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[SHORT COMMUNICATION]

Studies on Sex Pheromones of the Helmet Crab, *Telmessus cheiragonus* 1. An Assay Based on Precopulatory Mate-Guarding

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ABSTRACT—A new reliable bioassay method has been developed for detection of female sex pheromone(s) of the helmet crab *Telmessus cheiragonus* using artificial sponges based on the grasping behavior of male crabs. The seawater, male urine, and pre- and postmolt female urine were tested for pheromonal activity which resulted in the detection of activity only in urine from pre- and postmolt females.

INTRODUCTION

Chemical signals between male and female play important roles in copulation of crustaceans (Dunham 1988). In many aquatic brachyuran species, males initiate sexual behaviors in response to pre- and/or postmolt female urine as well as to waters in which these females have been maintained (Ryan, 1966; Gleeson, 1991; Bamber and Naylor, 1996). Because reactions of males towards pheromones released from conspecific females are different in each species, a reliable assay method to detect sex pheromone should be devised for each species. In order to detect sex pheromones, searching behavior, copulatory display, and chemotaxis of male crabs were used as criteria to determine sexual behaviors (Ryan, 1966; Eales, 1974; Gleeson, 1991; Bamber and Naylor, 1996).

The helmet crab, *Telmessus cheiragonus* is a medium-sized brachyuran of the family Atelecyclidae, widely distributed from east coast of Hokkaido and the neighborhood on the east coast of Asia to the west coast of North America (Urita, 1936). This crab is abundant in Usujiri, Hokkaido and the mating season is from March to July (Nagao, 1999). Since sexually mature individuals are readily available, this species is suitable for chemical studies of sex pheromone. Mating in *T. cheiragonus* is closely linked in time to the molt cycle of the female (Nagao, 1999) as seen in other fully aquatic brachyurans (Hartnoll, 1969). When a sexually-receptive male encounters a premolt female, the male grasps and guards her until ecdysis. The precopulatory guard takes place in a manner that ventral sides of both male and female are in contact. Immediately following ecdysis, copulation occurs (Sasaki,

1995; Asai, 1996). Such mating behaviors show a striking resemblance with those reported for portunide crabs, while distinctive courtship displays and searching behavior are missing in *T. cheiragonus* (Asai, 1996). The present study was conducted to devise a reliable bioassay method using sexual behaviors of competent males as criteria to assess pheromonal activity of the urine of pre- and postmolt female in *T. cheiragonus*.

MATERIALS AND METHODS

Pre-copula pairs and solo males of *T. cheiragonus* were collected during the months between May and June from pier walls in Usujiri, Hokkaido (N 41°57', E 140°58'). Paired males separated from their partner females, and solo males were maintained at 10.5 ± 1°C in an aquarium with recirculating seawater system for a minimum of 2 days prior to experiments at natural photoperiods. Females were housed in a separate flow through seawater system at ambient temperature.

For collection of urine, modified method of Bamber and Naylor (1997) was adopted. Crabs were restrained on a plastic board (5 x 12.5 cm) using rubber bands with their ventral surface uppermost. The third maxillipeds were lifted up using tightly rolled tissue paper which made the opening of the antennal gland exposed, while the gill cavity openings were blocked with absorbent paper (Fig. 1). As the operculum of the opening was lifted up using modified insectpin, urine flowing out of the opening was collected by a sharpened tip for pipettes which was connected to a silicon tube (φ 2.15 mm). Suction was done by a peristaltic pump. Pre-, postmolt female, and male urines were separately pooled and stored at -20°C until required.

Male crabs maintained in an aquarium with recirculating seawater system at 10.5 ± 1°C were individually transferred into a still water aquarium (31.5 x 18.5 x 24.4 cm) containing 12 L seawater and acclimated at 15.5 ± 1°C for 2–3 hr. Each male crab was separated visually by masking the aquarium with black vinyl sheet. A urethane sponge (2.5 x 2.5 x 4.0 cm) in which 7.5 g lead weight was inserted to prevent from floating was attached to a transparent plastic rod (φ 0.3 x 30 cm). An 20 µL aliquot of samples were pipetted onto sponges as a single

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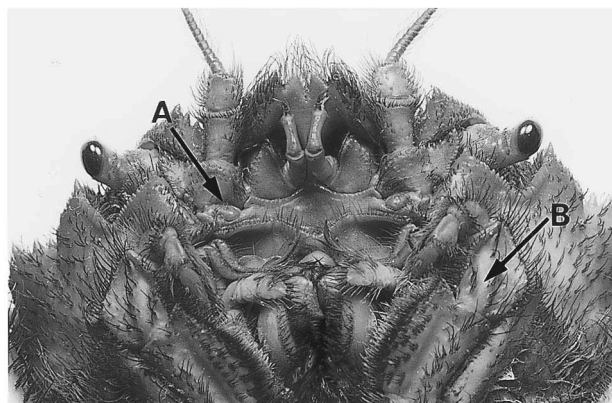


Fig. 1. Collection of urine from antennal gland opening. (A) operculum of antennal gland opening, (B) third maxilliped

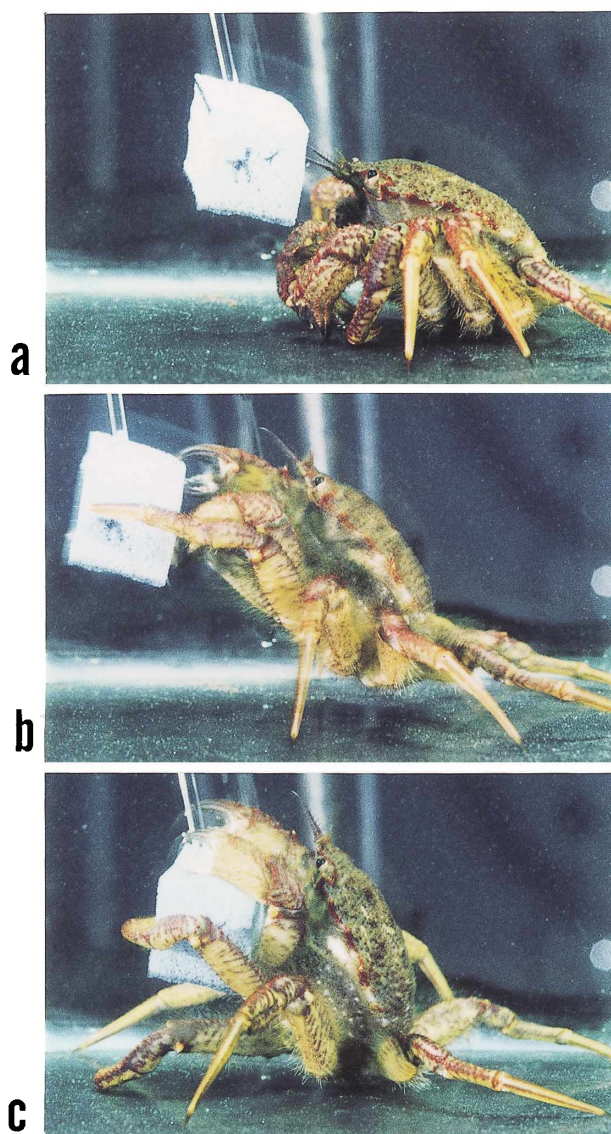


Fig. 2. Sequential images of grasping behavior of a male crab to a female urine-containing sponge (a–c). When a sponge with pre- or postmolt female urine was presented to a male crab, the male grasped the sponge with chelae and walking legs.

spot. Sponges were presented to males so as to make the first antennae of male crabs encounter the spot, and behaviors of the male crab were observed for 1 min, and the presence or absence of grasping behavior was recorded (Fig. 2). All observations were performed during night under artificial illumination (200 lx).

The assay system described above was used to detect sex pheromonal activity of female and male urines. Seawater was used as a control. Male urine was collected from 5 individuals which were paired with females when collected, while premolt female urine was collected from 4 individuals which were paired with males when collected and had not molted yet. Postmolt female urine was collected from 6 individuals that had molted 2–6 days earlier in laboratory in the absence of male. Behaviors of male crabs were noted for each operation. For postmolt female urine, male urine, and sea water, 40 males were used for each sample while premolt female urine was tested with 25 males. Individual male crab was tested only once in this experiment.

To test the threshold concentration of the pheromone in postmolt female urine to elicit grasping behavior of male crab the method described above was used. Postmolt female urine was collected from 6 individuals that had molted 2–6 days ago and pooled and four serial dilutions was made with sea water adjust to 1/2–1/1000 dilution. Each sample was tested with 5 males.

RESULTS AND DISCUSSION

Copulatory behavior in *T. cheiragonus* lacks distinctive copulatory display and searching behavior. Thus grasping behavior can be used as a criterion to define sexual response of male crabs. When a sexually receptive male encounters a premolt female, the male grasps and fumbles with her so as to orient properly (Sasaki, 1995; Asai, 1996). Similar behaviors were observed when sponges containing urine of pre- or postmolt female were presented to males so as to make the spots of urine encounter the first antennae of male crabs (Fig 2). The male grasps the sponge with chelae and walking legs (Fig. 2) and fumbles with the sponge. This grasping behavior of male crabs was used as a criterion to define sexual response. This response was observed in a high rate only under controlled condition; stock and test aquaria should be maintained at $10.5 \pm 1^\circ\text{C}$ and $15.5 \pm 1^\circ\text{C}$, respectively (data are not shown). When sponges containing food extracts (e.g., shrimp or crab extract) were given to the males, they immediately grabbed, tore pieces off, and ate them. Thus, the sexual behaviors are distinguished from feeding behaviors.

Grasping behavior-guided sponge assay clearly detected the sex pheromone in female urines. The responses of sexually receptive males to seawater, male urine, pre- and postmolt female urines are summarized in Table 1. Because intermolt or sexually inactive females were not available, urines collected from them were not tested. The grasping behavior was

Table 1. Pheromonal Activity in Female Urines

sample	No. of crabs showed grasping behavior	No. of crabs did not show grasping behavior
sea water	1	39
male urine	2	38
pre-molt female urine	16	9
postmolt female urine	29	11

Table 2. Sexual responses of male crabs to various concentration of postmolt female urine

concentration	No. of crabs showed grasping behavior	No. of crabs did not show grasping behavior
1/2	5	0
1/10	5	0
1/100	5	0
1/1000	0	5

observed at significantly higher percentages for pre- and postmolt female urines than for controls (χ^2 test, $P < 0.001$); 64 and 73% of male crabs grasped the sponges with pre- and post-molt female urines, respectively, whereas only 2.5% and 5% of male crabs grasped the sponges containing sea water and male urine, respectively. It is thus obvious that only pre- and postmolt female urines possess pheromonal activity; the pheromonal activity of intermolt and sexually inactive female urines remains to be examined. Thus, the present study clearly indicates that urines of pre- and postmolt females of *T. cheiragonus* contain sex pheromone to which conspecific males responded by grasping sponges containing female urine. Similar results were observed in the cases of *Portunus sanguinolentus* (Ryan, 1966), *Carcinus maenus* (Bamber and Naylor, 1997), and *Callinectes sapidus* (Gleeson, 1980). In further experiments with sponges containing postmolt urine at concentrations of 1/2, 1/10 and 1/100, five of five males exhibited grasping behavior. While none of five males grasped the sponges containing urine at a concentration of 1/1000 (Table 2). The data suggested that, using method described, the threshold concentration is between 1/100 and 1/1000. This concentration means potential for chemical communication at a distance in the helmet crab. Probably this pheromone is contained in female urine and released into surrounding water. Male may initiate a precopulatory guard in response to this pheromone released from premolt female. Although the copulation occurs immediately after ecdysis of female, the urine samples collected from postmolt females continued to

induce grasping behavior of male crabs for at least two weeks after ecdysis. Moreover, pooled urine collected from six females which had gonopore plug showed grasping behavior inducing activity in five of seven males. Because the plugs are present only in recently copulated females (Nagao 1999), the female of *T. cheiragonus* might copulate with more than one male in a mating season.

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