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 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 compounds was < 1kDa 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℃, and remained active in seawater at 12℃ even after 6 days from sample collection, which suggest a rather stable characteristic of the female sex pheromones of this species.

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

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 Terschak 2011; Kamio et al. 2014; Yano et al. 2016). However, there is no complete identification of sex pheromone candidate molecules in dacapod 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; Zang 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 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 the 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 has 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*-test 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℃. 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.

123

124 Bioassay procedures

125

126 Solitary males of the hermit crab often show assessment behavior toward mature
127 females, while no response behavior was observed toward immature females or shells
128 with no females. The assessment behavior is preliminary to guarding behavior, and it is
129 elicited by female sex pheromones (Imafuku 1986; Goshima et al. 1998; Minouchi and
130 Goshima 1998; Okamura and Goshima 2010). The male embraces the female with its
131 ambulatory legs, and rotates the female into a face-to-face position. The male then
132 brings the anterior parts of his cephalothorax close to the shell aperture of the female,
133 and examines it by touch with antennae, walking legs, and chelae on a part of the
134 female's body just protruding from the shell. We determined whether this assessment
135 behavior occurred or not as well as duration of the interaction when observed in
136 experiments to detect sex pheromonal activity in the samples of "female-conditioned"
137 water as follows.

138 Guarding males from the field were separated off the females, and each male
139 was placed in a plastic case (10.5 cm diameter, 2 cm high) full of natural seawater. Most
140 guarded females in Kattoshi were in *Batillaria cumingi* shells (Yoshino et al. 2002).
141 Two groups of empty *Batillaria* shells which were similar in size were plugged with
142 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 at 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, Mollipore) to separate each water sample into two fractions of different molecular size; < 1 or > 1kDa. The pheromonal activity of the remaining fraction on the filter (> 1kDa) and filtrate (< 1kDa) 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 > 1kDa) 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 (> 1kDa) was obtained by rinsing the YM-1 filters 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 pheromones 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 up to 90℃ 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℃ 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

223 (26.7%) were particularly dominant among them ($P < 0.05$ in all cases except for
224 between stage I and II, $P = 0.409$).
225
226 “Female conditioned” water sample collection and bioassay procedures
227
228 In both trials where artificial seawater and “female conditioned” water were presented
229 first, there was a significant difference in the duration of male assessment behavior
230 between seawater and the “female conditioned” water samples (Fig. 2A, Wilcoxon
231 signed-rank test, $T = 7.5$, $P < 0.01$, Fig. 2B, Wilcoxon signed-rank test, $T = 3$, $P < 0.01$).
232 When presented with the “female conditioned” water samples, males significantly
233 increased the duration of assessment behavior in this experiment, suggesting that the
234 “female conditioned” water contained sex pheromones. Furthermore, the order of the
235 treatments (“female conditioned” water first or second) had no effects on the
236 experimental results. Therefore, all the later tests were conducted in order of seawater
237 first and then samples second.
238
239 Experiment 1: “pheromone” molecular size
240
241 There was a significant difference in duration of male assessment behavior between
242 artificial seawater and the filtered fraction (Fig. 3A, Wilcoxon signed-rank test, $T = 6.5$,

243 $P < 0.01$), while no significant difference was observed between artificial seawater and
244 the unfiltered fraction (> 1kDa) (Fig. 3B, Wilcoxon signed-rank test, $T = 7$, $P > 0.05$).
245
246 Experiment 2: “pheromone” polarity
247
248 A significant difference was found in the duration of male assessment behavior between
249 seawater and the sample of the fraction adsorbed on ODS resins and eluted with 30%
250 MeOH (Fig. 4A, Wilcoxon signed-rank test, $T = 54.5$, $P < 0.05$), and between seawater
251 and the sample of the fraction eluted with 50% MeOH (Fig. 4B, Wilcoxon signed-rank
252 test, $T = 18$, $P < 0.01$), respectively. It is suggested that the sex pheromone of this
253 species is a low polar compound.
254
255 Experiment 3: stability
256
257 *Freeze-drying*: Figure 5 shows the duration of male assessment behavior toward
258 artificial seawater and the freeze-dried “female conditioned” water samples. There was
259 a significant difference in duration of male assessment behavior between artificial
260 seawater and the freeze-dried sample (Wilcoxon signed-rank test, $T = 3$, $P < 0.05$).
261 *Heating*: The compound acting as “sex pheromone” in this species was stable
262 at high temperature. Figure 6 shows the duration of male assessment behavior toward

artificial seawater and the sample which was heated at 90℃ 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 6 days at 12℃ 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.

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

301 The hermit crabs live on intertidal rocky shores where the water movement is
302 usually variable and complicated; rapid in rough sea conditions while calm during some

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

319 As the water movement is usually complicated and non-directional in intertidal
320 rocky shores of the habitat of the hermit crabs, it would be difficult for males to locate
321 reproductive females by using only olfactory cue over “long” distances. Indeed,
322 guarding males often climb up leafy algae to keep female sex pheromons away from

323 rival males beneath (Kawaminami and Goshima 2015), and in the case of the European
324 shore crab *Carcinus meanas* the pair formation that proceedes mating occurs earlier and
325 the male simply carries the female away from hot spots to ensure single paternity
326 (Meeren 1994). These male behaviors may indicate that keeping a female odor source a
327 certain distance away from rival males is effective in preventing them from detecting
328 receptive females emitting pheromones. Therefore males of the hermit crab may use
329 multiple cues (e.g. visual or olfactory) in a complementary fashion when searching for
330 receptive females in the field. It is also possible that males alternate the use of such cues
331 for locating and accessing females, depending on the distance from the emitting source
332 and habitat conditions. In our previous studies, males of *Pagurus filholi* were shown to
333 use both visual and olfactory stimuli to recognize potential mating opportunities
334 (Okamura and Goshima 2010; Kawaminami and Goshima 2015). A similar case has
335 been also observed in the blue crab *Callinectes sapidus* (Kamio et al. 2008; Kamio
336 2009; Baldwin and Johnsen 2009). Males of the blue crab perform courtship in
337 stationary paddling bouts to deliver his pheromone to inform females of his location
338 only when females are inaccessible due to location uncertainty from high refuge density
339 and low visibility. Thus, male blue crabs use both visual and olfactory cues effectively
340 to adapt their reproductive behavior to the habitat conditions (Kamio et al. 2008).
341 Much effort has been undertaken in the search for sex pheromones in decapod
342 crustaceans, but only a few compounds have been identified and reported as sex

343 pheromones or potential sex pheromones (Gleeson et al. 1984; Hardege et al. 2002,
344 2011; Kamio et al. 2000, 2002; Kamio 2009). If candidate substances appear, their
345 pheromonal activity could be confirmed by using the behavioral bioassay presented here.
346 However, male behavioral responses in bioassays were not constant but varied
347 depending on individuals, male condition, and timing of experiments during a
348 reproductive season (S. Okamura, personal observation). Such response variability was
349 also reported for the green crab *Carcinus maenas* because of impacts of maturity, social
350 hierarchy and etc. (Fletcher and Hardege 2009). Although using only males that
351 performed assessment behavior on the “female-conditioned” water samples solved this
352 problem (positive control) in this study, we need to control these factors more
353 effectively.

354 If identification of sex pheromones in the hermit crab is achieved, it may allow
355 us further studies dealing with tests for different hypotheses on manipulative female
356 mate choice. For example, which behavior would female hermit crabs adopt: an active
357 female mate choice in which females release pheromones depending on availability or
358 quality of potential mates or a passive female mate choice in which females do not
359 control pheromone release depending on mate availability or quality, but release sex
360 pheromones while guarded, attracting many rival males and inducing male-male combat,
361 and high quality males end up mating at high frequency (Yamanoi et al. 2006; Okamura
362 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 case and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

References

Agosta WC (1992) Chemical communication: the language of pheromones. WH Freeman, New York

Asai N, Fusetani N, Matsunaga S, Sasaki J (2000) Sex pheromones of the hair crab

Erimacrus isenbeckii. Part 1: Isolation and structure of novel ceramides. Tetrahedron 56: 9895-9899

Atema J (1995) Chemical signals in the marine environment: dispersal, detection, and temporal signal analysis. Proc Natl Acad Sci USA 92: 62-66

Baldwin J, Johnsen S (2009) The importance of color in mate choice of the blue crab *Callinectes sapidus*. J Exp Biol 212: 3762-3768

Bamber SD, Naylor E (1996) Sites of release of putative sex pheromones and sexual behaviour in the female common shore crab *Carcinus maenas*. Estuar Coast Shelf Sci 44: 195-202

Bouwma P, Hazlett BA (2001) Integration of multiple predator cues by the crayfish *Orconectes propinquus*. Anim Behav 61: 771-776

Breithaupt T (2001) Fan organs of crayfish enhance chemical information flow. Biol Bull 200: 150-154

Breithaupt T, Thiel M (eds.) (2011) Chemical communication in crustaceans. Springer, New York

Bublitz R, Sainte-Marie B, Newcomb-Hodgetts C, Fletcher N, Smith M, Hardege JD (2008) Interspecific activity of sex pheromone of the European shore crab (*Carcinus maenas*). Behaviour 145: 1465-1478

Christofferson JP (1978) Evidence for the controlled release of a crustacean sex pheromone. J Chem Ecol 4: 633-639

Dunham PJ (1978) Sex pheromones in Crustacea. Biol Rev 53: 555-583

Dunham PJ (1988) Pheromones and behaviour in Crustacea. In: Laufer H, Downer R (eds) Endocrinology of selected invertebrate Types. Alan R Liss, New York, pp 375-392

Dusenbery DB (1992) Sensory ecology: how organisms acquire and respond to information. W. H. Freeman, New York

413 Fletcher N, Hardege JD (2009) The cost of conflict: agonistic encounters influence
414 responses to chemical signals in the European shore crab. Anim Behav 77:
415 357-361
416 Gleeson RA (1980) Pheromone communication in the reproductive behavior of the
417 blue crab, *Callinectes sapidus*. Mar Behav Physiol 7: 119-134
418 Gleeson RA, Adams MA, Smith AB (1984) Characterization of sex pheromone in the
419 blue crab, *Callinectes sapidus*: crustecdysone studies. J Chem Ecol 10:
420 913-921
421 Goshima S, Kawashima T, Wada S (1998) Mate choice by males of the hermit crab
422 *Pagurus filholi*: Do males assess ripeness and/or fecundity of females? Ecol
423 Res 13: 151-161
424 Hardege JD, Jennings A, Hayden D, Mueller CT, Pascoe D, Bentley MG, Clare AS
425 (2002) Novel behavioral assay and partial purification of a female derived sex
426 pheromone in *Carcinus maenas*. Mar Ecol Prog Ser 244: 179-189
427 Hardege JD, Bartels-Hardege HD, Fletcher N, Terschak JA, Harley M, Smith MA,
428 Davidson L, Hayden D, Muller CT, Lorch M, Welham K, Walther T, Bublitz R
429 (2011) Identification of a female sex pheromone in *Carcinus maenas*. Mar Ecol
430 Prog Ser 436: 177-189
431 Hardege JD, Terschak JA (2011) Identification of crustacean sex pheromones. In:
432 Breithaupt T, Thiel M (eds) Chemical communication in crustaceans. Springer,
433 New York, pp 373-392
434 Hazlett BA, McLay C (2000) Contingencies in the cognitive architecture of the crab
435 *Heterozius rotundifrons*. Anim Behav 59: 965-974
436 Imafuku M (1986) Sexual discrimination in the hermit crab *Pagurus geminus*. J Ethol
437 4: 39-47
438 Kamio M (2009) Toward identifying sex pheromones in blue crabs. Ann NY Acad

Sci 1170: 456-461

Kamio M, Derby CD (2011) Approaches to a molecular identification of sex pheromones in blue crabs. In: Breithaupt T, Thiel M (eds) Chemical communication in crustaceans. Springer, New York, pp 393-412

Kamio M, Matsunaga S, Fusetani N (2000) Studies on sex pheromones of the helmet crab, *Telmessus cheiragonus*. Zool Sci 17: 731-733

Kamio M, Matsunaga S, Fusetani N (2002) Copulation pheromone in the crab *Telmessus cheiragonus* (Brachyura: Decapoda). Mar Ecol Prog Ser 234: 183-190

Kamio M, Reidenbach MA, Derby CD (2008) To paddle or not: context dependent courtship display by male blue crabs, *Callinectes sapidus*. J Exp Biol 211: 1243-1248

Kamio M, Schmidt M, German MW, Kubanek J, Derby CD (2014) 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*. J Exp Biol 217: 1286-1296

Kawaminami T, Goshima S (2015) Guarding males of the hermit crab climb up algae to avoid male-male competition. J Ethol 33: 25-33

Kittredge JS, Terry M, Takahashi FT (1971) Sex pheromone activity of the molting hormone, crustecdysone, on male crabs. Fish Bull 69: 337-343

Meeren GI (1994) Sex- and size-dependent mating tactics in a natural population of shore crabs *Carcinus maenas*. J Anim Ecol 63: 307-314

Minouchi S, Goshima S (1998) Effect of male/female size ratio on mating behavior of the hermit crab *Pagurus filholi* (Anomura: Paguridae) under laboratory conditions. J Crust Biol 18: 710-716

Okamura S, Goshima S (2010) Indirect female choice mediated by sex pheromones

in the hermit crab *Pagurus filholi*. J Ethol 28: 323-329

Raffaelli D, Hawkins S (1996) Intertidal ecology. Champman & Hall, London

Salmon M (1983) Courtship, mating systems and sexual selection in decapods. In: Rebach S, Dunham DW (eds) Studies in Adaptation; the Behavior of Higher Crustacea. Wileyliss, New York, pp 143-169

Wyatt TD (2010) Pheromones and signature mixtures: defining species-wide signals and variable cues for identify in both invertebrates and vertebrates. J Comp Physiol A 196: 685-700

Wyatt TD (2014) Pheromones and animal behavior: chemical signals and signatures 2nd edn. Cambridge University Press, Cambridge

Yamanoi T, Yoshino K, Kon K, Goshima S (2006) Delayed copulation as a means of female choice by the hermit crab *Pagurus filholi*. J Ethol 24: 213-218.

Yano H, Kamio M, Nagai H (2016) The molting biomarker metabolite *N*-acetylglucosamino-1, 5-lactone in female urine of the helmet crab *Telmessus cheiragonus*. Biol Bull 230: 143-151

Yoshino K, Goshima S, Nakao S (2002) Temporal reproductive patterns within a breeding season of the hermit crab *Pagurus filholi*: effects of crab size and shell species. Mar Biol 141: 1069-1075

Zhang D, Lin J, Harley M, Hardege JD (2010) Characterization of a sex pheromone in simultaneous hermaphroditic shrimp, *Lysmata wurdemanni*. Mar Biol 157: 1-6

Figure legends

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

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 (< 1kDa) (Wilcoxon signed-rank test, $n = 30$, $T = 6.5$, $P < 0.01$), and B: toward seawater and unfiltered fraction of ultrafiltration (> 1kDa) (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:

506 Toward seawater and the sample of the fraction adsorbed on ODS resins and eluted with
507 30% MeOH (Wilcoxon signed-rank test, $n = 38$ each, $T = 54.5$, $P < 0.05$), and B: toward
508 seawater and the sample of the fraction adsorbed on ODS resins and eluted with 50%
509 MeOH (Wilcoxon signed-rank test, $n = 28$ each, $T = 18$, $P < 0.01$)
510
511 Fig. 5: The results of the bioassay showing duration of male assessment behavior
512 toward seawater and freeze-dried pheromone water (Wilcoxon signed-rank test, $n = 14$
513 each, $T = 3$, $P < 0.05$)
514
515 Fig. 6 The results of the bioassay showing duration of male assessment behavior
516 toward seawater and the pheromone water heated at 90℃ for 15 min (Wilcoxon
517 signed-rank test, $n = 14$ each, $T = 6$, $P < 0.01$)
518
519 Fig. 7 The results of the bioassay showing duration of male assessment behavior
520 toward seawater and the pheromone water kept for 6 days at 12℃ (Wilcoxon
521 signed-rank test, $n = 22$ each, $T = 5.5$, $P < 0.05$)
522
523

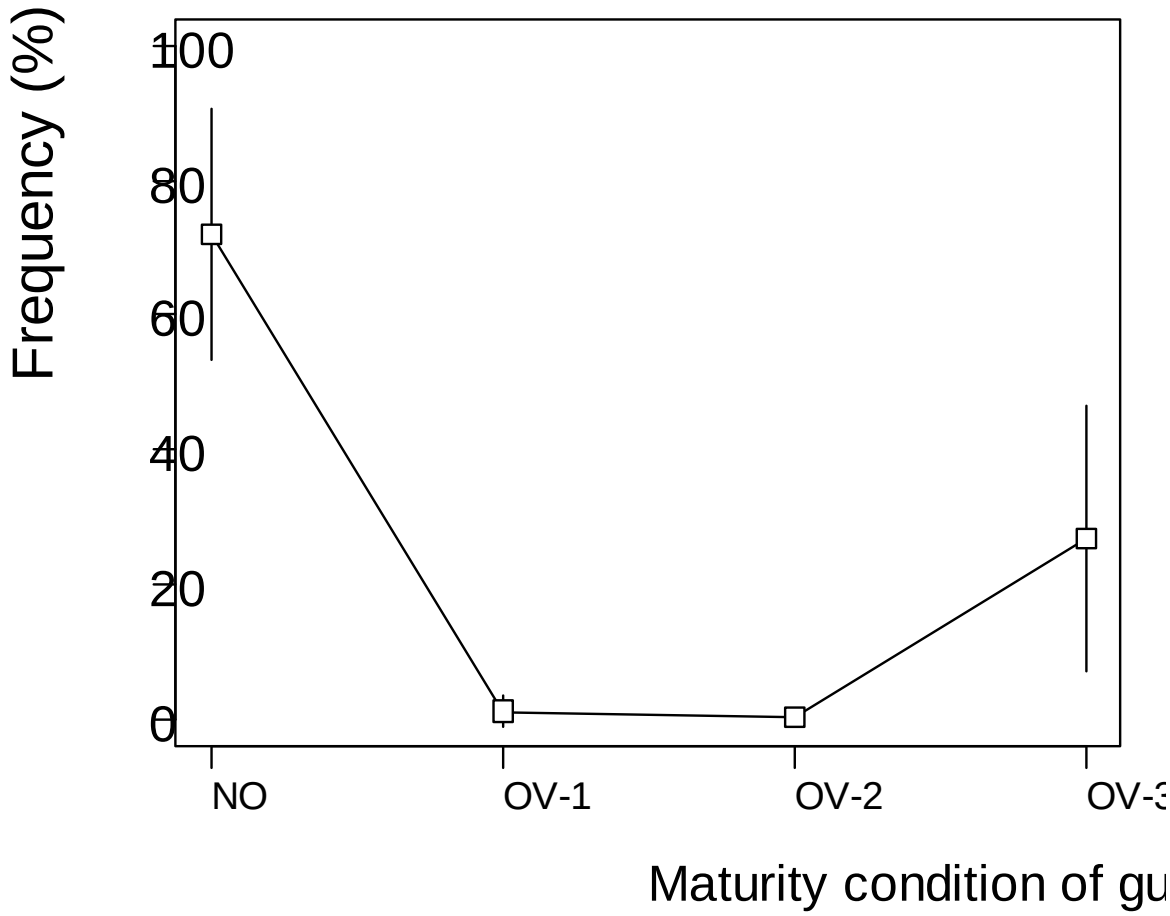
Frequency (%)
100
80
60
40
20
0
NO
OV-1
OV-2
OV-3
Maturity condition of gu

Fig. 1

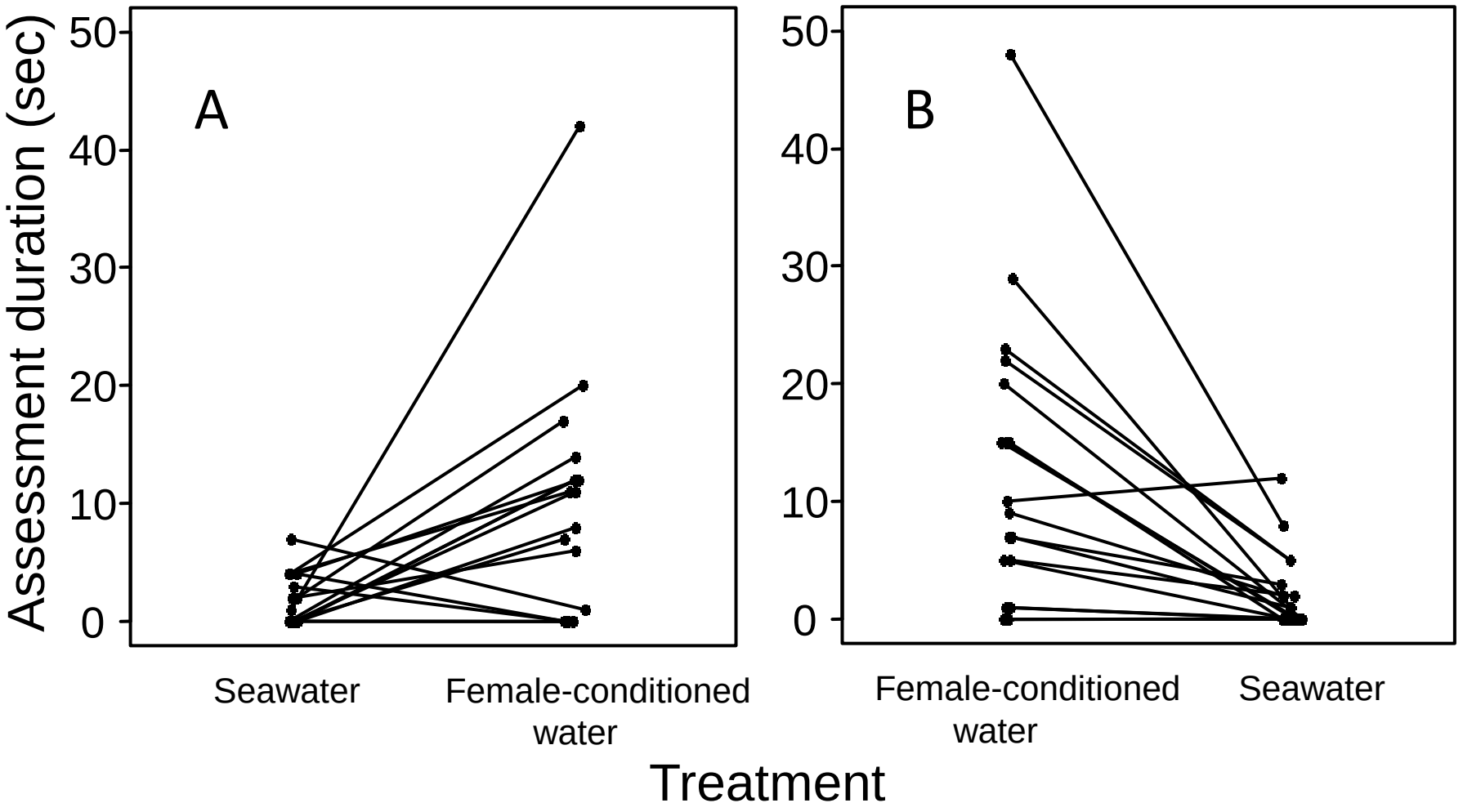
Assessment duration (sec)
50
40
30
20
10
0
A
B
Seawater
Female-conditioned water
Female-conditioned water
Seawater
Treatment

Fig. 2

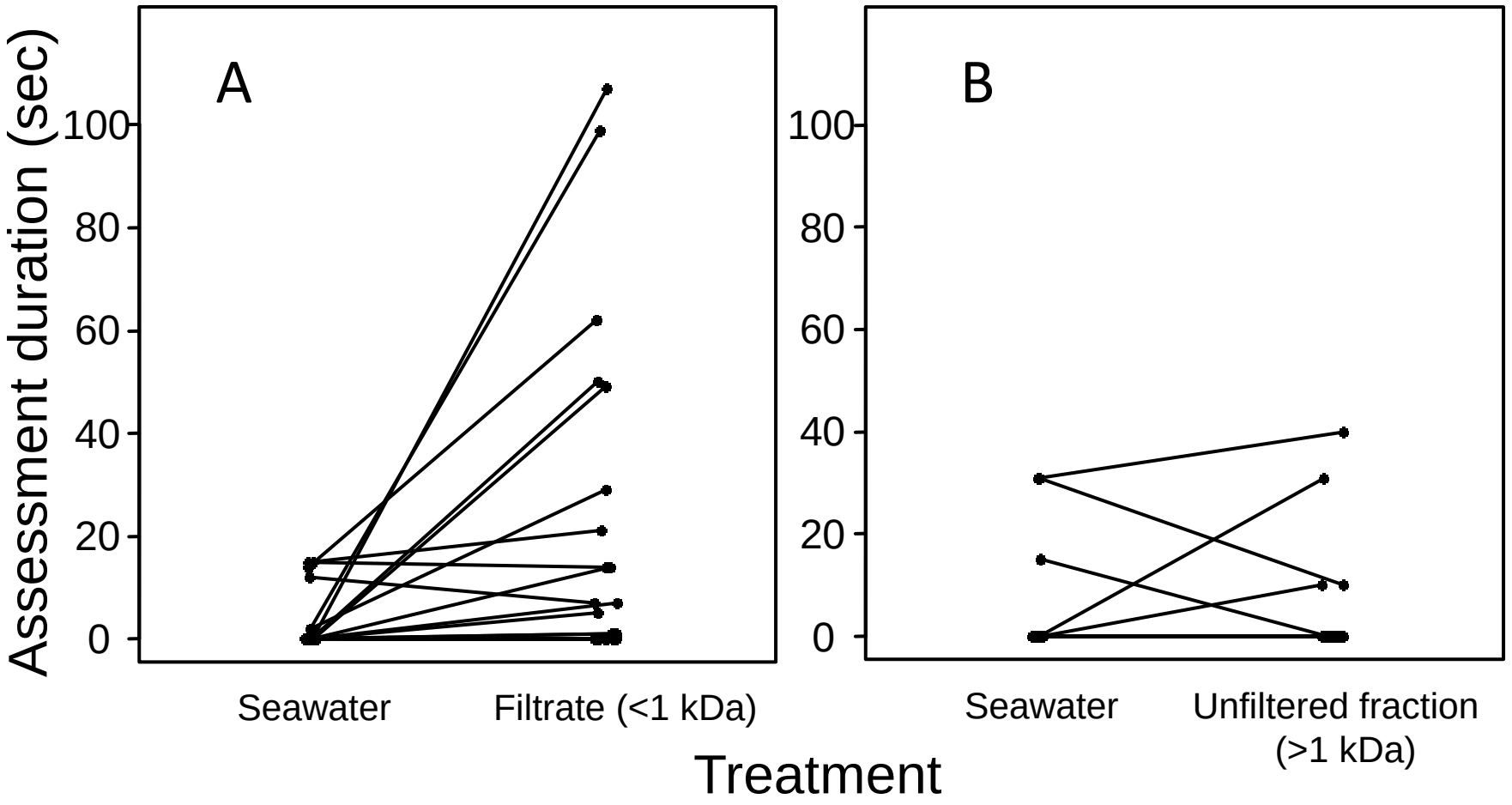
A
B
Assessment duration (sec)
0
20
40
60
80
100
Seawater
Filtrate (<1 kDa)
Seawater
Unfiltered fraction
(>1 kDa)
Treatment

Fig. 3

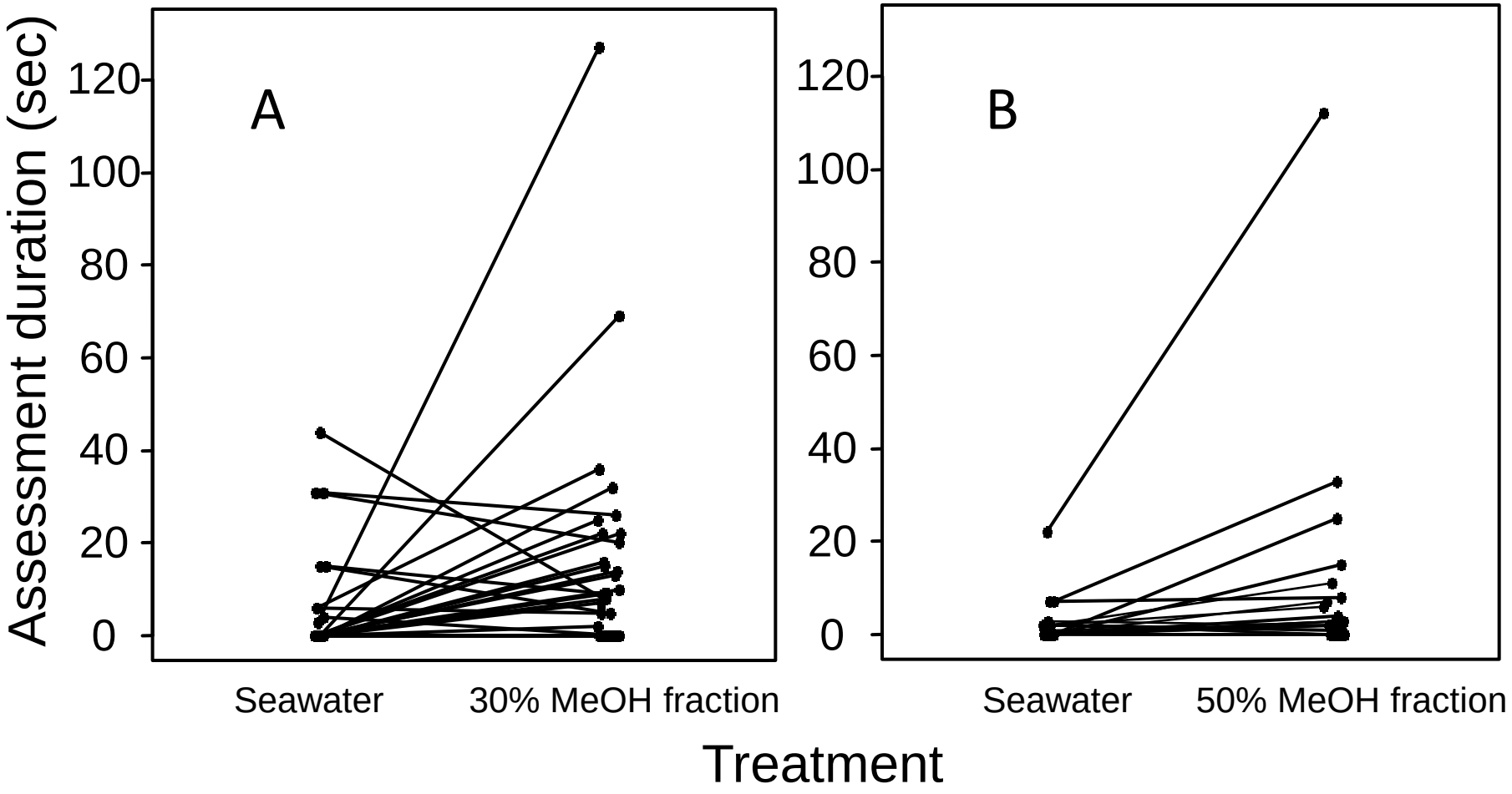
Assessment duration (sec)
120
100
80
60
40
20
0
A
B
Seawater
30% MeOH fraction
50% MeOH fraction
Treatment

Fig. 4

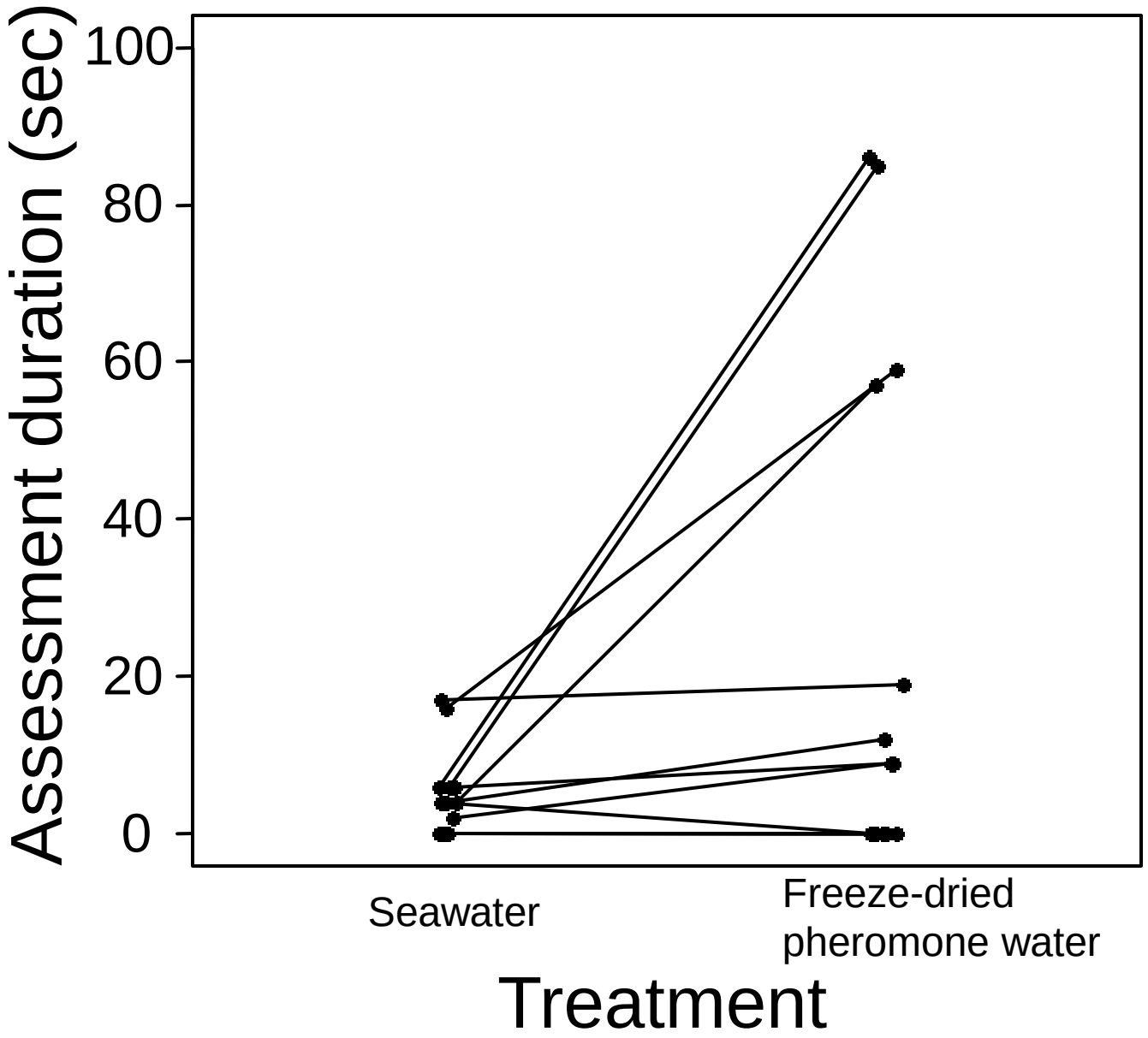
Assessment duration (sec)
100
80
60
40
20
0
Seawater
Freeze-dried
pheromone water
Treatment

Fig. 5

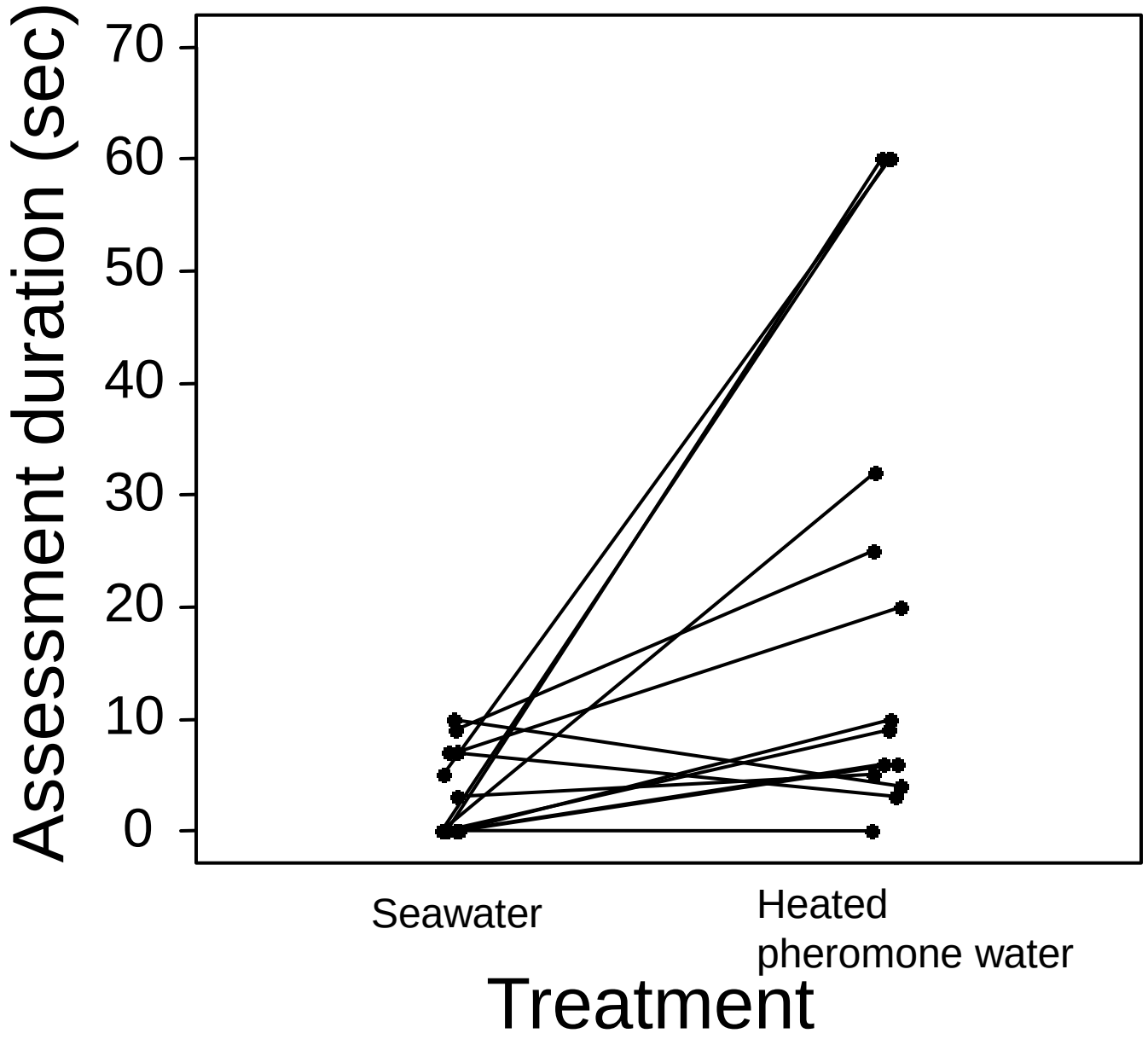
Assessment duration (sec)
70
60
50
40
30
20
10
0
Seawater
Heated
pheromone water
Treatment

Fig. 6

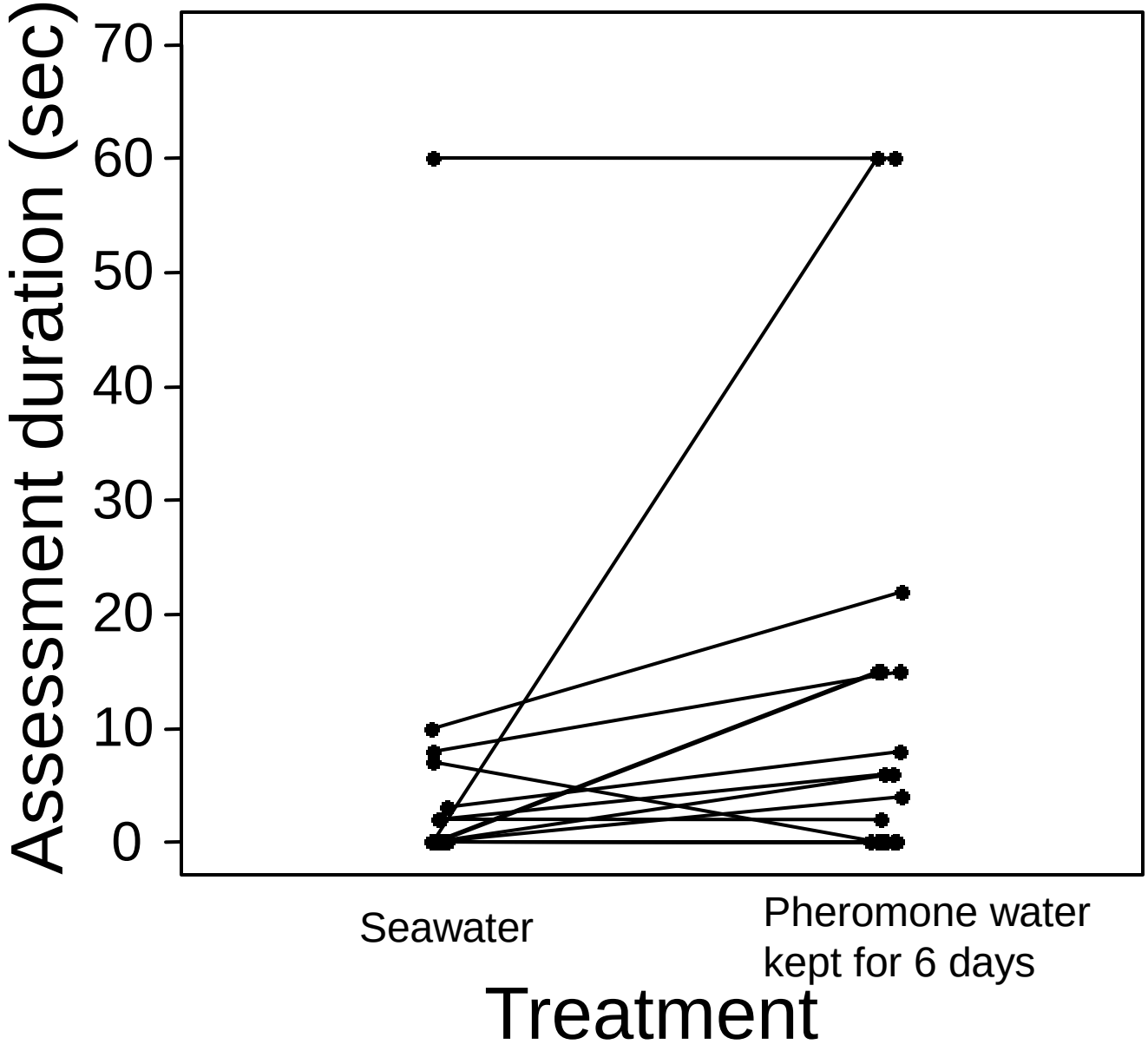
Assessment duration (sec)
70
60
50
40
30
20
10
0
Seawater
Pheromone water kept for 6 days
Treatment

Fig. 7