heating*

The effect of high temperature on pheromonal activity of "female conditioned" water was tested by increasing the sample temperature to 90 °C for 15 min. The behavioral

assay described under Bioassay procedures Section was performed for $n = 14$ samples.

*Stability at ambient temperature*

The objective of the bioassay for this item was to test the stability of effect of the compound involved in triggering male assessment behavior at ambient temperature. "Female conditioned" water samples (made using natural seawater in this experiment) were kept in plastic bottles for 6 days at 12 °C before presentation to the males in an assay as described in the Bioassay procedures Section for $n = 22$ samples.

## Results

### Maturity condition of guarded females

Figure 1 shows maturity condition of guarded females in the field. Although all stages were included in the guarded females, there was a significant difference in the percentage of guarded females among their maturity stages (repeated measure ANOVA, $F_{1.02,\ 7.15} = 36.62$, $P < 0.001$), and non-ovigerous females (71.9%) and stage III females (26.7%) were particularly dominant among them ($P < 0.05$ in all cases except for between stages I and II, $P = 0.409$).

### "Female conditioned" water sample collection and bioassay procedures

In both trials where artificial seawater and "female conditioned" water were presented first, there was a significant difference in the duration of male assessment behavior between seawater and the "female conditioned" water samples (Fig. 2a, Wilcoxon signed-rank test, $T = 7.5$, $P < 0.01$, Fig. 2b, Wilcoxon signed-rank test, $T = 3$, $P < 0.01$). When presented with the "female conditioned" water samples, males significantly increased the duration of assessment behavior in this experiment, suggesting that the "female conditioned" water contained sex pheromones. Furthermore, the order of the treatments ("female conditioned" water first or second) had no effects on the experimental results. Therefore, all the later tests were conducted in order of seawater first and then samples second.

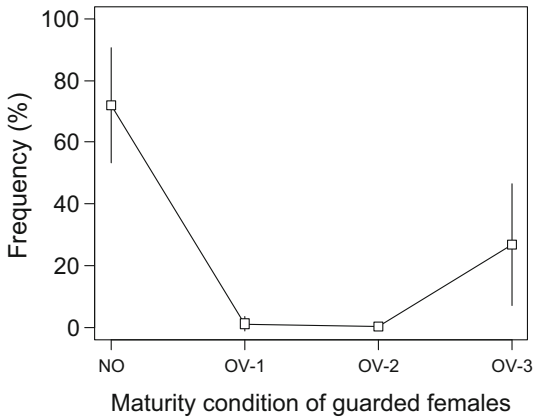


**Fig. 1** Percentages of guarded females with different reproductive conditions in the field (repeated measure ANOVA, $F_{1.02,\ 7.15} = 36.62$, $P < 0.001$). *NO* non-ovigerous females, *OV-1* females with eggs newly deposited and more than 70% of original yolk volume, *OV-2* females with eggs less than 70% of original yolk volume and some embryos having incomplete pigmented eyes visible, *OV-3* females with eggs of pigmented embryo having well-developed oval eyes visible. *Vertical bars* indicate SD

### Experiment 1: "pheromone" molecular size

There was a significant difference in duration of male assessment behavior between artificial seawater and the filtered fraction (Fig. 3a, Wilcoxon signed-rank test, $T = 6.5$, $P < 0.01$), while no significant difference was observed between artificial seawater and the unfiltered fraction (>1 kDa) (Fig. 3b, Wilcoxon signed-rank test, $T = 7$, $P > 0.05$).

### Experiment 2: "pheromone" polarity

A significant difference was found in the duration of male assessment behavior between seawater and the sample of the fraction adsorbed on ODS resins and eluted with 30% MeOH (Fig. 4a, Wilcoxon signed-rank test, $T = 54.5$, $P < 0.05$), and between seawater and the sample of the fraction eluted with 50% MeOH (Fig. 4b, Wilcoxon signed-rank test, $T = 18$, $P < 0.01$), respectively. It is suggested that the sex pheromone of this species is a low polar compound.

### Experiment 3: stability

*Freeze-drying*

Figure 5 shows the duration of male assessment behavior toward artificial seawater and the freeze-dried "female conditioned" water samples. There was a significant difference in duration of male assessment behavior between artificial seawater and the freeze-dried sample (Wilcoxon signed-rank test, $T = 3$, $P < 0.05$).

*Heating*

The compound acting as "sex pheromone" in this species was stable at high temperature. Figure 6 shows the duration of male assessment behavior toward artificial

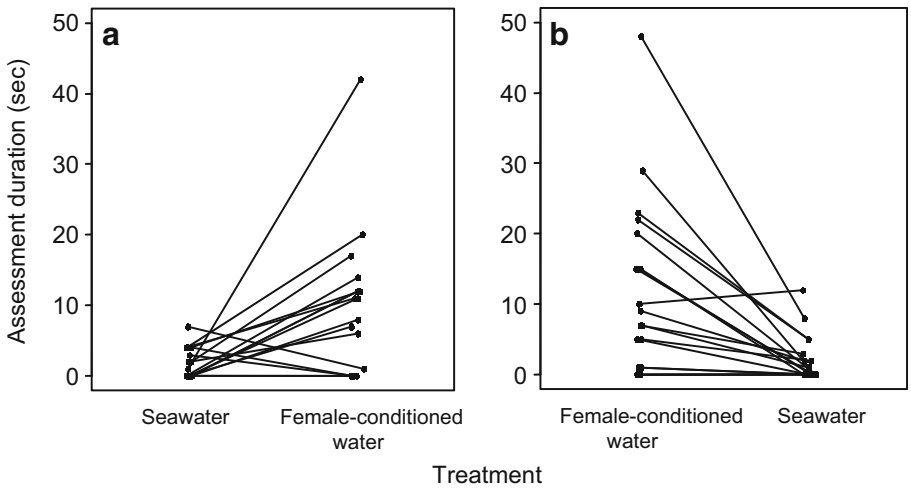


**Fig. 2** Duration of male assessment behavior toward seawater and "female-conditioned" water. **a** The order of treatment was seawater first and then "female-conditioned" water (Wilcoxon signed-rank test, $n = 17$ each, $T = 7.5$, $P < 0.01$), and **b** "female-conditioned" water first and then seawater (Wilcoxon signed-rank test, $n = 20$ each, $T = 3$, $P < 0.01$)

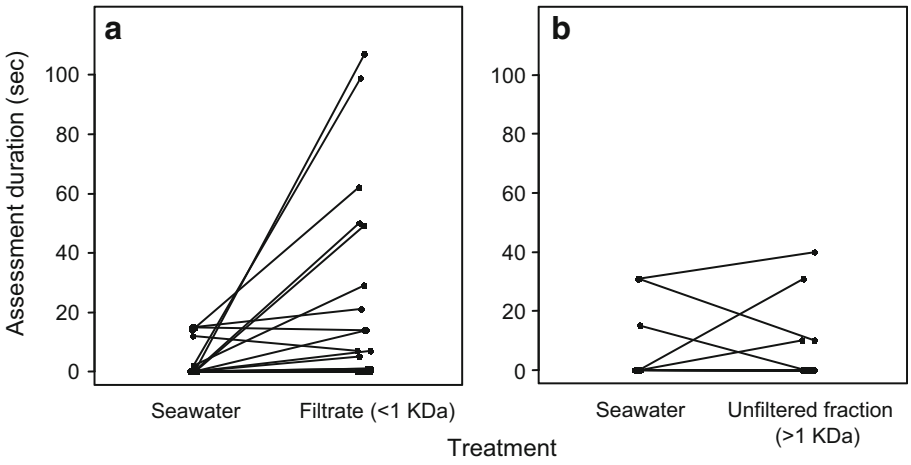


**Fig. 3** Duration of male assessment behavior. **a** Toward seawater and filtrate (<1 kDa) (Wilcoxon signed-rank test, $n = 30$, $T = 6.5$, $P < 0.01$), and **b** toward seawater and unfiltered fraction of ultrafiltration (>1 kDa) (Wilcoxon signed-rank test, $n = 30$ each, $T = 7$, $P > 0.05$)

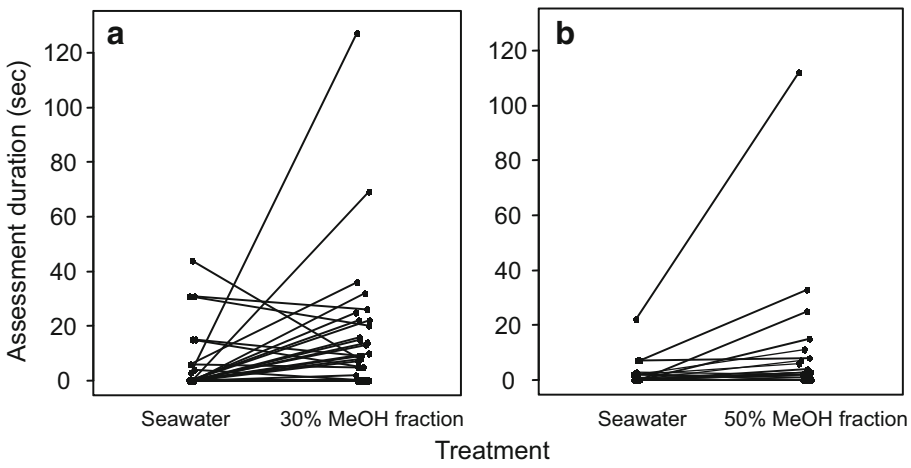


**Fig. 4** The results of the bioassay showing duration of male assessment behavior. **a** Toward seawater and the sample of the fraction adsorbed on ODS resins and eluted with 30% MeOH (Wilcoxon signed-rank test, $n = 38$ each, $T = 54.5$, $P < 0.05$), and **b** toward seawater and the sample of the fraction adsorbed on ODS resins and eluted with 50% MeOH (Wilcoxon signed-rank test, $n = 28$ each, $T = 18$, $P < 0.01$)

seawater and the sample which was heated at 90 °C for 15 min. There was a significant difference in duration of male assessment behavior between artificial seawater and the heated sample (Wilcoxon signed-rank test, $T = 6$, $P < 0.01$).

*Stability at ambient temperature*

Significant difference was found in the duration of male assessment behavior between artificial seawater and the "female conditioned" water samples which were kept for

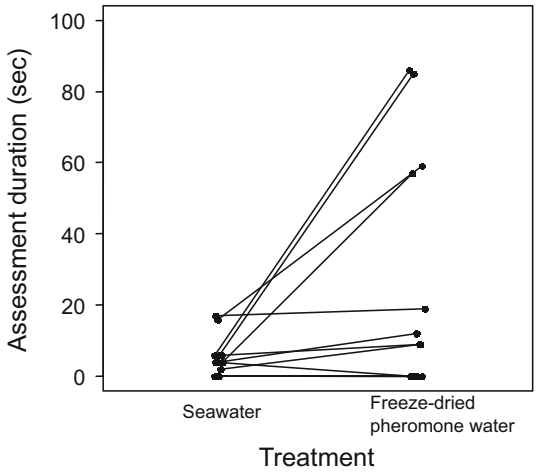


**Fig. 5** The results of the bioassay showing duration of male assessment behavior toward seawater and freeze-dried pheromone water (Wilcoxon signed-rank test, $n = 14$ each, $T = 3$, $P < 0.05$)

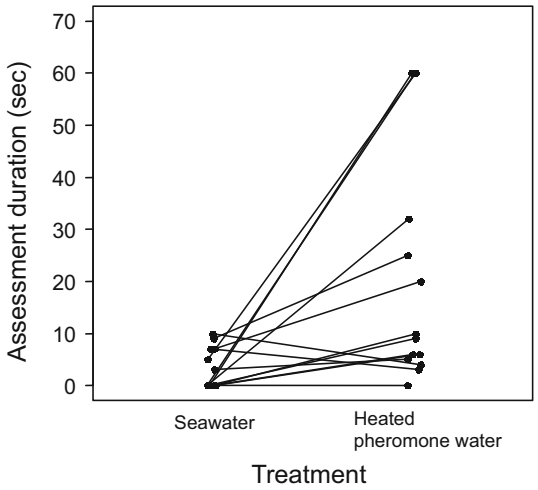


**Fig. 6** The results of the bioassay showing duration of male assessment behavior toward seawater and the pheromone water heated at 90 °C for 15 min (Wilcoxon signed-rank test, $n = 14$ each, $T = 6$, $P < 0.01$)

6 days at 12 °C before the assay (Fig. 7, Wilcoxon signed-rank test, $T = 5.5$, $P < 0.05$). The compound eliciting male assessment behavior was stable in natural seawater after 6 days from sample collection.

## Discussion

Females guarded by males mainly included non-ovigerous and ovigerous ones with well-developed eggs just prior to hatching, which suggests that the guarded females are mature and will soon spawn eggs within a few days (about 5 days in Hakodate Bay, Goshima et al. 1998). Guarding males can recognize such ripe females by sex pheromones emitted from the females (Imafuku 1986; Goshima et al. 1998; Okamura and Goshima 2010; Kawaminami and Goshima 2015). Sex pheromones play important roles in conducting reproductive behaviors in the hermit crabs; females release sex pheromones that induce male assessment behavior toward females as a preliminary step of precopulatory guarding.

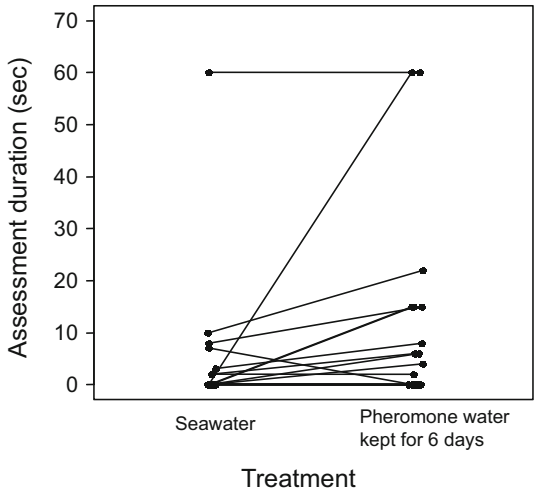


**Fig. 7** The results of the bioassay showing duration of male assessment behavior toward seawater and the pheromone water kept for 6 days at 12 °C (Wilcoxon signed-rank test, $n = 22$ each, $T = 5.5$, $P < 0.05$)

The hermit crab, *Pagurus filholi*, is a good model organism for studies on the effect of sex pheromones on behavior, because male response is easily detected, recorded and interpreted (Goshima et al. 1998; Minouchi and Goshima 1998). Collection methods of "pheromone water" samples from living individuals were effective, and some characteristics of the compound acting as a sex pheromone were revealed in the present study. These results provide valuable information for purification of the substances with pheromonal activity from living individuals that can be used in further chemical analysis. We determined that the "sex pheromone" is smaller than 1 kDa in molecular weight. This is a key finding in order to narrow the range of potential molecules with pheromonal activity in the hermit crab for future studies. Compounds eliciting male assessment behavior in *Pagurus filholi* were not volatile, as the result of freeze-drying "female conditioned" water samples shows. In addition, the sex pheromone was stable at high temperature (90 °C), and also effective after 6 days from sample collection when kept at 12 °C before the assay. These results suggest that the pheromonal molecules of the hermit crab may have high stability, which means it is convenient to treat them for further analysis along with purification procedures to identify potential compounds, because the compounds could be stored and handled for several days without any special facilities.

The hermit crabs live on intertidal rocky shores where the water movement is usually variable and complicated; rapid in rough sea conditions while calm during some low tides because of waves, winds, tides, and topography (Raffaelli and Hawkins 1996). The pheromonal molecules in this species rapidly diluted below detectable levels as a result of turbulent mixing and molecular diffusion (Atema 1995), particularly in rough sea conditions, and therefore concentration gradients of the sex pheromone are expected to be highly temporal. On the other hand, very slow molecular diffusion is expected in calm sea conditions because the dispersal of chemical stimuli depends on fluid flow (Dusenbery 1992). Many aquatic benthic animals are known to generate directed water currents by specialized appendages to acquire and send chemical signals in environments with stagnant flow conditions (e.g., Breithaupt 2001; Kamio et al. 2008). When they detect chemical stimulus, they may move upstream. Therefore, creating water currents in still water, or in water with little movement (such as tide pools or calm sea conditions), is an effective way to search for mates. Males of *Pagurus filholi* frequently beat their third maxillipeds, producing forward water currents, and also move their antennules up and down. These antennules are possible organs for detecting chemical signals (Imafuku 1986), probably for acquiring chemical stimuli from possible mates.

As the water movement is usually complicated and non-directional in intertidal rocky shores of the habitat of the hermit crabs, it would be difficult for males to locate reproductive females by using only an olfactory cue over "long" distances. Indeed, guarding males often climb up leafy algae to keep female sex pheromones away from rival males beneath (Kawaminami and Goshima 2015), and in the case of the European shore crab *Carcinus maenas* the pair formation that precedes mating occurs earlier and the male simply carries the female away from hot spots to ensure single paternity (Meeren 1994). These male behaviors may indicate that keeping a female odor source a certain distance away from rival males is effective in preventing them from detecting receptive females emitting pheromones. Therefore males of the hermit crab may use multiple cues (e.g. visual or olfactory) in a complementary fashion when searching for receptive females in the field. It is also possible that males alternate the use of such cues for locating and accessing females, depending on the distance from the emitting source and habitat conditions. In our previous studies, males of *Pagurus filholi* were shown to use both visual and olfactory stimuli to recognize potential mating opportunities (Okamura and Goshima 2010; Kawaminami and Goshima 2015). A similar case has been also observed in the blue crab *Callinectes sapidus* (Kamio et al. 2008; Kamio 2009; Baldwin and Johnsen 2009). Males of the blue crab perform courtship in stationary paddling bouts to deliver his pheromone to inform females of his location only when females are inaccessible due to location uncertainty from high refuge density and low visibility. Thus, male blue crabs use both visual and olfactory cues effectively to adapt their reproductive behavior to the habitat conditions (Kamio et al. 2008).

Much effort has been undertaken in the search for sex pheromones in decapod crustaceans, but only a few compounds have been identified and reported as sex pheromones or potential sex pheromones (Gleeson et al. 1984; Hardege et al. 2002, 2011; Kamio et al. 2000, 2002; Kamio 2009). If candidate substances appear, their pheromonal activity could be confirmed by using the behavioral bioassay presented here. However, male behavioral responses in bioassays were not constant but varied depending on individuals, male condition, and timing of experiments during a reproductive season (S. Okamura, personal observation). Such response variability was also reported for the green crab *Carcinus maenas* because of impacts of maturity, social hierarchy etc. (Fletcher and Hardege 2009). Although only using males that performed assessment behavior on the "female-conditioned" water samples solved this problem (positive control) in this study, we need to control these factors more effectively.

If identification of sex pheromones in the hermit crab is achieved, it may allow us further studies dealing with tests for different hypotheses on manipulative female mate choice. For example, which behavior would female hermit crabs adopt: an active female mate choice in which females release sex pheromones depending on availability or quality of potential mates or a passive female mate choice in which females do not control pheromone release depending on mate availability or quality but release sex pheromones while guarded, attracting many rival males and inducing male-male combat, and high quality males end up mating at high frequency (Yamanoi et al. 2006; Okamura and Goshima 2010). In addition, there are still some questions to be solved; the physiological pathway to pheromone synthesis and link in time between pheromone emission and female molt cycle. More studies will be needed to answer these questions.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

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