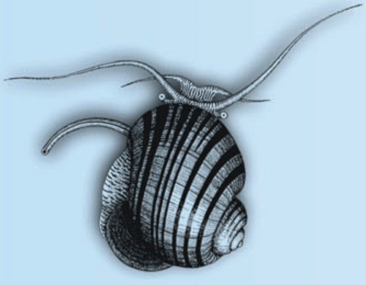

Journal of
Molluscan Studies

The Malacological Society of London

Journal of Molluscan Studies (2015) **81**: 96–103. doi:10.1093/mollus/eyu060
Advance Access publication date: 8 September 2014

The smell of sex: water-borne and air-borne sex pheromones in the intertidal gastropod *Littorina littorea*

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(Received 29 January 2014; accepted 16 June 2014)

ABSTRACT

We investigated whether individuals of the intertidal snail *Littorina littorea* may be attracted by water-borne and air-borne pheromones from conspecifics. The potential relative role of water-borne and air-borne pheromone in the behavioural and reproductive biology of *L. littorea* was assessed through chemotactic assays. These involved a series of choice experiments with snails, snail smell and snail mucus as stimuli in a T-maze under conditions of immersion and emersion, and during the nonmating and mating seasons. During the nonmating season, *L. littorea* did not choose the snail-conditioned side of the T-maze significantly more frequently than the control side under conditions of both immersion and emersion. During the mating season, under condition of immersion both males and females chose the side of the T-maze conditioned with individual of the opposite sex significantly more frequently than the control side, and did not show any preference for the side conditioned with individuals of the same sex. Under conditions of emersion, only males were significantly more attracted by cues from the opposite sex than by the control. These results indicate that *L. littorea* are not attracted by conspecifics during the nonmating season; the cues of conspecifics are then unlikely to play a role in the orientation or the aggregation behaviour of this species. The results also suggest that males and females are attracted by the odour of conspecifics of the opposite sex, which contains both water-borne and air-borne pheromones in females and only water-borne pheromones in males. Also the similar behaviours observed with snails, water-borne and air-borne snail smell, and snail mucus, as stimuli indicate that male and female sex pheromones are both present in mucus. Temperature manipulations conducted during both seasons did not affect any of the observed behavioural changes, hence suggesting that the chemoreceptive abilities of *L. littorea* may be driven by exogenous and/or endogenous factors that are decoupled from direct control by temperature. This is in sharp contrast to the locomotor activity of *L. littorea*, which consistently increased with temperature. We also report the possibility of mate-tracking in females, a behaviour that has yet to be reported in gastropods, since males are typically believed to be the active sex that searches for females. Our results build on previous evidence of the function played by mucus-trail following in the reproductive success of intertidal gastropods, through the ability of *L. littorea* to remotely detect the water-borne and air-borne sex-specific cues that are incorporated in their mucus.

INTRODUCTION

The role of water-borne chemicals in triggering behavioural responses to, e.g. the presence of prey, predators, injured conspecifics and potential sexual partners, and the related implications in orientation and navigation strategies, has received a considerable amount of attention in organisms ranging from bacteria to fish (Webster & Weissburg, 2009; Paul, Ritson-Williams & Sharp, 2011; Weissburg, 2011; Stocker, 2012). Water-borne pheromone-mediated chemotaxis is a known mechanism of aggregation, mate location and choice, and communication in a range of aquatic gastropods (see Chase, 2002, for a review). Specifically, mate location is mediated by water-borne chemicals in many aquatic gastropods, such as the marine caenogastropods *Mitra idea* (Cate, 1968) and *Ilyanassa obsoleta* (Moomjian, Nystrom & Rittschof, 2003), the marine opisthobranchs *Aplysia californica* and *A. brasiliana* (Audesirk, 1977; Painter *et al.*, 1998; Cummins *et al.*, 2009), and the freshwater caenogastropod *Pomacea canaliculata* (Takeichi, Hirai & Yusa, 2007).

It has also frequently been suggested that, beyond the potential saving in mucus production related to trail-following behaviour (for instance, *Littorina littorea* following fresh conspecific mucus trails produced only 27% of the volume of mucus produced by the trail layer, and hence expended less energy; Davies & Blackwell, 2007), the incorporation of species-specific and sex-specific cues in gastropod trails is advantageous in facilitation of mate-searching in both hermaphroditic and dioecious gastropods, and may play a crucial role in the reproductive success of at least some species (Ng *et al*., 2013). Trail-following has indeed been reported in many freshwater and marine gastropods (Edwards, 1968; Wells & Buckley, 1972; Townsend, 1974; Trott & Dimock, 1978; Cook, 1992; Nakashima, 1995), including a range of littorinids such as the mangrove-dwelling *Littoraria* (Chapperon & Seuront, 2011; Ng *et al*., 2011) and rocky-shore *Littorina* (Struhsaker, 1966; Dinter, 1974; Erlandsson & Kostylev, 1995; Davies & Beckwith, 1999; Edwards & Davies, 2002; Davies & Blackwell, 2007; Johannesson *et al*., 2008, 2010). In addition, recent empirical evidence that males of the mangrove snails *Littoraria ardouiniana* and *L. melanostoma* prefer to follow conspecific females during the mating season, but not at other times (Ng *et al*., 2011), suggests that trail-borne chemicals may not be produced continuously.

Mucus-trail following has recently been acknowledged as an alternative or a complementary mate-searching strategy to water-borne chemicals, because trail-borne cues might be more effective than water-borne cues in locating mates in high-shore environments that are infrequently submerged by seawater (Ng *et al*., 2013). Very limited information is, however, available on the potential role of both water-borne and air-borne chemicals in triggering chemotaxis towards conspecifics and/or conspecific trails, even in the well-studied genus *Littorina*. Note that while *L. littorea* is more active during immersion (Newell, 1958; Lubchenco, 1978), it is also potentially active during emersion when the rocks are moist (e.g. Little, 1989). It may hence also be influenced by air-borne cues from either conspecifics and/or their mucus trails, as observed in a range of terrestrial gastropods such as *Limax pseudoflavus* (Cook, 1980, 1992), *Achatina fulica* (Chase *et al*., 1978), *Anguispira alternata* (Atkinson, 2003) and *Helix aspersa* (Farkas & Shorey, 1976). Air-borne chemotaxis has, however, not yet been demonstrated in intertidal gastropods, and one aim of this study was to discover whether air-borne attractants may also be used by marine gastropods.

In this context, we assessed, during both the nonmating and mating seasons, whether *L. littorea* individuals are attracted by conspecifics, by pheromones released from conspecifics, and by the mucus of conspecifics. To determine the potential relative roles of water-borne and air-borne pheromones in the behavioural and reproductive biology of *L. littorea*, we conducted chemotactic assays under conditions of immersion and emersion, and during the nonmating and mating seasons.

MATERIAL AND METHODS

Snail collection and acclimation

Littorina littorea individuals were collected during the nonmating season (August-September 2012) and the mating season (April-May 2012) from Fort de Croy (50°45′48″N, 1°35′58″ E), an intertidal reef typical of the rocky habitats found along the French coasts of the eastern English Channel (Chapperon & Seuront, 2009). All individuals were sampled from mid-intertidal rock pools, and were in the range 12–16 mm in shell length. None of the 50 individuals haphazardly sampled from our sampling location, both during the nonmating and mating seasons, exhibited either patent or nonpatent parasitic infections, as no swimming cercariae were found in 100-ml seawater jars that held individual snails overnight and no parasites were microscopically observed in dissected visceral mass tissue.

During the nonmating season, *L. littorea* snails were not sexed because the penis is usually shed (Grahame, 1969; Fretter & Graham, 1980); hence we assumed that males and females were represented in roughly equal numbers (Saur, 1990; Erlandsson & Kostylev, 1995). During the mating season, the snails were sexed by letting them crawl on a glass sheet and then observed from below to determine whether or not a penis was present.

Littorina littorea snails were acclimatized in the laboratory in transparent PVC cylinders (50 cm tall and 20 cm in inner diameter, riddled with holes 5 mm in diameter) held in 120-l (90 × 50 × 30 cm) tanks of running natural seawater, aerated at temperatures representative of *in situ* conditions, i.e. 10 °C during the mating season and 18 °C during the nonmating season. These perforated ‘acclimation towers’ allow both seawater to be continuously renewed and captive snails to move freely in and out of the water without being able to escape. Behavioural experiments were run at water and air temperatures representative of *in situ* conditions during the nonmating season (10 °C) and the mating season (18 °C) to assess seasonal differences in behavioural responses. However, temperature is a confounding factor as many snails, including *L. littorea*, move significantly faster at higher temperature (e.g. Erlandsson & Kostylev, 1995; Ng *et al*., 2011). Therefore we further assessed the potential effect of temperature on the behavioural responses of *L. littorea* to chemical cues through behavioural experiments conducted at 18 °C during the nonmating season, and at 10 °C during the mating season. These experiments were specifically designed to disentangle the potential seasonal nature of the behavioural and sensory abilities of *L. littorea* from their temperature-related components.

During both the nonmating and mating seasons, test and stimulus snails were kept separately in distinct acclimation towers held in different aquariums, and both were acclimatized for 24 h before the experiments took place. During the mating season, males and females were also acclimatized in separate acclimation towers held in distinct aquariums. Before each experiment, in order to destroy potential chemical cues related to, e.g. biofilm formation, the shells of both test and stimulus snails were carefully wiped with a sterile cotton bud soaked with 10% HCl. Special care was taken not to cover the operculum with acid. The shell was then thoroughly rinsed in control seawater, the snail immersed twice in control seawater for 1 min, and left for 5 min in an aquarium filled with fresh control seawater before the beginning of the behavioural assay. No sign of behavioural impairments (e.g. prolonged withdrawal into the shell, loss of motility, lack of tentacle motility) was noticed in any of the snails, which immediately recovered their normal behaviour.

Experimental device

We ran choice experiments in a T-maze (Fig. 1) made of transparent Perspex tubes (3 mm thick and 40 mm inner diameter). Each arm of the T-maze was 150 mm long, and connected to 50 mm long removable chambers that were extended by PVC wells (inner diameter 30 mm) designed to introduce stimuli and test individuals in the maze. Two ‘stimulus chambers’ were connected to the left and right arms and a ‘test chamber’ to the end of the bottom chamber. The stimulus chambers were separated from the T-maze by a silk net of 500-μm mesh.

Experimental procedure

The T-maze was lit from above (75-W diffuse cold light) to ensure a uniform dim illumination of about 500 lux. Before each trial, the maze was rinsed with 95% (*v*/*v*) acetone, rinsed with distilled water twice and then with filtered (Whatman GF/C glass-fibre filters, porosity 0.45 μm) and autoclaved natural

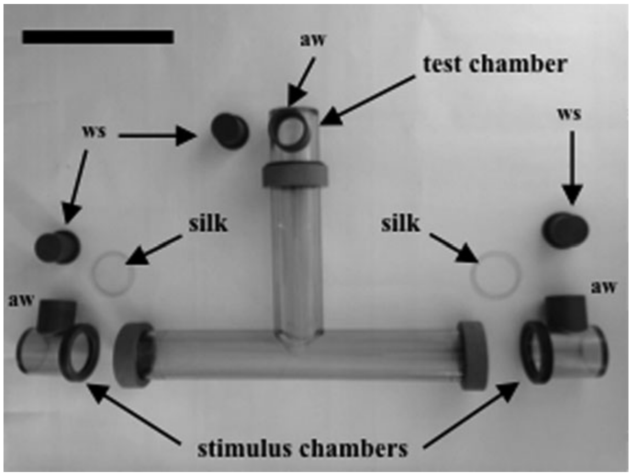

Figure 1. T-maze used in behavioural assays, shown disassembled. Abbreviations: aw, access well; ws, well stopper. Scale bar = 150 mm.

seawater (referred to hereafter as control seawater). The maze was then either filled or sprayed with a mist of control seawater, for the experiments conducted under conditions of immersion and emersion to infer the responses of *L. littorea* to water-borne and air-borne cues, respectively. A stimulus and a control were introduced haphazardly into each of the stimulus chambers (see below for details on each experimental condition), and left for 15 min before the introduction of a test individual at the bottom of the test chamber.

Each experimental condition was replicated 20 times and each snail was only used once, whether as a test snail or as a stimulus. Each trial lasted for 30 min and we subsequently recorded (1) the number of motile and nonmotile snails, (2) the side of the T-maze chosen by the snail (we considered that a snail chose one arm of the maze, when its tentacles touched the mesh of the stimulus chamber or control chamber within 30 min) and (3) the time T_s and T_c taken to reach respectively the mesh of the stimulus and the control chambers. After each trial, the maze was emptied, wiped dry to dislodge mucus present, cleaned with 95% (*v*/*v*) acetone, rinsed in distilled water twice and wiped dry, to remove any chemical scents. This cleaning procedure was validated through control experiments that consisted of letting three *L. littorea* crawl for 15 min in a haphazardly chosen arm of the T-maze, cleaning the T-maze as described above, and assessing the preference of fresh snails introduced at the bottom of the test chamber for the left or right arm; no significant difference was found in the number of *L. littorea* choosing the right and left sides of the T-maze (binomial test, $n = 25$, $P > 0.05$).

During the mating season, we conducted experiments to determine the responses of males and females to the presence of snails of both the same sex and the opposite sex, hence we consistently considered four test snail–stimulus combinations, i.e. male snail–male stimulus, male snail–female stimulus, female snail–male stimulus, and female snail–female stimulus, for each of the three sets of experiments described hereafter. These experiments were conducted in the T-maze either filled or sprayed with control seawater to infer the responses of *L. littorea* to water-borne and air-borne cues, respectively. Note that the T-maze was placed on a flat surface hence, when sprayed, no water motion was ever observed at the bottom of the T-maze, and no directional bias could have affected the behaviour of the test snails.

Control experiments

Control experiments were run during the mating and nonmating seasons and under conditions of immersion and emersion to ensure that the choice of snails of either the left or right arm of the T-maze was random, and that no accidental cues such as light, vibration or magnetic cues would affect the outcome of our experiments. Specifically, 20 control snails (i.e. unsexed individuals during the nonmating season, and either males or females during the mating season; Table 1) were introduced at the bottom of the test chamber, with no cues present in the stimulus chamber, and left undisturbed for 30 min. At the end of each control trial, we recorded the number of motile and nonmotile snails, the side of the T-maze chosen by the snail, and the time $T_{C_{\mathrm{Left}}}$ and $T_{C_{\mathrm{Right}}}$ needed to reach respectively the mesh of the left and right stimulus chambers.

Experiment 1: response to the presence of conspecifics

Three *L. littorina* (i.e. unsexed individuals during the nonmating season, and either three males or three females during the mating season) were placed inside one haphazardly chosen stimulus chamber, and three shells of similar length free of organic matter (held for 48 h at 75 °C, immersed for 1 min in 10% HCl acid after removing the operculum to destroy the organic matter remaining inside and outside the shell, rinsed with deionised water and dried) were considered as a control in the other stimulus chamber. Note that the potential adverse effect induced by the smell of empty shells on the behaviour of test snails is very unlikely to have biased our results as no significant difference was ever found in the number of *L. littorea* choosing the right and left sides of the T-maze in control choice experiments run with three shells free of organic matter in one haphazardly chosen stimulus chamber and nothing in the other stimulus chamber (Binomial test, $P > 0.05$).

Experiment 2: response to snail-conditioned substrate

During the nonmating season, snail-conditioned seawater was prepared for each replicate by keeping three *L. littorea* in 100 ml of control seawater for 1 h. During the mating season, male-conditioned and female-conditioned seawater solutions were prepared by keeping respectively three males and three females in 100 ml of control seawater for 1 h. We then immersed two spherical pieces of sterile cotton wool as a cue-carrying substrate (40 mm in diameter) for 10 min in conditioned seawater or control seawater, and subsequently haphazardly introduced them in the stimulus chambers of the T-maze, previously filled or sprayed with control seawater.

Experiment 3: response to snail mucus

To condition one of the stimulus chambers with mucus, we let three *L. littorea* (i.e. unsexed individuals during the nonmating season, and either males or females during the mating season) crawl freely in one stimulus chamber for 1 h, and subsequently removed them before haphazardly allocating it to the left or right arm of the T-maze, which was subsequently either filled or sprayed with control seawater.

Statistical analysis

Due to the relatively low number of *L. littorea* individuals used in each experiment (i.e. 15 to 20; Table 1), nonparametric statistics were used throughout. Specifically, the frequency of choosing both the right or left side of the T-maze in control experiments and the control or stimulus side in each of the three experiments was assessed using the binomial test (Zar, 1999). The experimentwise error related to multiple testing was not corrected following the so-called Bonferroni correction for k independent tests, because this correction is conservative and results in tests with low power (see, e.g. Legendre & Legendre, 2012, for

Table 1. Behavioural response of *Littorina littorea* to water-borne and air-borne stimuli, under conditions of immersion and emersion, respectively, during the nonmating and mating seasons.

Season	Condition	Test	Stimulus	N_+	N_-	N	n	T (min)	Binomial test
Nonmating (18 °C)	Immersion	Snail	Control	10	7	17	3	16.1–20.2	ns
			Snail	10	8	18	2	17.1–21.6	ns
			Snail smell	11	9	19	1	18.2–22.3	ns
			Snail mucus	10	7	17	3	16.3–19.9	ns
	Emersion	Snail	Control	8	8	16	4	17.2–20.8	ns
			Snail	10	9	19	1	17.8–22.1	ns
			Snail smell	10	6	16	4	16.7–22.7	ns
			Snail mucus	9	7	16	3	15.9–23.0	ns
Nonmating (10 °C)	Immersion	Snail	Control	8	7	15	5	19.1–23.8	ns
			Snail	6	8	14	6	20.2–27.2	ns
			Snail smell	8	6	14	6	21.3–28.1	ns
			Snail mucus	6	6	12	8	19.8–24.2	ns
	Emersion	Snail	Control	7	8	15	5	22.2–28.4	ns
			Snail	7	6	13	7	22.9–27.9	ns
			Snail smell	5	8	13	7	23.1–29.6	ns
			Snail mucus	6	6	12	8	20.5–25.6	ns
Mating (10 °C)	Immersion	Male	Control	10	9	19	1	22.4–28.5	ns
			Male	11	8	19	1	21.5–26.5	ns
			Male-conditioned water	9	10	19	1	22.0–27.2	ns
			Male mucus	8	11	19	1	23.1–27.9	ns
			Female	15	3	18	2	22.2–27.5	$P < 0.01$
			Female-conditioned water	16	2	18	2	21.2–26.7	$P < 0.01$
			Female mucus	17	2	19	1	20.7–27.2	$P < 0.01$
		Female	Control	10	8	18	2	23.7–29.1	ns
			Male	15	4	19	1	20.5–24.0	$P < 0.05$
			Male-conditioned water	16	3	19	1	21.7–24.5	$P < 0.05$
			Male mucus	14	4	18	2	22.2–26.9	$P < 0.05$
			Female	10	9	19	1	21.1–27.3	ns
			Female-conditioned water	8	11	19	1	20.9–24.9	ns
			Female mucus	8	11	19	1	21.6–25.8	ns
	Emersion	Male	Control	9	10	19	1	22.1–27.4	ns
			Male	11	9	20	0	21.5–26.9	ns
			Male smell	10	8	18	2	20.9–27.2	ns
			Male mucus	12	7	19	1	21.7–28.0	ns
			Female	14	4	18	2	20.4–27.6	$P < 0.05$
			Female smell	14	4	18	2	21.4–27.4	$P < 0.05$
			Female mucus	14	5	19	1	20.5–27.1	$P < 0.05$
		Female	Control	9	10	19	1	23.5–29.5	ns
			Male	9	9	18	2	22.7–28.6	ns
			Male smell	10	8	18	2	22.1–28.4	ns
			Male mucus	12	7	19	1	23.1–27.9	ns
			Female	8	11	19	1	23.2–27.5	ns
			Female smell	6	12	18	2	22.2–28.1	ns
			Female mucus	11	8	19	1	23.1–27.7	ns
Mating (18 °C)	Immersion	Male	Control	12	8	20	0	15.1–21.4	ns
			Male	13	7	20	0	15.4–22.2	ns
			Male-conditioned water	13	7	20	0	16.1–23.6	ns
			Male mucus	11	9	20	0	15.0–21.8	ns
			Female	18	2	20	0	14.9–22.8	$P < 0.001$
			Female-conditioned water	17	2	19	1	15.5–21.2	$P < 0.005$
			Female mucus	16	3	19	1	14.4–22.1	$P < 0.005$
		Female	Control	11	8	19	1	17.3–21.5	ns
			Male	16	4	20	0	15.3–22.3	$P < 0.01$
			Male-conditioned water	15	5	20	0	15.5–21.1	$P < 0.05$
			Male mucus	14	6	20	0	16.2–22.2	$P < 0.05$
			Female	9	11	20	0	15.5–21.4	ns

Continued

Table 1. *Continued*

Season	Condition	Test	Stimulus	N_+	N_-	N	n	T (min)	Binomial test
			Female-conditioned water	12	8	20	0	15.8–22.4	ns
			Female mucus	11	8	19	1	16.2–21.9	ns
	Emersion	Male	Control	8	7	15	2	16.7–21.2	ns
			Male	9	10	19	1	16.1–22.7	ns
			Male smell	11	8	19	1	16.9–21.3	ns
			Male mucus	12	8	20	0	17.0–22.5	ns
			Female	16	4	20	0	16.5–23.1	$P < 0.01$
			Female smell	14	5	19	1	16.8–21.2	$P < 0.05$
			Female mucus	14	5	19	1	17.6–22.5	$P < 0.05$
		Female	Control	7	9	16	1	17.7–21.1	ns
			Male	10	10	20	0	16.9–23.2	ns
			Male smell	9	11	19	1	17.2–22.8	ns
			Male mucus	8	12	20	0	18.1–23.2	ns
			Female	11	9	20	0	16.9–22.2	ns
			Female smell	7	13	20	0	17.2–21.8	ns
			Female mucus	8	12	20	0	15.9–20.2	ns

N_+ and N_- are respectively the numbers of *L. littorea* that showed positive and negative responses to the stimulus. N is the number of individuals that reached the edge of the stimulus chamber of either the left or right arm of the T-maze within 30 min, while n is the number of individuals that showed signs of narcosis (lack of motility). T is the time taken to reach the edge of the stimulus or control chamber.

rationale). The relevance of Bonferroni correction in ecological and behavioural sciences has hence been heavily criticised from mathematical, logical and practical standpoints (Moran, 2003; Nakagawa, 2004). Instead, we used the more powerful correction proposed by Hochberg (1988), which has the desired overall error rate for tests that do not share part of their data, and has the further characteristic that no adjusted P-value can be larger than the largest unadjusted P-value or exceed 1. For each experiment, comparisons of the size of *L. littorea* and the times T_c and T_s between control and experimental treatments were conducted using the Wilcoxon-Mann-Whitney U-test. Multiple comparisons between experimental treatments were conducted using the Kruskal-Wallis (KW) test (Zar, 1999).

RESULTS

Control experiments

In all the control experiments conducted during the nonmating and mating seasons, no significant difference was found in the number of *Littorina littorea* choosing the right and left sides of the T-maze (binomial tests, $P > 0.05$; Table 1). There was no significant difference in the shell length of individuals choosing the right or left side of the T-maze ($P > 0.05$), and no significant difference in the time $T_{C_{Left}}$ and $T_{C_{Right}}$ needed to reach respectively the mesh of the left and right control chambers ($P > 0.05$). No significant difference was found between the shell length of active and inactive snails ($P > 0.05$). Significant differences were found, however, in the time needed to reach the mesh of the control chamber, T_c, between temperature treatments during both the nonmating and the mating seasons ($P < 0.05$). Specifically, the time T_c was consistently significantly greater at 10 °C than at 18 °C during both seasons (Table 1).

Nonmating season

In all three experiments, no significant difference was found in the shell length of *L. littorea* choosing the stimulus or control sides of the T-maze (U-test, $P > 0.05$). Similarly, no significant difference was found in the times T_s and T_c taken to reach the mesh of the stimulus and control chambers, respectively, in all experiments ($P > 0.05$). The time T_s was, however, significantly shorter at 18 °C than at 10 °C ($P > 0.05$; Table 1), and the proportion of inactive snails significantly decreased with temperature ($P > 0.05$; Table 1). Specifically, *L. littorea* never chose the stimulus arm more frequently than the control arm under conditions of both immersion and emersion; this applied whether the stimulus chamber contained conspecific snails, snail-conditioned seawater, or snail mucus, regardless of the experimental temperature (Table 1).

Mating season

In all three experiments, there was no significant difference in the shell length of *L. littorea* choosing the stimulus or control sides of the T-maze (U-test, $P > 0.05$). The time T_s taken to reach the mesh of the stimulus chamber was not significantly different from the time T_c taken to reach the mesh of the control chambers under conditions of both immersion and emersion (U-test, $P > 0.05$). No significant difference was found in the time T_s and T_c between treatments (KW test, $P > 0.05$). The time T_s was significantly smaller at 18 °C than at 10 °C ($P < 0.05$) and did not significantly differ from the time T_s observed during the nonmating season at both temperatures ($P > 0.05$). In same-sex experiments, *L. littorea* never chose the arm holding the stimulus chamber that contained snails, snail-conditioned seawater, or mucus more often than the arm holding the control stimulus chamber under conditions of immersion or emersion (Table 1).

In experiments involving *L. littorea* of different sexes, males chose significantly more often the arm conditioned by female cues (i.e. actual females, female-conditioned water or female mucus) than the control arm of the T-maze ($P < 0.05$), under conditions of both immersion and emersion (Table 1). Females chose the arm of the T-maze conditioned with actual males, male-conditioned water or male mucus significantly more frequently than unconditioned seawater under condition of immersion ($P < 0.05$). In contrast, under conditions of emersion, females did not choose water conditioned with male cues more frequently than control water (Table 1).

DISCUSSION

During the nonmating season, individuals of *Littorina littorea* were not attracted by any of the water-borne or air-borne cues investigated here, in sharp contrast to observations conducted during the mating season (Table 1). This result may imply that no sex pheromones are produced during the nonmating season, as suggested by the behaviour of the males of the mangrove littorinids *Littoraria ardouiniana* and *L. melanostoma*, which prefer to follow the mucus trail of conspecific females during the mating season, but not at other times (Ng *et al.*, 2011). This is supported by the lack of responsiveness of *L. littorea* to any cues at both 10 °C and 18 °C during the nonmating season. This observation also suggests that the chemoreceptive abilities of *L. littorea* may be driven by exogenous and/or endogenous factors that are decoupled from temperature. This contrasts with the locomotor activity of *L. littorea*, which consistently moves faster at 18 °C than at 10 °C during both seasons (Table 1). Previous reports of trail-following behaviour in *L. littorea* during the nonmating season (Erlandsson & Kostylev, 1995) and the lack of responsiveness to conspecific water-borne and air-borne cues observed here (Table 1) may alternatively indicate that pheromones are mucus-bound, hence insoluble and nonvolatile, and are only detected by direct contact of the tentacles with the mucus trail, as observed in a range of littorinids (Stirling & Hamilton, 1986; Ng *et al.*, 2011), including *L. littorea* (Erlandsson & Kostylev, 1995). This hypothesis is consistent with observations conducted on the trail-following behaviour of *Littoraria ardouiniana* and *L. melanostoma* (Ng *et al.*, 2011), and *L. littorea* (L. Seuront, unpubl.). Specifically, when a tracker snail encountered a deviation in the trail followed, the snail consistently had one tentacle touching the mucus trail while the other tentacle reached outside the trail; this behaviour has been suggested as a strategy for tracker snails to deal with the geometric complexity of mucus trails while trail following (Ng *et al.*, 2011) and an indication of how *L. littorea* sense mucus-bound pheromones.

During the mating season, *L. littorea* males were consistently attracted to female water-borne and air-borne cues (Table 1). In contrast, females were only attracted to male water-borne cues (Table 1). Hence, *L. littorina* males and females detect the odour of the opposite sex, which contains both water-borne and air-borne pheromones in females and only water-borne pheromones in males. In addition, *L. littorea* males and females were never attracted by the odour of conspecifics of the same sex under conditions of either immersion or emersion (Table 1). This implies that (1) the odour and the mucus of males and females both contain sex pheromones, (2) these pheromones are soluble and volatile in females, but only soluble in males, (3) these pheromones are sex-specific and (4) *L. littorea* can use both water-borne and air-borne cues to navigate. The ability to detect water-borne and air-borne cues is consistent with behavioural observations conducted on *L. littorea* on mucus-free surfaces, under conditions of both immersion and emersion, that showed snail tentacles flapping up and down between contact with the substrate at a frequency of about 10–20 and 25–35 flaps min^{-1} in the absence and presence of conspecific cues, respectively (L. Seuront, personal observation). This behaviour is comparable with the signal scanning behaviour observed in a range of aquatic and terrestrial arthropods—see e.g. reviews by Weissburg (2000), Ryan (2002), Koehl (2006) and Webster & Weissburg (2009)—and further supports the role of *L. littorea*'s tentacles in detecting water-borne and air-borne cues. Observations conducted during the mating season showed that artificially displaced *L. littorea* individuals use water-borne cues from both soft and hard substrates to find their way back to the original substrate where they have been shown to form large aggregates (Chapperon & Seuront, 2009). This result generalizes early work showing that a range of homing limpet species can navigate back to their original location without trail following (Stephenson, 1936; Beckett, 1968; Cook *et al.*, 1969; Thomas, 1973). Finally, the hypothesis of sex-specific sex pheromones in *L. littorea* is consistent with previous evidence for species-specific trail cues in the mud snail *Ilyanassa obsoleta* (Trott & Dimock, 1978) and the mangrove snails *Littoraria ardouiniana* and *L. melanostoma* (Ng *et al.*, 2011), and the ability of the rocky-shore snail, *Littorina saxatilis*, to discriminate between mucus trails laid by conspecific females of different ecotypes (Johannesson *et al.*, 2008).

Our results have implications for the trail-following behaviour reported in *L. littorea* (Davies & Beckwith, 1999; Edwards & Davies, 2002; Davies & Blackwell, 2007) during both the mating and nonmating seasons (Erlandsson & Kostylev, 1995), and more generally in a range of aquatic and terrestrial gastropod species (see Ng *et al.*, 2013, for a review). In particular, the hypothesis that sex pheromones may be present in *L. littorea* mucus as nonsoluble, and as soluble and volatile molecules, during the nonmating and mating seasons, respectively, is consistent with previous evidence for soluble and volatile low molecular weight substances in mucus that have been shown to trigger conspecific trail following in the land slugs *Mariaella dussumieri* (Ushadevi & Krishnamoorthy, 1980) and *Limax pseudoflavus* (Cook, 1994), the freshwater snail *Biomphalaria glabrata* (Bousfield *et al.*, 1981) and the abalone, *Haliotis asinina* (Kuanpradit *et al.*, 2012), and predatory trail following in the land snail *Euglandina rosea* (Clifford *et al.*, 2003). The effect of these substances appears to be species-specific, because they did not trigger trail following in *Ilyanassa obsoleta* (Dunn, 1982). Trail following has also been suggested to provide a simple mechanism for self-organization in groups of snails, hence promoting aggregation, and potentially relieving desiccation and predation pressures (Stafford, Davies & Williams, 2012). The lack of chemotropism towards cues from conspecifics of the same sex during both nonmating and mating seasons (Table 1) suggests, however, that water-borne and air-borne sex pheromones are essentially an adaptation to optimize reproductive success, and therefore an unlikely candidate mechanism to elicit the local formation of very large *L. littorea* aggregations, e.g. 3900 to 4500 m^{-2} observed in our sampling site during the mating season (Chapperon & Seuront, 2009).

These results generalize previous evidence of the function played by mucus-trail following in the reproductive success of intertidal gastropod, through the ability of *L. littorea* to detect remotely the water-borne and air-borne sex-specific cues that are incorporated in their mucus. As such, during the mating season, trail following may not be the primary means by which *L. littorea* locates and tracks its mates. Though an important component in mate-searching strategies, trail-following relies on mucus-bound pheromones to be detected by direct contact of the tentacles with the mucus trail (Chase & Croll, 1981; Stirling & Hamilton, 1986; Erlandsson & Kostylev, 1995; Ng *et al.*, 2011) and hence considerably limits the ability of a searcher to locate a potential mate remotely. The release of water-borne and air-borne pheromones and the related odour plumes may either be seen as an alternative mechanism of pheromone transmission, or an addition to trail following, which might contribute to enhance the probability of a male to locate a female. Note that the opposite, i.e. females searching for males, may also hold true as they exhibited positive chemotactic behaviour towards male water-borne cues (Table 1). To our knowledge the possibility of mate-tracking in females has yet to be reported in gastropods, in which males are typically believed to be the active sex that searches for females. However, this finding contradicts previous observations conducted during the mating season showing that (1) *Littorina planaxis* males actively search for females (Gibson, 1965), and (2) females of *L. littorea* and the mangrove snails *Littoraria ardouiniana* and *L. melanostoma* did not prefer to follow mucus trails laid by conspecific males (Erlandsson & Kostylev, 1995; Ng *et al.*, 2011). In addition, it appears from our results (Table 1) that the observed *P*-values were lower when males responded to female cues than when females responded to male cues, which may indicate either that females are less sensitive to the stimulus or that male cues are less concentrated than female cues. The resolution of this particular issue goes beyond the scope of the present work, and further work is clearly needed to disentangle the mechanisms behind the sensory and behavioural ecology of *L. littorea*. These mechanisms, in particular of females, may be more complex than previously thought.

The use of pheromone plumes by *L. littorea* males and females to increase the probability of encountering a mate is conceptually analogous to the strategies developed by other organisms, such as moths and other insects, relying on olfactory cues to locate females; sex pheromones from grounded females disperse downwind and males can track these plumes upwind to locate females through pheromone-mediated anemotaxis or chemotaxis (Murlis, Elkinton & Cardé, 1992; Mafra-Neto & Cardé, 1994; Visser, 1998). However, in the wave-swept intertidal the probability of a downstream searcher intersecting the odour plume of a conspecific is dependent upon the effects of mixing and the concentration of the signal. Specifically, slow flows prevent odour signals from propagating downstream, while the cross-stream expanse of plumes increases with the degree of mixing, so that slow flows produce narrower plumes that expand more slowly as they propagate downstream. The probability of a downstream searcher intersecting a plume may thus be diminished in less turbulent conditions, but the potentially positive increase in downstream and cross-stream propagation is also opposed by a negative effect due to odour dilution (Webster & Weissburg, 2009). Alternatively, in the case of extreme mixing events, such as wave breaking, that characterize rocky intertidal environments, a searcher is more likely to experience, and hence react to, a bulk-phase water pheromone concentration than a well-defined pheromone plume (Seuront & Stanley, 2014). As a consequence, the chance of success in locating a target may be controlled and increased by subtle behavioural changes that are triggered by the detection of the odour of a conspecific of the opposite sex, as recently shown for the copepods *Temora longicornis* and *Eurytemora affinis* (Seuront & Stanley, 2014). This hypothesis is supported by empirical evidence that artificially displaced *L. littorea* have the ability to navigate back to their original substrate in a wave-swept and tidally mixed environment (Chapperon & Seuront, 2009). Further work is needed to disentangle the potential adaptive and evolutionary advantages of the distinct mechanisms of pheromone transmission discussed here in terms of mating encounter rates and fitness.

ACKNOWLEDGEMENTS

This research was supported by the Australian Research Council's Discovery Projects funding scheme (Projects DP0664681 and DP0988554 to L. Seuront), Flinders University, the South Australian Government through the Marine Innovation South Australia Initiative, and the Centre National de la Recherche Scientifique, and the Université Lille 1, Sciences et Technologies. Dominique Menu is acknowledged for constructing the T-maze, Dr C. Luczak for his valuable comments and recommendations on Bonferroni corrections, and Dr Dorine Seuront for her help in setting up the light levels used in the T-maze experiments.

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