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The influence of contact chemical signals on mate recognition in a harpacticoid copepod

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Among copepods, reproduction is facilitated by a combination of sensory modalities, such as mechano- and chemoreception. The role of chemical communication in copepod mate recognition was assessed using behavioural bioassays that were based on precopulatory behaviours of an estuarine harpacticoid, *Coullana canadensis*, and the sibling species *Coullana* sp. Intra- and interspecific crosses demonstrated that males recognize genetically distinct conspecific and heterospecific females, indicating that prezygotic isolation remains incomplete. There was no association between the frequency of mate-guarding behaviour and geographic distance between populations of *C. canadensis*. However, reduced levels of interspecific mate guarding relative to intraspecific frequencies suggest the existence of a species-specific mate-recognition system. Lectins, which possess strong affinities for specific carbohydrate groups, were used to confirm that glycoproteins on the surface of females function as mate-recognition factors. Information regarding the chemical composition of these molecules was derived from observed effects of lectin binding to females on male mate-guarding behaviour. Mate guarding was inhibited within all tested populations when treated with *Triticum vulgaris*, a lectin that possesses an affinity for carbohydrates of the N-acetylglucosamine group. Surface glycoproteins responsible for mate recognition in the two species of *Coullana* may be glycosylated with monosaccharides from this group. Differential responses to lectin treatments suggested that composition of the contact chemical cues vary among populations of *C. canadensis* and between species. Yet, populations that appeared most similar based on shared lectin responses successfully discriminated against each other in mate-selection experiments. These findings indicate that contact chemical cues probably act in concert with additional factors to promote effective mate recognition and thereby contribute to reproductive success.

Keywords: chemical communication; Copepoda; glycoprotein; lectin; mate recognition

1. INTRODUCTION

The role of sex pheromones in reproductive isolation has been widely documented in terrestrial invertebrates (Roelofs & Comeau 1969; Scott 1994). But, their overall influence on aquatic counterparts, particularly zooplankton, remains unclear. It is generally accepted that for successful courtship and copulation most copepods rely on sensory modalities other than vision, such as mechanoreception and chemoreception (Blades & Youngbluth 1980; Blades-Eckelbarger 1991; Lonsdale *et al.* 1998). Katona (1973) asserted that diffusible pheromones elicit well-defined, mate-seeking behaviours, but that actual mate recognition occurs upon contact of male and female. Surface-bound molecules may promote mate recognition in copepods (Snell & Morris 1993).

A surface glycoprotein that acts as a functional mate-recognition pheromone for the rotifer *Brachionus plicatilis* has been isolated (Snell *et al.* 1995). Surface molecules, localized along body regions thought to be important for mating within several copepod species, also may serve as species-specific mate-recognition factors (Snell & Carmona 1994). Preliminary behavioural bioassays, in which surface glycoproteins were blocked, resulted in a significant reduction in the amount of mate guarding in the genus *Coullana* (Lonsdale *et al.* 1996). Mate guarding, in which an adult male positions himself with an immature female to form a paired complex, is maintained until the female's terminal moult when copulation takes place (Boxshall 1990). Given that mate recognition occurs upon contact, a mate-recognition system based on distinct contact chemical cues may serve as a prezygotic barrier among species of *Coullana*.

Coullana canadensis (Willey), formally known as *Scottolana canadensis*, is an estuarine harpacticoid copepod that maintains a wide geographic range extending along the east coast of North America until a break point near Florida where the sibling species *Coullana* sp. occupies the range southward through the Gulf of Mexico (Coull 1972; Por 1984; Lonsdale *et al.* 1988; Huys 1993). The amount of range overlap between these species remains unclear. Failure to produce viable offspring in laboratory crosses indicates that *Coullana* sp. is reproductively isolated from *C. canadensis* (Lonsdale *et al.* 1988). In addition, differences in several life-history traits between latitudinally separated populations of *C. canadensis* and *Coullana* sp. have a genetic basis (for examples, see Lonsdale & Levinton

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Table 1. *Location and environmental conditions in which populations of* Coullana canadensis *and* Coullana *sp. were collected*

population	locale	latitude (°N)	longitude (°W)	date	temperature (°C)	salinity (p.p.t.)
Coullana canadensis						
NY	Quantuck Creek Quoque, NY	41	73	1994	22	19
NJ	Wading River Wading River, NJ	39.5	74.5	1994	22	10
MD	Patuxent River Lusby, MD	38.5	76.5	1994	23	10
SC	Sampit River Georgetown, SC	33	79	1988	—	—
Coullana sp.						
FL	St Sebastian River Sebastian, FL	28	80.5	1995	30	21
MS	Graveline Bay Ocean Springs, MS	30.5	88.5	1994	—	—

Table 2. *Per cent of females mate guarded by adult males within and between species of* Coullana

(Calculated from number of stage CV–CVI females in precopula/total number females observed × 100. Sample sizes indicated in parentheses.)

	males					
	Coullana canadensis				*Coullana* sp.	
females	NY	NJ	MD	SC	FL	MS
Coullana canadensis						
NY	96 (22)	—	100 (8)	83 (6)	—	—
NJ	100 (13)	94 (17)	88 (17)	85 (13)	13 (24)	—
MD	85 (20)	79 (24)	73 (30)	88 (16)	8 (12)	—
SC	74 (23)	88 (16)	74 (23)	89 (27)	5 (21)	22 (9)
Coullana sp.						
FL	—	56 (9)	44 (9)	38 (13)	39 (18)	—
MS	—	44 (16)	35 (20)	58 (12)	40 (10)	69 (16)

1985; Lonsdale *et al.* 1993). Although the degree of mate-guarding behaviour is greatly reduced in crosses involving heterospecifics relative to conspecifics, prezygotic barriers are not fully developed between these sibling species (Lonsdale *et al.* 1988; Frey 1996).

The purpose of this work was to evaluate the role of chemical cues, namely contact molecules, in mate recognition and reproductive isolation among several populations in the genus *Coullana*. We tested the hypothesis that a mate-recognition system, unique to each species of *Coullana*, involves contact chemical cues.

2. MATERIALS AND METHODS

(a) *Maintenance of populations and behavioural bioassays*

Populations of *Coullana canadensis* and *Coullana* sp. were collected from geographically distinct estuaries along the east coast of North America (table 1). In the laboratory each population was reared in several batch cultures under constant environmental conditions of 20–21 °C, 15 p.p.t. salinity, and a 14 h : 10 h light–dark cycle. In each batch culture, an algal concentration, equally comprised *Isochrysis galbana* (ISO; chrysophyte) and *Thalassiosira pseudonana* (3H; diatom), was maintained at 2.5×10^5 cells ml^{-1} (after Lonsdale & Levinton 1985). Each month culture flasks within each geographic locale were mixed to maintain maximum genetic heterogeneity within a population. Under these laboratory conditions the following bioassays were conducted in which prospective mates were removed from culture, placed in a 1-ml well of a multidepression dish (2–3 individuals ml^{-1}), and observed for behavioural interactions indicative of mate recognition. Mate recognition was based on precopulatory behaviours, mate contact and mate guarding. Mate contact was defined as the seizing of a female, usually at the caudal rami, by a male using antennules. This behaviour was often followed by mate guarding.

(b) *Reproductive compatibility within and between species*

Intra- and interspecific crosses were compared to assess reproductive compatibility among populations of *C. canadensis* and the sibling species *Coullana* sp. Juvenile females, mostly early stage copepodids (CII–CIII), were individually exposed to two adult males, observed twice daily, and scored for precopulatory behaviours. An index of prezygotic compatibility was calculated as the percentage of

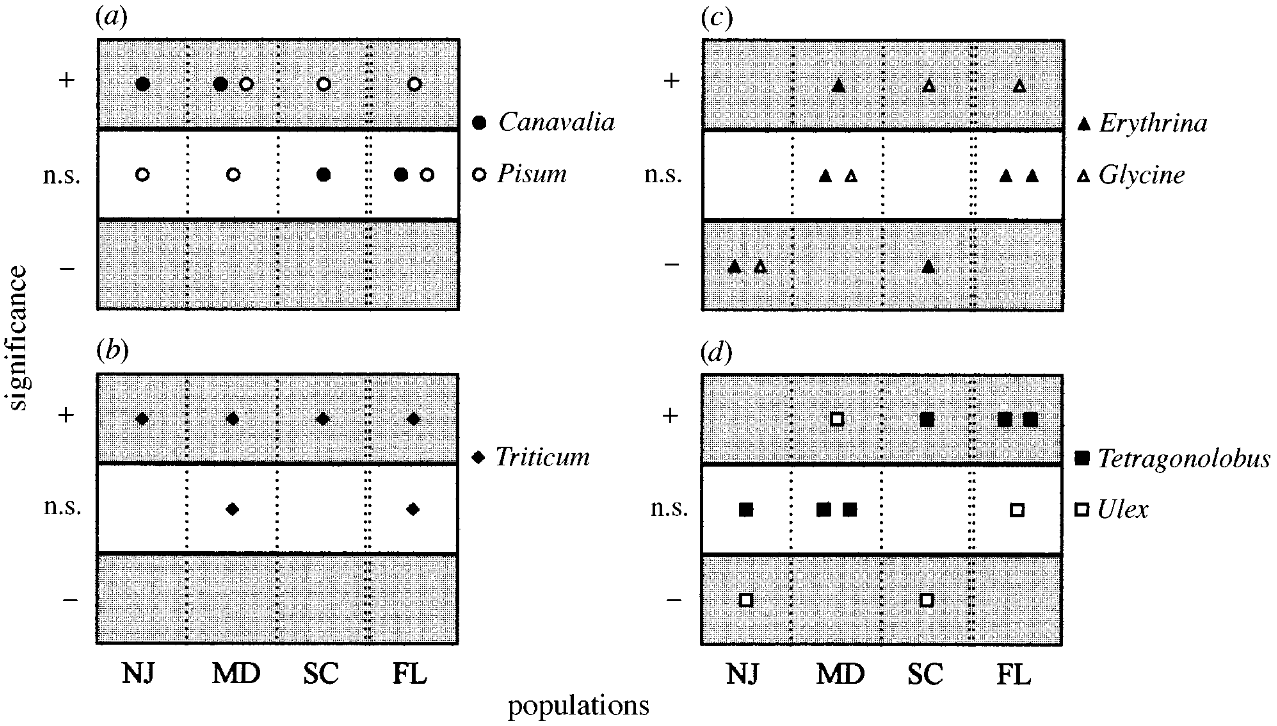

Figure 1. Effect of lectins, divided by respective carbohydrate classes, on populations of *Coullana canadensis* and *Coullana* sp. (+, significance at the 0.05 level, i.e. precopulatory behaviours of lectin treated females observed less than in control females; n.s., no significant difference between treatment and control groups; −, significance at the 0.05 level, but behavioural response in reverse direction of that expected, i.e. precopulatory behaviours of lectin treated females observed more than in control females). Results of replicated experiments done within MD and FL populations presented separately. (*a*) Glucose–mannose group; (*b*) N-acetlyglucosamine group; (*c*) N-acetylgalactosamine–galactose group; (*d*) L-fucose group.

females engaged in mate contact or mate guarding just before the time at which copulation can occur (i.e. number CV–CVI females mate guarded/total number females × 100). The significance between indices of reproductive compatibility and geographic distance among source locales of *C. canadensis* was tested by using the Mantel test of association (Smouse *et al.* 1986; Sokal & Rohlf 1995). The female data set was based on interlocale percentages to the left of the matrix diagonal, whereas the male set was based on data to the right of the diagonal (table 2).

(c) *Mate recognition and the role of contact chemical cues*

Behavioural assays using lectins were conducted to establish the importance of contact chemical cues in mate recognition and to determine the degree of structural variation in these putative chemicals that may contribute to mate discrimination among populations of *C. canadensis* and *Coullana* sp. Lectins are proteins that bind to carbohydrates specifically and noncovalently (Sharon & Lis 1989). Based on results of lectin-binding intensities to *Coullana* sp. females (Snell & Carmona 1994), seven lectins representing four main carbohydrate groups were selected: *Canavalia ensiformis* and *Pisum sativum* (glucose–mannose group), *Triticum vulgaris* (N-acetylglucosamine group), *Glycine max* and *Erythrina corallodendron* (N-acetylgalactosamine–galactose group), and *Tetragonolobus purpurea* and *Ulex europaeus* (L-fucose group). Each lectin was tested in populations representing *C. canadensis* and *Coullana* sp.

Binding of a lectin to the oligosaccharide on surface glycoproteins was expected to block the contact chemical signal produced by a female, and thus reduce the associated behavioural response by a male. To test the effect of each lectin, 18 late-stage juvenile females (mostly CV copepodids) were exposed to a 0.1 mg ml^{-1} lectin treatment for 15 min, washed twice in 15 p.p.t. autoclaved, filtered sea water, and transferred to an algal concentration of 2.5×10^5 cells ml^{-1}. Control females did not undergo lectin treatment, but were washed and transferred in the same manner as treatment females. Each female was placed in the presence of two males and scored for mate contact and mate guarding over the following 24 h. Because of low mating propensities, experiments involving *Coullana* sp. were extended to 48 h.

To determine the effect of each lectin on each population, the percentage of precopulatory behaviour of treatment and control was compared by using the Wilcoxon's matched-pairs signed-ranks test (Sokal & Rohlf 1995). Data from previous experiments (Lonsdale *et al.* 1996) that also tested the effect of lectins on premating behaviour were reanalysed to include mate-contact behaviour. Significance levels were compared to determine repeatability of results. A similarity index based on the number of shared lectin effects between populations was calculated. Populations were scored and weighted for similar responses to each lectin, either significant or nonsignificant (score of weighted similar responses/number lectins tested).

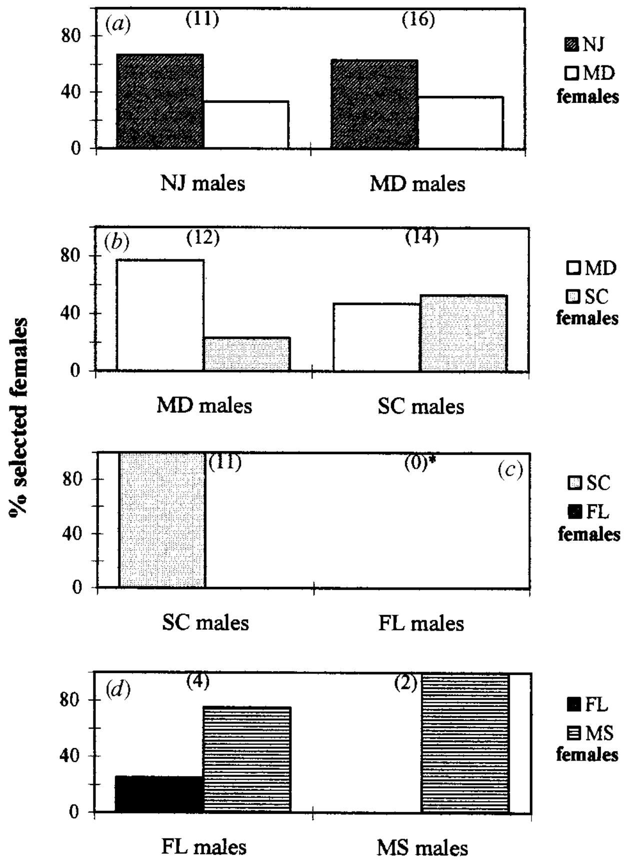

Figure 2. Frequency of precopulatory behaviour displayed by males when in the presence of females of same population and that of nearest neighbouring populations. (*a*, *b*) Conspecific crosses between populations of *Coullana canadensis*: (*a*) NJ × MD; (*b*) MD × SC. (*c*) Heterospecific crosses between populations of *Coullana canadensis* (SC) and *Coullana* sp. (FL). (*d*) Conspecific crosses between populations of *Coullana* sp. (FL × MS). Samples size indicated in parentheses. Asterisk indicates that crosses involving FL males were done; however, no selection of females occurred.

(d) *Mate discrimination within and between species*

Mate-selection experiments were conducted to assess the degree of mate discrimination between populations of *C. canadensis* and *Coullana* sp. Each adult male was presented with a choice of females by exposing them to a female from the same population and a female from a neighbouring population, both CV copepodids ($n=18$). To distinguish between females, females from one population were stained with a $0.01\,\mathrm{g\,l^{-1}}$ Neutral Red solution for 30 min (Anstensrud 1989). Observations of mate contact and mate guarding were made over a 24-h period, except for tests which involved *Coullana* sp., when experiments were extended to 48 h. For each population the total number of selected females was compared by using a *G*-test of independence (Sokal & Rohlf 1995).

3. RESULTS

(a) *Reproductive compatibility within and between species*

Conspecific mates were more reproductively compatible compared with heterospecific pairings based on precopulatory behaviours (table 2). There was little variation in precopulatory frequencies among either conspecific crosses of *C. canadensis* or *Coullana* sp. Among populations of *C. canadensis* measures of prezygotic compatibility appeared to be inversely related to geographic distance between source locales, but this relation was not significant ($R=-0.62$ and $p=0.09$, $R=-0.55$ and $p=0.13$). In contrast, males of *C. canadensis* and *Coullana* sp. mate guarded heterospecific females at lower frequency than conspecific females.

(b) *Mate recognition and the role of contact chemical cues*

Based on the assumption that lectin treatment effectively binds and blocks putative glycoproteins, the proportion of precopulatory behaviour exhibited in the control group was expected to be significantly higher than in the treatment group. Lectins were considered to have a positive effect if mate contact or mate guarding in the treatment groups was significantly reduced, and a negative effect if behaviour was significantly enhanced compared with the controls. Similar levels of significance of *Canavalia*, *Pisum* and *Tetragonolobus* lectin treatments were shared between NJ and MD populations of *C. canadensis* and between SC *C. canadensis* and FL *Coullana* sp. (figure 1*a*,*d*). *Triticum*, representing the N-acetylglucosamine carbohydrate class, showed a significant positive effect in at least one case for each population (figure 1*b*). In contrast, *Erythrina*, *Glycine*, and *Ulex* yielded unique responses when comparing NJ and MD populations (figure 1*c*,*d*). *Erythrina* and *Ulex* were also unique in comparing SC and FL; however, *Glycine* proved to be an effective blocker of precopulatory behaviour for both of these populations (figure 1*c*,*d*). Similarity indices, based upon these shared lectin responses, indicated that NJ and MD were more similar. Interestingly, MD and SC appeared to be least comparable, whereas SC and FL proved to be the most alike (table 3). The level of similarity appears to decrease with increased distance between populations of *Coullana*.

(c) *Mate discrimination within and between species*

Signs of mate discrimination, assessed as mate selection of females from the same population over females from neighbouring populations, were detected both within and between species. In comparing *C. canadensis* males from neighbouring populations, males from northern populations were more selective for females from the same population (67–77%) than southern females. Males from more southerly populations were less selective, choosing females of the neighbouring northern population in about the same frequency as from their own population (figure 2*a*,*b*). Despite this trend, within *C. canadensis* (NJ × MD and MD × SC) mate selection by males was independent of female population ($G=0.04$ and $G=2.68$, respectively, p was n.s.). Mate discrimination was most evident between heterospecifics. During experiments in which SC males were given a choice between a conspecific (SC) and a heterospecific (FL) female, mate guarding was observed only between conspecifics (figure 2*c*). Thus, mate selection was highly dependent upon species ($G=38.83$ and $p=0.001$). Adult males of *Coullana* sp. rarely displayed any precopulatory behaviour even among conspecific females, making it

Table 3. *Index of similarity between populations of* Coullana canadensis *and* Coullana *sp. based on shared responses to lectin treatment of females*

	Coullana canadensis			*Coullana* sp.
population	NJ	MD	SC	FL
Coullana canadensis				
NJ	1.00	—	—	—
MD	0.57	1.00	—	—
SC	0.43	0.14	1.00	—
Coullana sp.				
FL	0.14	0.21	0.71	1.00

difficult to assess the extent of mate discrimination between populations (figure 2*d*).

4. DISCUSSION

Chemical communication between sibling species may be critical considering the few morphological differences that possibly serve as premating barriers (Knowlton 1986, 1993). The findings from this study suggest that contact chemical cues influence precopulatory behaviours between *C. canadensis* and the sibling species *Coullana* sp., and may serve as mate-recognition factors for prospective mates.

Reduced levels of interspecific mate guarding relative to intraspecific frequencies suggests the existence of a species-specific mate-recognition system. These findings are consistent with similar experiments involving different populations of *C. canadensis* and *Coullana* sp. (Lonsdale *et al.* 1988). Differences in precopulatory-behaviour frequencies among populations may be attributed to either mechanical or chemical cues. Studies involving other copepod species conclude that sex–size ratios can be a significant factor for effective spermatophore transfer (DeFrenza *et al.* 1986; Grad & Maly 1988). However, variation in body size proved to be independent of the propensity to engage in precopulatory behaviours, and therefore, was not assumed to be an important determinant of mate guarding between populations of *C. canadensis* and the smaller sibling species *Coullana* sp. (Lonsdale *et al.* 1988). Other factors such as contact molecules may be more influential in determining successful mate recognition and extent of reproductive isolation.

The behavioural bioassays using lectins provide evidence that surface glycoproteins are involved in mate recognition. Treatment of females with certain lectins produces significant effects that reduce precopulatory behaviours. Mate recognition is inhibited within all populations when females are treated with *Triticum vulgaris*, a lectin that possesses an affinity for carbohydrates of the N-acetylglucosamine group. Thus, the glycoproteins responsible for mate recognition within species of *Coullana* may be glycosylated with some monosaccharides from this group. The isolated glycoprotein of *Brachionus plicatilus* also contained N-acetylglucosamine subunits (Snell *et al.* 1995; Snell & Rico-Martinez 1996).

Yet, variation within intersexual signals and receptors is critical for successful mate discrimination (Butlin 1995). An oligosaccharide composition unique to each population or species seems necessary for glycoproteins to serve as effective mate-recognition factors. Differential lectin binding indicates that some neighbouring populations (NJ and MD, SC and FL) are more similar, sharing several of the same responses to certain lectins. However, there also exist some differences between these populations. For example, NJ and MD differ by their responses to *Erythrina corallodendron*, *Glycine max*, and *Ulex europaeus*, indicating either an absence or presence of the respective carbohydrate group within each population. Discrimination among populations of *C. canadensis* and *Coullana* sp. may be dependent upon such slight variations in the composition of mate-recognition factors. Based on shared lectin responses, populations seem to be increasingly different with increased geographic distance.

Reduced similarity of chemical cue composition with increased distance between tested populations is in agreement with those of reduced reproductive compatibility among distant populations of *Coullana*. However, discrepancies between lectin blocking tests and mate-selection experiments exist. Whereas neighbouring populations of *C. canadensis* and *Coullana* sp. share similar lectin responses, males fail to mate guard heterospecific females when given a choice involving conspecifics and heterospecifics. The lack of mate recognition between *C. canadensis* and *Coullana* sp. provides further evidence of a species-specific mate-recognition system.

These results contrast with studies that have documented interspecific matings between several freshwater copepod species (Maier 1995; Chen *et al.* 1997). Within laboratory crosses males of both *C. canadensis* and *Coullana* sp. erroneously mate guard when exposed only to heterospecific females, albeit at lower frequencies relative to conspecifics. Whether such mate guarding among genetically distinct populations or between species of *Coullana* occurs in nature remains unclear. As *C. canadensis* and *Coullana* sp. are reproductively isolated based upon postzygotic barriers (Lonsdale *et al.* 1988), investment into precopulatory behaviours with an individual that is genetically incompatible dramatically decreases fitness. If these species and distinct populations are able to distinguish conspecifics from heterospecifics, then hybrid zone formation between distinct populations may be prevented, thereby precluding contiguous populations and maintaining reproductively isolated populations.

Given the variation of physical transport processes over different spatial and temporal scales within the geographic range of *Coullana* (Bumpus 1969, 1973), there may be the potential for encounters between populations of *C. canadensis* and *Coullana* sp. Based on mate-selection experiments there appears to be no dependence of mate choice upon the population of females within *C. canadensis*. However, the pattern of mate selection between neighbouring populations suggests that northern populations are more discriminative relative to southern populations. Whereas southern males readily mate guard northern females, the reverse situation is less frequent. Thus, there appears to be some directional mate discrimination among populations of *C. canadensis*. Similar asymmetry in mating preferences have been found for spatially separated populations of *B. plicatilis* (Snell & Hawkinson 1983).

Rather than invest energy and time into mating attempts that may prove unsuccessful, populations of *C. canadensis* and *Coullana* sp. probably rely on a species-specific mate-recognition system, involving contact chemical cues, to enhance reproductive success. By serving as a reproductive barrier and preventing gene flow, these mate-recognition systems may further reinforce the level of genetic divergence and reproductive isolation between these sibling species and perhaps distinct populations. Evidence for the existence of such a system has been presented here.

Discrepancies between the results of lectin treatments and behavioural bioassays suggest that a single molecule may not be the sole factor determining mate recognition (see, for example, Blades & Youngbluth 1980). Additional surface glycoproteins and/or diffusible pheromones may also serve as signals. The nature of lectins and glycoproteins may offer further explanations for such discrepancies. Although lectins have proven to be a useful tool for obtaining information about carbohydrate structure (Snell & Carmona 1994), the list of potential lectin treatments was not fully exhausted in this study. Information about the degree of variation in chemical composition of both oligosaccharide and protein signals may have been missed. Thus, blocking one molecule on the female may not be enough to eliminate precopulatory behaviours. Further research, such as determining the precise chemical composition and location of these putative contact pheromones, is needed to clarify the role of these cues and other factors that may be involved in mate recognition in *Coullana*.

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