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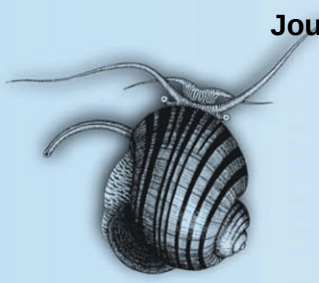

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Chemical cues correlate with agonistic behaviour and female mate choice in the southern blue-ringed octopus, *Hapalochlaena maculosa* (Hoyle, 1883) (Cephalopoda: Octopodidae)

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ABSTRACT

Chemoreception cues potentially influence intraspecific interactions of cephalopods, including mate choice. However, at present there is limited empirical evidence demonstrating whether cephalopods can use olfaction to identify the sex or identity of conspecifics. This study examined the responses of the southern blue-ringed octopus, *Hapalochlaena maculosa* (Hoyle, 1883), to conspecific odours during controlled laboratory trials. The ventilation rates in aquaria of 25 wild-sourced animals were measured during four treatments: baseline, sea water, sea water containing male conspecific odour and sea water containing female conspecific odour. When used as 'receivers' in trials, female *H. maculosa* significantly increased their ventilation rates in response to male odours, but not to female odours. However, female response decreased significantly with the receiver's size during female-odour treatments. The ventilation rates of male *H. maculosa* were statistically similar in all treatments. However, their ventilation rates showed a significant progressive increase over the observation period during male and female-odour treatments. Eighteen of these animals (nine females and nine males) were used in focal-animal trials 1 week after odour-cue experiments. Of these individuals, females were significantly more receptive to copulation attempts, and spent significantly more time per day in copulation, with males whose odours had elicited a weaker ventilation response in prior trials. These results suggest that female *H. maculosa* can use chemosensory cues to discriminate the sex, and possibly identity, of conspecifics and that this information might influence their mate choice. However, the mechanisms underlying these responses and subsequent copulatory access to females by males remain unknown.

INTRODUCTION

Social recognition among members of the animal kingdom has been proposed as a necessary prerequisite for the avoidance of unnecessarily competitive or aggressive interactions within species (Colgan, 1983; Wilson, 2000). The coleoid cephalopods have been reported to have relatively complex intraspecific interactions compared with other marine invertebrates (Hanlon, Smale & Sauer, 1994; Hanlon, Maxwell & Shashar 1997; Hanlon & Messenger, 1998; Norman, Finn & Tregenza, 1999; Hall & Hanlon, 2002; Huffard, Caldwell & Boneka, 2008, 2010; Godfrey-Smith & Lawrence, 2012; Caldwell *et al.*, 2015). Social recognition has so far been observed in several cephalopod species, with the ability to identify and signal the sex (Hanlon *et al.*, 1994; Hall & Hanlon, 2002; Huffard *et al.*, 2008), mating status (Cigliano, 1995; Norman *et al.*, 1999; Wada *et al.*, 2010) and dominance (Cigliano, 1993; Boal, 1996; Huffard *et al.*, 2010) of conspecifics. These forms of social recognition are thought to influence both mate choice (Cigliano, 1995; Hall & Hanlon, 2002; Huffard *et al.*, 2008, 2010) and competition for resources (Cigliano, 1993; Huffard *et al.*, 2010; Scheel, Godfrey-Smith & Lawrence, 2016). While individual recognition based on visual cues has currently been demonstrated for only one cephalopod species (Tricarico *et al.*, 2011), many of them are suspected to use visual context to inform behavioural interactions with conspecifics (Boal, 2006). Explicit chromatophore patterning and signals are used by some species of octopus, cuttlefish and squid to identify the sex, mating strategy and agonistic intent of interacting individuals (Corner & Moore, 1981; Hanlon *et al.*, 1994; Hall & Hanlon, 2002; Huffard, 2007; Huffard & Godfrey-Smith, 2010; Scheel *et al.*, 2016). The size of individuals has also been reported to aid in establishing hierarchies and recognizing dominance of conspecifics in several octopods (Boyle, 1980; Mather, 1980; Cigliano, 1993; Huffard *et al.*, 2010). Additionally, the location of individuals (e.g. den or egg clutch) is thought to

help some male octopods and cuttlefish recognize recent mates and to facilitate mate-guarding behaviours (Boal, 1996; Huffard *et al.*, 2008).

While the use of visual cues in intraspecific interactions appears widespread among cephalopods, increasing evidence indicates that chemosensory cues might also be important (Boal, 1997; Boal & Golden, 1999; Walderon *et al.*, 2011). Cuttlefish, squid and octopods can sense chemical stimuli both from a distance using olfactory organs close to the eyes and upon contact with objects using chemoreceptor cells located on the lips and suckers (Budelmann, 1996), and cephalopods are known to use chemosense to aid in locating food items (Wells, 1963; Chase & Wells, 1986). Some cephalopods have also been reported to react to odour from conspecifics, although the reasons for these responses are unclear (Boal & Golden, 1999; Buresch *et al.*, 2003; King, Adamo & Hanlon, 2003; Walderon *et al.*, 2011). For example, *Sepia officinalis* increases its ventilation rate when exposed to sea water containing odour from conspecifics, suggesting that it can detect them by chemical stimuli from a distance (Boal & Golden, 1999). However, *S. officinalis* does not display any change in approach behaviour based solely on odours from conspecifics of different sex or mating history, suggesting that this species might not use odour cues in sex discrimination or mate choice (Boal & Marsh, 1998). The use of non-tactile chemoreception to detect and interpret information about conspecifics has not yet been investigated for squids, although it has been demonstrated that these animals have the capacity to obtain information from chemical stimuli in the water (Lucero, Horrigan & Gilly, 1992). Tactile chemoreception has been demonstrated in *Loligo pealei* and it has been suggested that a pheromone present in its egg capsules triggers males to engage in male–male agonistic behaviour to compete over females (Buresch *et al.*, 2003; King *et al.*, 2003). The use of odour cues in social recognition also appears possible in octopods. Laboratory trials with *Octopus bimaculoides* revealed that it can detect conspecifics based on odour cues and that ventilation rates of individuals were different depending on the sex of conspecifics that were detected (Walderon *et al.*, 2011). Given that sex discrimination based on chemical stimuli is supported for at least this species, it seems possible that odour cues might play a role in locating or discriminating between potential mates within the mating systems of some cephalopods.

There is some indirect evidence for the use of odour cues in cephalopod mating behaviours. Boal (1997) found that female *S. officinalis* were more likely to choose to mate with newly introduced males that had previously mated with another female than with unmated males. Since females in this experiment could not have seen whether a male had already mated, Boal (1997) hypothesized that females might have used chemical cues to discern recent male mating history in order to mate preferentially with sexually mature, healthy males that had already proved capable of copulation. In an earlier study, Boal (1996) showed that recently-mated male *S. officinalis* were more likely to mate-guard recently-mated females than unmated females, regardless of whether they were the male that had mated with the female. This behaviour suggests that male *S. officinalis* might also depend on chemical cues to identify the recent mating history of females and (presuming the female was not switched) use this information to limit the risk of sperm competition. Additionally, laboratory observations of *S. lycidas*, an unidentified pygmy octopus and *Hapalochlaena maculosa* all showed that males adjusted their copulation times with females based on whether they were the last male to have mated with her (Cigliano, 1995; Wada *et al.*, 2010; Morse *et al.*, 2015). It is possible in the first two of these studies that males assessed the recent female mating history of females by visual means (Cigliano, 1995; Wada *et al.*, 2010). However, the experimental design in the third (nocturnal) study ensured that visual assessment of female mating history was unlikely, suggesting that odour cues could have been responsible for this behaviour in *H. maculosa* (Morse *et al.*, 2015).

To date, the role of odour cues in mate-choice behaviours of octopods has not been formally investigated. *Hapalochlaena maculosa* serves as a good model species for research of this nature for a variety of reasons. As noted above, males of this species might use odour cues to recognize the recent mating histories of females (Morse *et al.*, 2015). Also, females might be selective of potential male partners. Female *H. maculosa* can reject the copulation attempt of one male and within hours be receptive to another male (P. Morse, personal observation). However, patterns of female receptivity appear independent of measured male physical traits including wet weight, mantle length (ML), inter-ocular width and ligula length (Morse *et al.*, 2015). Additionally, *H. maculosa* is a nocturnal octopod that lives in subtidal or turbid environments, where light and therefore visual cues are limited (Tranter & Augustine, 1973). If social recognition is important within the mating system of *H. maculosa*, as it is in some other cephalopods (Hanlon *et al.*, 1994, 1997; Hanlon & Messenger, 1998; Norman *et al.*, 1999; Hall & Hanlon, 2002; Huffard *et al.*, 2008, 2010), then this species would most likely have to rely on forms of sensory input that are useful at a distance, like odour cues, to gain information about conspecifics. Finally, *H. maculosa*, like other octopods, has a ventilation action that is easily observable (Walderon *et al.*, 2011). Mantle ventilation in octopods serves to bring oxygen to the gills, as well as to move chemical signals in the water over olfactory cells (Woodhams & Messenger, 1974).

Therefore, this study was designed to determine whether *H. maculosa* can recognize the scent of conspecifics and to assess whether female ventilatory response to male odours correlates with the performance of males during mate-choice trials, thus attempting to clarify the potential role of odour cues within the social behaviour of this species. Specifically, this study aimed to answer the following three questions: (1) Does *H. maculosa* change ventilation rate in response to odour from conspecifics? (2) Can either male or female *H. maculosa* discriminate the sex of conspecifics based on odour cues, as determined by differences in ventilation rate? (3) Are female responses to individual male odours correlated with copulation patterns?

METHODS

Animal acquisition and maintenance

Ten female and 15 male *Hapalochlaena maculosa* were obtained from the by-catch of commercial fishermen (under the license of the Fremantle Octopus Company) between Mandurah and Cockburn Sound in Western Australia (WA) from November 2013 to June 2014. Female size ranged from 1 to 12 g, and male size from 1 to 7 g (Supplementary Material Table S1). All animals had a ML of at least 20 mm, the minimum size at which both males and females have been observed to copulate during pilot studies (P. Morse, personal observation). All animals were housed in individual 1-l plastic containers connected to a closed flow-through system with a 1,000-l sump at Fremantle Octopus Company facilities in O'Conner, WA. Sea water was obtained from Cockburn Sound and maintained at 22 °C and salinity of 34–35 ppt before and during experiments. Male and female containers were separated by an opaque divider and activated carbon was used to neutralize odours in the sea water entering individual containers to limit each animal's exposure to conspecific odours prior to trials. Each animal was given an appropriately-sized shell for use as a den and was fed *ad libitum* with pieces of thawed, frozen prawns and occasional live crabs. No animals were fed in the 24 h leading up to trials to avoid any effect of recent feeding on ventilation rates. ReefOne^{TM} biOrb LED aquarium lights were used to simulate daylight for 14 h per day, which corresponded to local daylight hours when trials began. Animals were obtained under WA Department of Parks and Wildlife permit SF00963.

The use and treatment of the animals were approved by the James Cook University Ethics Committee (approval no. A1850).

Odour preparation

All sea water was obtained from Cockburn Sound the day before each set of odour-response trials. Ten litres of sea water were placed in each of three clean plastic buckets in the laboratory. One was the source of the seawater control; a male *H. maculosa* was put in the second bucket and a female in the third to prepare the male and female-odour treatments, respectively. A clean aerator was placed in each bucket and the three buckets were left in an air-conditioned part of the laboratory, continuously maintained at 22 °C for 18 h prior to use in odour-response trials. As not all experiments were conducted at the same time and different animals were available at different times, a total of 15 males were used individually in the male-odour treatments and ten females in the female-odour treatments. No animals were fed within 24 h leading up to being used as odour sources; however, all animals fed immediately after experiments. All buckets and aerators used in odour preparation were cleaned with fresh water using a high-pressure hose and left to air-dry overnight in a clean section of the laboratory between trials.

Odour-response trials

All ten female and eight of the male *H. maculosa* were used as 'receivers' (i.e. animals whose ventilation rates were being recorded) in odour-response trials after 2 d to 1 week of acclimation in the laboratory. All observations were made in three ReefOne biOrb Life 30-l square aquaria with opaque sides and an opaque barrier that blocked any view from the back of the aquaria. Aquaria were filled with 10 l of clean sea water. All animals were left to acclimate in the observation aquaria for a minimum of 30 min before observation. A CCTV camera in front of the aquaria was used to count ventilations without disturbing the animals.

Odour-response trials entailed counting receiver ventilations for 30 s once each minute for 5–10 min in each of a set of three treatments. The 'baseline' treatment was the receiver's normal resting rate without any odour stimulus. The 'seawater' treatment followed immediately after the baseline treatment and was the response after 1 l of sea water was gently poured into the corner of the observation tank in a 30-s action. The seawater treatment was followed immediately by either a 'male-odour' or 'female-odour' treatment, each applied in the same manner as the seawater treatment, but using sea water with male or female odour, poured from a separate, clean plastic watering can. Ventilation rates were scored for 10 min for all treatments with female receivers. However, due to time constraints during data collection, ventilations were only scored for 5 min during baseline and seawater treatments with male receivers.

No receiver had more than one male or female-odour treatment per day. Sometimes a receiver had both a male and a female-odour treatment in the same day, but with a minimum of 90 min between trials and only if the ventilation rate had returned to its previous baseline rate. If this was the case, male and female treatments were applied in random order and each included initial baseline and seawater treatments. Observation aquaria were cleaned and filled with new sea water for each new receiver, but not otherwise (to avoid excessive disturbance of the animals).

Each of the ten female receivers was used in at least two male-odour treatments. The number of male-odour treatments varied between female receivers, due to animal availability and to ensure that each of the females had given a response to every male that would later be used in the same focal animal trial, explained below. Seven of these female receivers were also used in one female-odour treatment and an eighth female in two. In total, there were 39 observations of female response to male odours and nine observations of female response to female odours (Supplementary Material Table S1). The eight male receivers each had one male-odour treatment and one female-odour treatment. However, the baseline ventilation rate of one male preceding a male-odour treatment was almost twice all other recorded observations, so this trial was omitted from analyses. This gave a total of seven observations of male response to male odour and eight observations of male response to female odour (Supplementary Material Table S1).

Analyses of odour-response trials

An initial analysis of all raw 30 s observations of receiver ventilation rates used a linear mixed-effects model (LMEM) to determine which factors were correlated with ventilation rate during trials. Ventilation rates (as ventilations per 30 s) were square-root transformed to normalize the distribution (Jones *et al.*, 2013). As each animal was exposed to multiple treatments, 'Receiver ID' was set as a random effect. This enabled an animal's ventilation rate to be compared between different treatments of unequal sample sizes, while eliminating the variance caused by measuring the response of different individuals (Jones *et al.*, 2013). The fixed-effects used in this analysis were 'treatment' (baseline, sea water, male or female), 'receiver sex' (male or female), 'mass' (receiver wet weight: 1–12 g) and 'min' (minute of recorded ventilation: 1–10). These fixed effects were represented by the S+ model: square-root ventilation~receiver sex+treatment+mass+min. Preliminary results indicated that all animals reacted strongly to the addition of any water to the observation aquaria for the first 1–2 min of observation (Fig. 1). This reaction was thought to be associated with the physical disturbance of adding water, so ventilation recorded during the first 2 min of all treatments were omitted from further analyses.

Next, a separate LMEM was applied within each treatment type among both female and male receivers to assess the effects of both time (min) and receiver mass on ventilation rates within individual treatments (square-root ventilation~mass+min). Finally, additional LMEMs were used to compare the change in ventilation rate of animals between each treatment type for both female and male receivers (square-root ventilation~treatment*mass*min). Where interactions between variables were nonsignificant, the analysis was repeated with the interaction terms removed in order to maximize statistical power (square-root ventilation~treatment+mass+min).

Focal-animal observations

One week after odour-response observations, nine of the 15 males that had had their odours given to females and nine of the ten female receivers were used in focal animal trials addressing mate-choice behaviour. These animals were split into three separate trials, each containing six animals (Supplementary Material Table S1). As one of the initial objectives had been to assess differences in behaviour with different operational sex ratios (OSR), the numbers of males and females differed among the three trials. The first trial had four females and two males, the second two females and four males and the third three females and three males.

The focal-animal trials took place in a 1-m^2 observation tank, with a water depth of 50 cm. The bottom of the tank was lined with sandy rubble, and 12 shells of varying shapes and sizes were haphazardly placed in the tank for animals to shelter in. Water was maintained at 34–35 ppt and 22 °C. A ReefOne™ biOrb LED aquarium light was used to provide 14 h of daylight per 24 h period and animals were fed *ad libitum* with pieces of prawn throughout the trials. The six animals were allowed to interact freely for the duration of the trial and observed using CCTV with infrared-recording capability. The first two trials each ran for 5 d. However, the third trial (with equal OSR) was terminated after 3.28 d as one of the males had died from excessive copulation (Morse *et al.*, 2015).

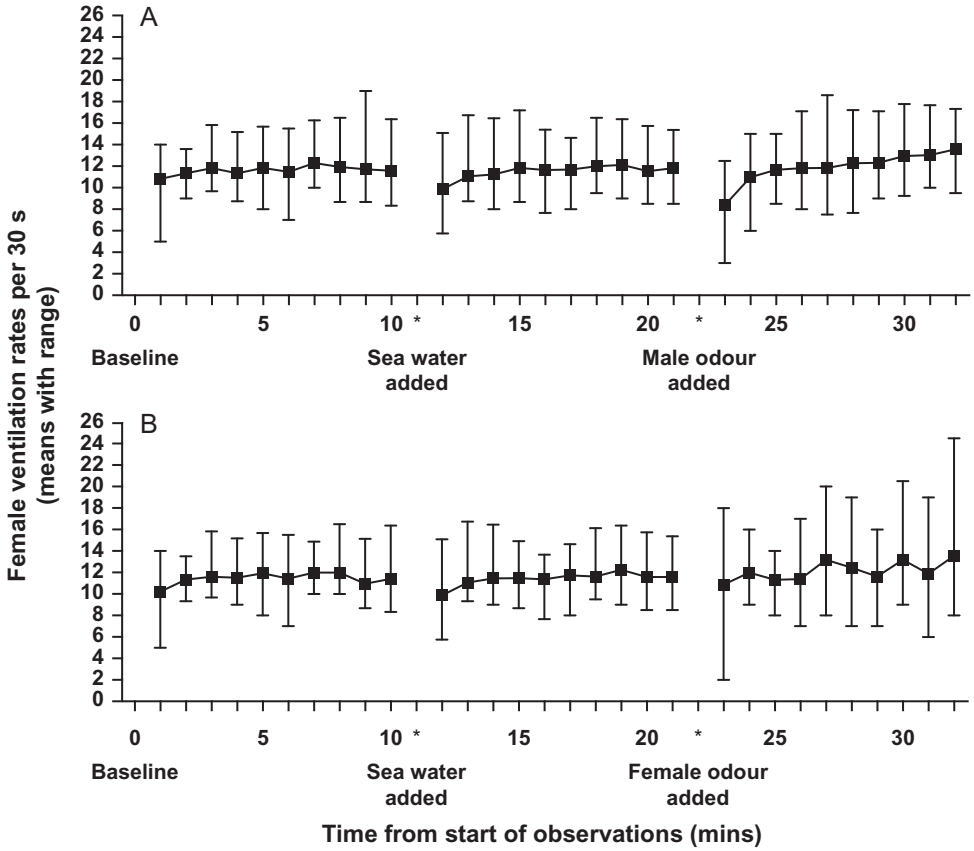

Figure 1. Mean ventilation rates per 30 s interval of female *Hapalochlaena maculosa* for each minute of observation. **A.** Male-odour trials ($N = 10$ animals, 39 trials). **B.** Female-odour trials ($N = 8$ animals, 9 trials). Asterisks indicate time of additions.

Behaviours of each animal were scored during video playback, to quantify the time each pair of animals spent in copulation per day (pair copulation time) and female receptivity to the males' mount attempts (female receptivity). A mount attempt was defined as any attempt by a male to climb onto a female's mantle. Any mount that lasted for more than 30 s was considered a copulation (Morse *et al.*, 2015). Pair copulation time was defined as the average time per day that a male–female pair spent in copulation. Females were considered receptive to male mount attempts if there was no rejection, i.e. a grappling phase or obvious attempt to retreat between male contact and a successful male mount. Copulations were often successful even when females were not receptive, but in this study female receptivity referred to the reaction of a female to a male, and not to whether males actually succeeded in copulating.

Comparison of female response to male odours with mating behaviour

As each female had previously been exposed to the odour of each male that was included with her in the same focal trial, it was possible to compare each female's response to individual male odours with observed mating interactions. First, a response value (RV) was calculated for each female–male pair. This was defined as the average female ventilation rate from minutes 3 to 10 after exposure to a male's odour, minus her average ventilation rate during minutes 1–10 of her immediately preceding baseline treatment. Analyses of the difference in magnitude of behaviours before and after exposure to an experimental stimulus are common in the literature on chemical ecology (e.g. Ferrari, Wisenden & Chivers, 2010; Walderon *et al.*, 2011). In this way, RV represented a relative measure of biological response of females to individual male odours. In some cases this calculation yielded an RV less than zero. However, even negative values were considered meaningful for the purpose of this study.

During focal animal trials, not all of the 25 potential female–male pairs copulated with each other. If the male never attempted to mount the female then this pair was omitted from analyses. However, if a male attempted to copulate with a female, and was unsuccessful due to rejection by the female, the pair copulation time was scored as zero. Values of pair copulation time were normalized using a log (x + 1) transformation (Jones *et al.*, 2013) and linearly regressed on female RV to the corresponding male odours and on female wet weight.

To assess any correlation between female receptivity and female response to male odours, a logistic regression was used to compare both female mass and pair RVs to the proportion of male mount attempts to which the female was receptive during focal-animal trials. As the number of mount attempts was not consistent among all pairs, the 'cbind' function in S+ was used to weight the effect of each proportion by its sample size for this analysis (Jones *et al.*, 2013). For one of the pairs, a copulation began inside a shell and so it was unclear whether the female was receptive to this mount attempt. This pair was consequently omitted from this analysis. Additionally, in order to ensure it was not a confounding variable, male wet weight was compared with female receptivity using a separate logistic regression, and against pair RV using a linear regression.

RESULTS

Baseline ventilation rates

Mean female and male ventilation rates during baseline observations were 12.5 ± 0.28 SE ventilations/30 s ($n = 10$ females; 48

trials) and 9.6 ± 0.38 ventilations/30 s (n = 8 males; 15 trials), respectively. Female ventilation rates at baseline were significantly faster than those of males (ANOVA of LMEM: $F_{1,15} = 4.697$, $P = 0.047$). Baseline rates were not significantly affected by animal size for either females (ANOVA of LMEM: $F_{1,8} = 2.693$, $P = 0.139$) or males (ANOVA of LMEM: $F_{1,5} = 0.441$, $P = 0.536$).

Female response to treatments

All female receivers reduced their ventilation rates immediately after addition of sea water or odour to their aquaria (Fig. 1). After omitting the first 2 min of observation from treatments, female ventilation rate was statistically independent of time for baseline (ANOVA of LMEM: $F_{1,303} = 0.013$, $P = 0.909$), seawater (ANOVA of LMEM: $F_{1,302} = 0.175$, $P = 0.676$) and female-odour treatments (ANOVA of LMEM: $F_{1,63} = 2.436$, $P = 0.124$). However, within male-odour treatments, female receivers increased their ventilation rates significantly with time between minutes 3 and 10 of observation (ANOVA of LMEM: $F_{1,301} = 5.653$, $P = 0.018$; Fig. 1).

Additionally, female ventilation rates during male-odour treatments were significantly faster than female baseline rates (Table 1; Fig. 2). As there was no statistical difference between female ventilation rates during baseline and seawater treatments (Table 1), these could be combined into a 'non-odour' treatment and compared with female ventilation during male-odour treatments for greater statistical power. Female ventilation rates during male-odour treatments were significantly different from the combination of baseline and seawater treatments (ANOVA of LMEM: $F_{1,926} = 5.682$, $P = 0.017$). Female response to female odour was highly variable and there was no significant difference in female ventilation rates between male and female-odour treatments (Table 1; Fig. 2).

Female ventilation rates during female-odour treatments were also statistically similar to female ventilation rates during both baseline and seawater treatments (Table 1). However, there was a significant interaction between receiver size and treatment type when comparing female ventilation rates between female-odour treatments and either baseline (ANOVA of LMEM: $F_{1,373} = 5.933$, $P = 0.015$) or seawater treatments (ANOVA of LMEM: $F_{1,372} = 4.785$, $P = 0.029$). Female ventilation rates during female-odour treatments decreased significantly with the wet weight of the female receiver (ANOVA of LMEM: $F_{1,6} = 17.429$, $P = 0.006$; Fig. 3). Female ventilation rates were statistically independent of female size for all other treatments (ANOVA of LMEM: $F_{1,8} = 1.815$, $P = 0.215$).

Male response to treatments

Like the females, most male receivers also decreased their ventilation rate for the first 2 min of observation after the disturbance caused by the addition of sea water or odour to their aquaria (Fig. 4). After excluding these first 2 min from analyses, male ventilation rates were statistically independent of time in baseline (ANOVA of LMEM: $F_{1,44} = 2.338$, $P = 0.133$), seawater (ANOVA of LMEM: $F_{1,45} = 2.823$, $P = 0.1$) and female-odour treatments (ANOVA of LMEM: $F_{1,48} = 2.794$, $P = 0.101$). However, male receivers increased their ventilation rates significantly with time over observations from minutes 3 to 10 during male-odour treatments (ANOVA of LMEM: $F_{1,48} = 9.524$, $P = 0.003$; Fig. 4).

Overall, male ventilation rates were highly variable (Fig. 4) and there were no statistically significant differences between any of the treatments (Table 1). However, there was an interaction between treatment type and time when comparing male ventilation rates between the seawater treatment and both the male-odour (ANOVA of LMEM: $F_{1,96} = 9.193$, $P = 0.003$) and female-odour treatments (ANOVA of LMEM: $F_{1,96} = 7.297$, $P = 0.008$). The males were observed to increase ventilation rates over the observation periods when exposed to odour from either a male or female conspecific, relative to rates during either a baseline or seawater treatment (Fig. 4). Male ventilation rates were statistically independent of the receiver's size for all treatments (ANOVA of LMEM: $F_{1,5} = 0.431$, $P = 0.541$).

Correlations between odour cues and female mate-choice patterns

Despite the different OSR in the three focal-animal trials, preliminary analyses (not shown) revealed that neither pair copulation time nor male mount success was significantly affected by the trial setup. The results were therefore analysed together. Among the 16 pairs of males and females that had mating interactions during focal-animal trials, there was a significant negative relationship between RV of females to the specific male's odour and the average time per day that the female spent in copulation with that

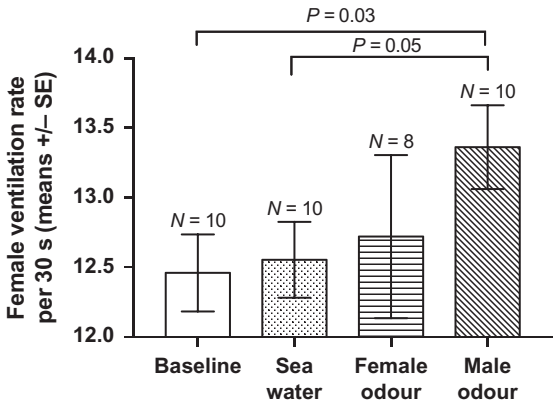

Figure 2. Mean ventilation rates of female *Hapalochlaena maculosa* during baseline, and in response to addition of sea water and sea water containing odour from female and male conspecifics. *P*-values are indicated for treatment types where receiver ventilations approached significantly different rates. *N*, number of receiver animals used in trials.

Table 1. Results for ANOVAs of linear mixed-effect models comparing ventilation rates of male and female *Hapalochlaena maculosa* between treatments of 'baseline', addition of 'sea water', 'female odour' and 'male odour'.

	Baseline			Sea water			Female odour			Male odour		
Compared Treatments	*F*	df (1, x)	*P*	*F*	df (1, x)	*P*	*F*	df (1, x)	*P*	*F*	df (1, x)	*P*
Baseline		–		0.039	613	0.844	1.452	373	0.229	**4.724**	**613**	**0.030***
Sea water	2.448	92	0.121		–		1.433	372	0.232	**3.852**	**612**	**0.050***
Female odour	0.061	95	0.805	3.176	96	0.078		–		0.016	371	0.899
Male odour	0.082	95	0.775	3.548	96	0.063	0.008	99	0.931		–	

Results for female receivers are given above diagonal, and for male receivers below diagonal. Treatment pairs that yielded differences in receiver ventilation rates at a significance level of 0.05 or less are shown in bold and indicated with an asterisk.

male (ANOVA of linear regression: $F_{1,13} = 22.754$, $P < 0.001$). However, in this analysis there was also a trend for pair copulation time to increase with the female's wet weight (ANOVA of linear regression: $F_{1,13} = 2.814$, $P = 0.117$).

Two of the females, which were less than 5 g in wet weight, gave high RV during odour-response trials and later went on to copulate very little during focal animal observations. Excluding these two females from analyses resulted in pair copulation time being slightly less affected by female wet weight (ANOVA of linear regression: $F_{1,11} = 2.589$, $P = 0.136$). Among the remaining 14 pairs, in which all females weighed at least 5 g, RV was the only measurement that correlated with pair copulation time, supporting the finding that females spent significantly more time per day in copulation with males for which they showed a lower RV during odour trials (ANOVA of linear regression: $F_{1,11} = 8.028$, $P = 0.016$; Fig. 5).

Among 13 of these same pairs where the female was at least 5 g in wet weight and female receptivity could be observed, there was a significant negative relationship between the proportion of mount attempts by a male that females were receptive to, and the extent to which the same females previously responded to his odour during odour trials (logistic regression: $\chi^2_{11} = 6.384$, $P = 0.012$; Fig. 6). In this analysis, female receptivity to male mount attempts was not significantly correlated with the wet weight of the female (logistic regression: $\chi^2_{10} = 1.005$, $P = 0.316$). Male wet weight was also compared with both female receptivity and the RV shown to him by females to test if male size was a confounding variable. Male wet weight was found to be independent of both female receptivity (logistic regression: $\chi^2_{11} = 0.373$, $P = 0.541$) and RV (linear regression: $F_{1,11} = 0.008$, $P = 0.923$).

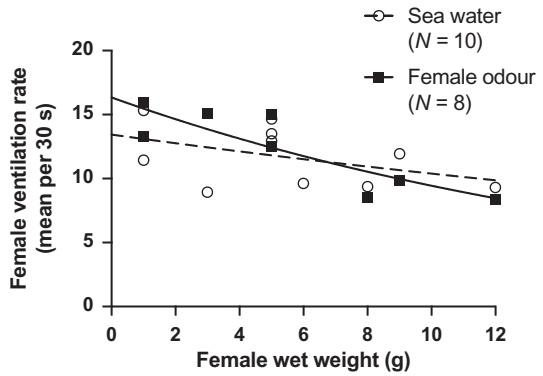

Figure 3. Change in ventilation rates of female *Hapalochlaena maculosa* with respect to wet weight, following treatments with seawater controls and female odours. There was a significant interaction between female wet weight and treatment type between these two treatments ($P = 0.029$). Female ventilation rates significantly decreased with receiver size during female-odour treatments (solid line shows LMEM fitted to square-root transformed ventilation rates: $y = (-0.093x + 3.696)^2$; $P = 0.006$). However, a female's ventilation rate was statistically independent of her size during seawater treatments (broken line shows LMEM fitted to square-root transformed ventilation rates: $y = (-0.036x + 3.522)^2$; $P = 0.317$).

DISCUSSION

Increased ventilation and heart rates in response to social stressors have been documented in a variety of animal taxa (Barreto & Volpato, 2006; von Borell *et al.*, 2007). It is hypothesized that these behavioural mechanisms increase oxygenation of the blood, thus

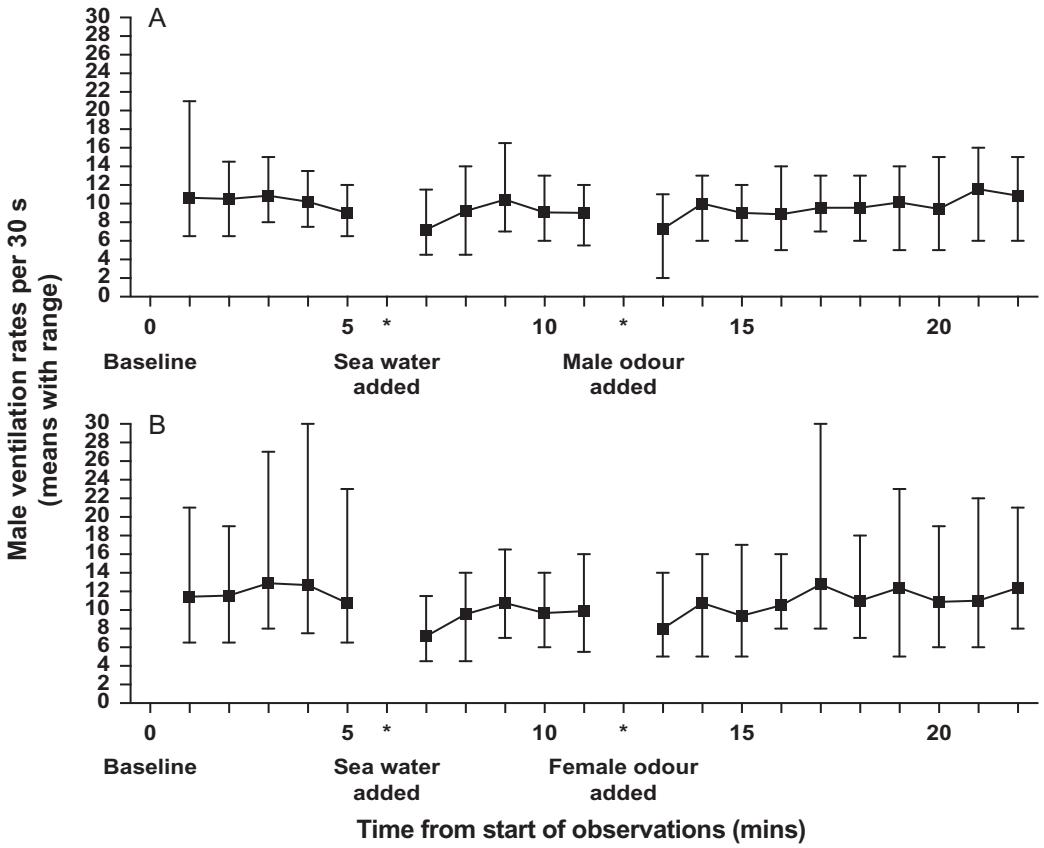

Figure 4. Mean ventilation rates per 30 s interval of male *Hapalochlaena maculosa* for each minute of observation. **A.** Male odour trials ($N = 7$ animals, 7 trials). **B.** Female odour trials ($N = 8$ animals, 8 trials). Asterisks indicate time of additions.

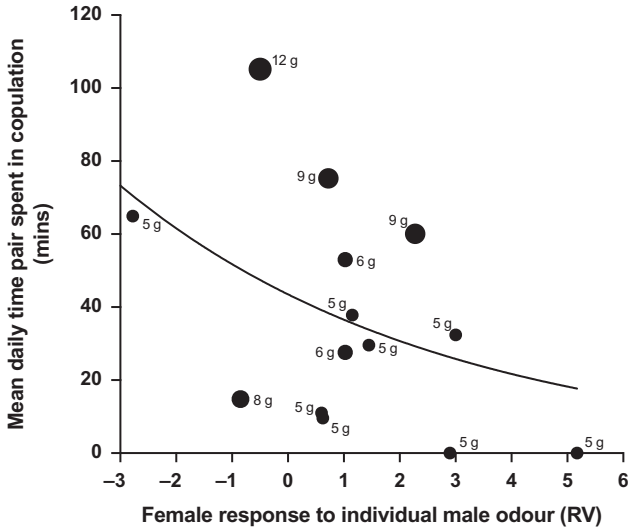

Figure 5. Mean time that pairs of males and females (females ≥5 g wet weight) spent in copulation given response of the female to odour of corresponding male tested 1 week previously. Response value was calculated as mean female ventilation rate (per 30 s) after exposure to the male's odour minus mean ventilation rate of female during preceding baseline trial. Female size is represented by proportionately sized circles labelled according to wet weight (effect of female size not significant; see text). Solid line shows linear regression fitted to log (x + 1) transformed data: $y = e^{((-0.395x + 1.977)-1)}$; $P = 0.016$ ($N = 14$ pairs).

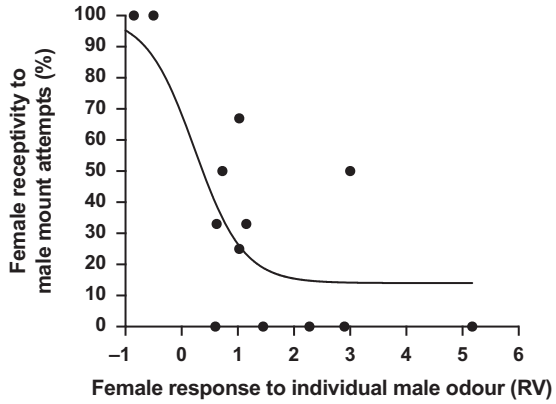

Figure 6. Female receptivity to males (measured as proportion of mount attempts by a male to which the female was receptive) as a function of female response to the same male's odour. Response value was calculated as mean female ventilation rate (per 30 s) after exposure to male's odour minus mean ventilation rate of female during preceding baseline trial. Females were significantly more likely to try to reject copulation attempts from males to whom they had previously reacted strongly during odour-cue trials. Solid line shows logistic regression: $y = 1 / (1+e^{-(-0.755x - 1.33)})$; $P = 0.012$ ($N = 13$ pairs).

aiding in a 'fight or flight' response (Barreto *et al.*, 2003). In cephalopods, increased ventilation would also aid water movement over olfactory cells, enhancing detection of odour cues in the water (Woodhams & Messenger, 1974). Thus, the *Hapalochlaena maculosa* in this study that increased their ventilation rate after exposure to conspecific odours, might have done so as an alarm response in the same manner that *Sepia officinalis* increase ventilation rate when presented with odour from a potential predator (Boal & Golden, 1999).

Additionally, the large reduction of ventilation, observed with nearly all receivers in the first 2 min immediately following the addition of sea water or odours (Figs 1, 4) is consistent with field observations of *Abdopus aculeatus* (Huffard, 2007). This species has been documented to use 'freezing' behaviour to avoid potential predators (Huffard, 2007). It seems likely that many receivers in the present study might have frozen, in a similar manner to *A. aculeatus*, after sudden movement in the water as a defensive response to reduce visual stimulus and water movement in order to minimize detection by a predator or agonistic conspecific. Therefore, the present observations of both increased ventilation rates and freezing behaviour are consistent with defensive behaviours previously recorded in cephalopods (Boal & Golden, 1999; Huffard, 2007). This being the case, it is likely that changes to ventilation rates observed here were alarm responses, and their interpretation as evidence for conspecific recognition should be regarded with this in mind.

Despite the above limitation and the high variability in individual receiver responses, the data presented here show some evidence that female *H. maculosa* are capable of detecting the odours of male conspecifics. Female ventilation rates were significantly faster after exposure to male-conspecific odour than during baseline, and showed a (nonsignificant) trend to be greater than their ventilation rates during seawater trials. Additionally, female receivers showed a progressive increase in their ventilation rate over the 10-min observation period following the introduction of a male's odour, whereas this pattern was not shown in response to other treatments.

The female receivers showed no clear response to odours from other females, as also found in a study of *Octopus bimaculoides* (Walderon *et al.*, 2011). However, female response to female odours did decline significantly with the size of the receiving female. This finding is consistent with the interpretation that higher ventilation rates represent an alarm response, because smaller females might be at greater risk of cannibalism or aggression from conspecific females. Paradoxically, female responses to male and female odours were not statistically different, a pattern consistent with the high levels of unexplained variance within the female-odour trials.

Although ventilation rates of male *H. maculosa* were statistically similar among all treatments, male receivers did show a significant pattern of progressively increasing their ventilation rates over time, after exposure to conspecific odour of either males or females. This suggests that the males might be capable of detecting conspecific odours, but there was no evidence of their ability to discriminate the sex of conspecifics. Regrettably, the sample size for males was low and male ventilation rates were highly variable in all treatments. Therefore, the capacity of male *H. maculosa* to detect conspecifics via chemical signals remains unresolved.

It is possible that males of *H. maculosa* do not use odour cues in social recognition. This finding would be consistent with previous observations that males of this species approach both sexes equally (Morse *et al.*, 2015) and with observations that males of both *H. maculosa* and *H. lunulata* frequently attempt copulations with other males (Cheng & Caldwell, 2000; Morse *et al.*, 2015). However, *H. maculosa* has a limited breeding season (Tranter & Augustine, 1973), is nocturnal and male–male mounts are both time-wasting and can lead to aggressive interactions (Morse *et al.*, 2015). Therefore, it remains a mystery why males do not appear to use odour cues either to locate potential mates or to avoid same-sex mounts, especially given that females in this study seemed to detect conspecific odours.

Female 'masking' of sex-specific chemical cues has previously been documented in an abundant marine snail (Johannesson *et al.*, 2010). This behaviour has been hypothesized to benefit females by enabling them to reduce the predation risks associated with excessive copulations (Johannesson *et al.*, 2010). It is possible that females of the genus *Hapalochlaena* might employ a similar strategy of masking their scent from conspecific males in order to avoid unwanted copulations, but this possibility remains to be investigated. We

have no evidence of a mechanism by which copulations might reduce female fitness. Additionally, several observations of octopod mating behaviour report a male tactile phase prior to copulation (Wells & Wells, 1972; Voight, 1991; Morse, 2008). It is possible that males rely on tactile chemoreception for the recognition of conspecifics or their sex. This aspect also remains to be investigated further, but might explain why male–male mounts are typically shorter than male–female mounts in *Hapalochlaena* (Cheng & Caldwell, 2000; Morse *et al.*, 2015).

Interestingly, female response to individual male odours was negatively correlated with both female receptivity and the average time that females spent in copulation with the same males. Females were thus more receptive to copulate, and copulated for longer, with males to whose odour they had previously displayed a lower response. Female rejection of male copulation attempts frequently leads to grappling and often to forced copulations in this species (Morse *et al.*, 2015). Therefore, although sample sizes were small and the evidence limited, these correlations at least suggest that the response to conspecific odours could be linked to defensive or agonistic behaviour, or some other form of stress.

It is also possible that the patterns observed here reflect an ontogenetic shift in the sexual behaviour of females. Smaller females (<5 g) showed the greatest magnitude of response to male odours, as well as being less receptive to copulation attempts. This is consistent with previous research on *H. maculosa*, which revealed that females less than 5 g almost always tried to reject male copulation attempts, while males as small as 1 g (but at least 20 mm ML) made frequent attempts to mount conspecifics (Morse *et al.*, 2015). The observed shift in female response to male odours at around 5 g might coincide with the size of most females when they reach sexual maturity. However, it is noteworthy that female response still correlated with mate choice patterns even when females <5 g were omitted from analyses.

The results reported here are in agreement with the work of Boal (1997), who found that female *S. officinalis* consistently spent more time with males that had recently mated with a different female, even though they could not have visually assessed the mating history of the male. If female use of odour cues in *S. officinalis* operates in a similar manner to that in *H. maculosa*, then it is possible that the females were not reacting to the male's recent mating history, but were rather choosing to mate with the males with more attractive chemical cues. This pattern could theoretically evolve through a 'Fisherian' mechanism (Kirkpatrick, 1982); females would benefit from mating with males that emit odours less likely to result in female agonistic behaviour and by also having sons that have similar odours to their fathers. This would increase the reproductive success of the mother (Kirkpatrick, 1982). However, the chemical signal to which females might be responding remains unknown.

Evidence for the detection of conspecifics and the discrimination of their sex via odour cues in *H. maculosa* remains weak. The behaviour of octopods in general can be unpredictable (Mather & Anderson, 1993), so it is often a challenge to explain the high variance in their observed behaviours. Nevertheless, the data presented here show some significant correlations between odour response and defensive or agonistic behaviour. Although further studies with larger samples and greater statistical power are needed to verify these patterns, our results add to the growing evidence that chemosensory systems play a role in cephalopod cognition and social recognition (e.g. Boal, 1996, 1997; Boal & Golden, 1999; Walderon *et al.*, 2011).

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Molluscan Studies* online.

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