Responses of mud snails from low and high imposex sites to sex pheromones

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Abstract

Imposex, male secondary sexual characteristics in female snails, is a morphological indicator of sub-lethal exposure to organotin compounds. The relation between imposex and behavioral responses to sex pheromones was studied. Responses of snails to sex specific pheromones were determined in laboratory assays. Females and males from a low imposex site and females, imposex females and males from a high imposex site were tested. The snails from the low imposex site showed the expected strong behavioral dimorphism with females responding to male pheromone and males responding to female pheromone. In assays using snails from the high imposex site, female and imposex females showed no dimorphism in response to pheromones and males showed a relatively weak dimorphism. As there was no breeding or egg capsule deposition at the high imposex site, we conclude that even the morphologically normal snails were behaviorally and reproductively compromised.

Keywords: Environmental pollution; Breeding and spawning behavior; Imposex; Behavioral toxicology; Mud snails; Sex pheromones

1. Introduction

The focus of our study is responses of mud snails (Ilyanassa obsoleta (Say, 1826) = Nassarius obsoletus = Nassa obsoleta) from low and high imposex sites to sex pheromones. Imposex is the presence of male secondary sexual characteristics in female snails (c.f. Jenner, 1979) and is a morphological consequence of sub-lethal exposure to organotin compounds. While there are abundant data supporting the assertion that the morphological condition of imposex can result in sterility in gastropods (Oehlmann et al., 1991; Gibbs et al., 1990a,b; Oehlmann et al., 1996), there are no reports of alteration of behavioral responses. Here we report tests of behavioral response of mud snails from low and high imposex sites to sex pheromones.

Mud snails are extremely abundant intertidal prosobranch gastropods (Dimon, 1905). They occur in often spectacular aggregations (Jenner, 1957) on soft bottom sediments on the Atlantic seaboard from Cape Cod to Florida. While mud snail responses to chemicals have been studied for about a century and include scavenging (Dimon, 1905; Curtis, 1985) feeding stimuli (Carr, 1967); trail following (Crisp, 1969; Trott and Dimmock, 1978; Bretz and Dimock, 1983) alarm (Atema and Burd, 1975); Imposex (Smith, 1971; Smith, 1980; Obendorster et al., 1998) and predator odors (Rahman et al., 2000), responses to sex pheromones have only recently been reported (Moomjian et al., 2002).

Combined spawning and breeding aggregations in mud snails are particularly impressive and reported to occur from February to May in North Carolina (Sastry, 1971) The aggregations often contain tens of thousands of snails. Egg capsule deposition, which is dependent upon water temperature (Scheltema, 1967), is spectacular (Jenner, 1957; Sastry, 1971). By the end of the spawning season, every surface in a breeding area (including algae such as Ulva spp. and Codium spp.), save sand, and mud is coated with a continuous fuzzy layer of egg capsules.

Over the last several years, laboratory assays have been developed that enable experimental testing of mud snails to water containing pheromones and kairomones.

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Laboratory results (Moomjian et al., 2002) complement laboratory and field experiments (Rittschof et al., 2002) and show that spawning and breeding aggregations are organized by at least two kairomones and three pheromones. Two of the pheromones originate from sexually active snails. Pheromones released by sexually active males attract sexually active females while pheromones released by sexually active females attract sexually active males (Moomjian et al., 2002).

Here we report results of laboratory assays in which we tested responses of snails from low and high imposex field sites to male and female sex pheromones. Our goal was to compare responses of females and males from the low imposex site to responses of morphological female, male, and imposex snails from the high imposex site.

2. Materials and methods

2.1. Snail collections

Sexually active female and male mud snails (17–25 mm in shell height) were collected as needed from late March to May 2003 from a low imposex site (historically 0–8%) Carrot Island, Beaufort, NC (34°43.625′ N, 76°40.230′ W). Copulating snails were collected in the act and separated by gender. Snails with a high probability of imposex (historically 60–80%) were collected immediately west of Olympus Dive Center in Morehead City, NC (34°43.172′ N, 76°42.783′ W). Because there was no copulation or egg capsule deposition at the Olympus site, snails were gathered randomly, used in experiments and then morphological gender and imposex status determined by shell removal followed by examination of the gonad and determination of the presence or absence of a penis or penis bud. All snails were collected at low tide and experiments were performed around the time of high tide, when snails would normally be active.

2.2. Preparation of pheromones

Pheromones were prepared by incubating individual sexually active snails in 50–100 ml filtered aged seawater for 1.5 h (Moomjian et al., 2002). Female snails ranged in total mass from 1.28 to 1.39 g (shell plus tissue). Male snails ranged in total mass from 0.75 to 0.84 g. At the end of the incubation, pheromone water was decanted into a beaker for immediate use in bioassays.

2.3. Ring bioassay

Mud snail responses to sea water and to pheromone waters were determined in ring bioassays (Duval et al., 1994; Rittschof et al., 2002; Moomjian et al., 2002). Briefly, a 0.5 cm wide by 4.0–4.5 cm diameter ring of pheromone water was placed in the bottom of an 8.5 cm in diameter finger bowl. A snail was then placed in the center of the ring and its initial response upon contact with the ring recorded. The initial response of a snail to contact with the ring was recorded as either follow or not follow. If the snail turned and followed the ring, the response was scored as a follow response. If the snail crossed through the ring without hesitation, the response was considered to be not follow. Avoidance or alarm responses, stopping and backing up or freezing in place observed to crushed conspecifics and predator odors (Duval et al., 1994; Rahman et al., 2000) were not observed in this study. A minimum of thirty snails of each morphological gender were tested with sea water and with each pheromone water. With the exception of snails used in Experiment 3 (see below) each snail and each finger bowl were used only once in each experiment. Snails were returned to the environment and finger bowls were soaked in running seawater overnight and then washed with sea water and dried with a paper towel.

2.4. Experiments

Three experiments were conducted. The first experiment tested responses of 60 sexually active female and 60 sexually active male snails from the low imposex site to sea water, female and male pheromone. The second experiment tested responses of 90 snails from the high imposex site to sea water, female and male pheromones. After initial responses were recorded, snail shells were removed and snails were classified as female, imposex, or male.

In the third experiment we tested snails from both sites with the same pheromone preparations and at the same time. Every snail was tested in seawater, female pheromone and male pheromone. Responses to each odor were recorded, and snail shells were then removed to identify morphological sex. We tested 30 sexually active female and male snails from the low imposex site. We tested responses of snails from the high imposex site until we had collected data on at least 30 morphological female, 30 imposex and 30 morphological male snails. The Z test of proportions (Walpole, 1968) and contingency analysis (Sokal and Rohlf, 1995) using frequencies of response categories were used to analyze data.

3. Results

3.1. Observations of breeding aggregations

By the time this study was initiated in March, breeding and egg capsule deposition activity at the low imposex site were near peak levels. Thousands of aggregated mating snails and egg capsules were appar-
ent at a glance. Copulation was easily detected whenever snails were completely submerged. Penis withdrawal was apparent in a very high percentage of paired snails when manually separated. In contrast, snails at the high imposex site were numerous, but were not aggregated. Although we actively searched for copulating snails and egg capsules during each snail collection and at the end of the study, none were found from March through mid-May. Thus, copulation and egg capsule deposition were not observed at the high imposex site, although many snails appeared to be sexually mature when examined after shell removal.

3.2. Bioassay results low imposex site

In the first experiment, sexually active female and male snails from the low imposex site were tested for responses to sea water, female pheromone and male pheromone (Fig. 1). Low and statistically similar percentages of sexually active females followed seawater and female pheromone \((Z = 0.3, P > 0.05)\). Over 60% of sexually active females followed male pheromone. Significantly more females followed male pheromone than followed seawater \((Z = 11.7, P < 0.001)\) or female pheromone \((Z = 11.6, P < 0.001)\). Low percentages of sexually active males followed sea water and male pheromone and responses were statistically similar \((Z = 1.2, P > 0.05)\). Over 65% of males followed female pheromone. This response was significantly higher than that to sea water \((Z = 14.9, P < 0.001)\) or male pheromone \((Z = 15.9, P < 0.001)\).

3.3. Bioassay results high imposex site

As was the case for the low imposex site, ring assays were used to test the follow responses of female, imposex, and male mud snails to sea water, female pheromone and male pheromone (Fig. 2). Over 30% of morphological females followed seawater. The follow response of female snails to female pheromone was not significantly different than that to sea water \((Z = 1.52, P > 0.05)\) and was also not significantly different than the response of females to male pheromone \((Z = P > 0.05)\). However, female responses to male pheromone were significantly greater than that to sea water \((Z = 2.13, P < 0.05)\). Female and imposex snails both significantly followed male pheromone in comparison to sea water. \((Z = 2.13, P < 0.05, Z = 4.3, P < 0.001, \text{ respectively})\). Imposion and male snail responses to female pheromone were similar \((Z = 0.38, P > 0.05)\) and significantly different than responses to sea water \((Z = 3.5, P < 0.001, Z = 5.8, P < 0.001)\), respectively, male snails from the high imposex site also significantly followed male pheromone when compared to seawater \((Z = 3.4, P < 0.001, \text{ Fig. 2})\).

3.4. Third experiment

In the third experiment, snails from both sites were tested simultaneously for responses to sea water, female and male pheromone. Every snail was tested with every odor and results are summarized in Fig. 3. Contingency analysis showed responses to seawater were similar in all test groups \((G = 1.5, 3 \text{ df}, P > 0.05)\). Responses to
female pheromone were significantly different for some groups but not others \((G = 61.3, \text{ 3 df, } P < 0.001)\). Decomposition of the \(G\) statistic into significant and nonsignificant subsets indicated low imposex site females were different from low imposex site males, statistically similar to high imposex site females and different than imposex females and high imposex site males. Responses of all morphological groups of snails from the high imposex site to female pheromone were statistically similar.

Contingency analysis showed responses to male pheromone were significantly different for some groups but not others \((G = 33.4, \text{ 3 df, } P < 0.001)\). Decomposition of the \(G\) statistic into significant and nonsignificant subsets indicated low imposex site males were different from low imposex site females, imposex females, high imposex site females and statistically similar to high imposex site males. Responses of all morphological groups of snails from the high imposex site to male pheromone were statistically similar.

3.5. Responses of individual snails to sea water, female and male pheromone

Because we tested all of the last groups of snails in all solutions and kept track of the responses of the individuals to the odors, we were able to compare responses of individuals from each group to determine if there were patterns of responsiveness. There were striking differences between patterns of responsiveness between low and high imposex sites (Fig. 4a–e). The most distinctive pattern was for low imposex site females in which 60% of the snails responded only to male pheromone (Fig. 4a) with about 8% responding to both pheromones. One female responded exclusively to female pheromone. In contrast, only 16% of the females from the high imposex site responded exclusively to male pheromone (Fig. 4b). The response pattern for females from the high imposex site was dominated by snails that did not respond to either pheromone and with snails that responded to both male and female pheromone. Similar numbers of these female snails responded exclusively to female pheromone as to male pheromone.

The pattern of imposex females (Fig. 4c) was intermediate between that of females and males (Fig. 4d) from the high imposex site. Most notable was the decrease in the proportion of snails responding to both pheromones and concomitant increase in snails responding exclusively to either female or male pheromone. There was no difference in proportion of imposex snails that responded more to male than to female pheromones \((Z = 1.4, P > 0.05)\).

The majority of male snails from the high imposex site either did not respond to pheromones or responded exclusively to one or the other pheromone (Fig. 4d). Similar proportions of morphological males did not respond to pheromones and responded to one pheromone. This pattern of responsiveness was in contrast to responses of males from the low imposex site (Fig. 4e). The pattern of the low imposex site males was dominated by responses exclusively to female pheromone followed by responses to both pheromones, no response and finally responses exclusively to male pheromone. In summary, the low imposex site male and female response patterns were those we expected for animals responding to gender specific pheromones while the response patterns of the snails from the high imposex site were not what would be expected.

4. Discussion

The results of our initial experiment verified that reproductively active snails produce and respond to sex specific pheromones (Moomjian et al., 2002). Responses of all the morphological types of snails from the high imposex site were different than responses of snails we tested from the low imposex site and reported for sexually active snails. Tracking of responses and comparison of patterns of individuals from the high imposex and low imposex site suggests most snails from the high imposex site do not respond to gender specific pheromones.

There are two likely possibilities explaining the differences in results of the studies: (1) the snails at the imposex site were in a pre-sexually active state; (2) high imposex site snails were behaviorally castrated. Although reproduction at the low imposex site began in
mid January and continued through the first two weeks in May, there was no evidence of reproduction at the high imposex site. Many snails from the high imposex site appeared reproductively mature. These observations support the hypothesis that the snails at the high imposex site are behaviorally castrated.

Observed breeding activity and egg capsule deposition at the low imposex site and absence of breeding and egg capsule deposition at the high imposex site when combined with bioassays using male and female pheromones support the hypothesis that even morphologically normal snails at high imposex sites may be functionally sterile. If correct, this conclusion would extend the observations of morphological sterility of gastropods of Gibbs et al. (1988, 1990a,b), Gibbs and Bryan (1994) and Oehlmann et al. (1996) to sterility of morphologically normal snails from high imposex sites.

Most snails from the high imposex site were impacted whether or not they showed morphological effects. The observation that behavior is a sensitive indicator of toxic compounds is well known in larval invertebrates and is gaining recognition in lower vertebrates (Bridges, 1999; Weis et al., 2001; Park et al., 2001; Beauvais et al., 2001). The ability to combine morphological imposex data with behavioral responses to pheromones provides a unique opportunity to use mud snails to develop a more clear understanding of the relation between morphological changes in secondary sexual characteristics and disruption of sexual behavior. Our measures of imposex were crude compared to those used to assess TBT

Fig. 4. (a–e) Responses of different classes of snails to male and female pheromones. (a) Responses of morphological females from the low imposex site. These snails show dimorphism in their response to pheromones, responding most strongly to male pheromones. (b) Responses of morphological females from the high imposex site. The highest response category for this group was no response to either pheromone. (c) Responses snails that were morphologically imposex from the high imposex site. The pattern of responses of these snails were similar to those of morphological females from the high imposex site. (d) Responses of morphological males from the high imposex site. This group did not show a dimorphism in its responses to pheromones. (e) Responses of morphological males from the low imposex site. As for the females for the low imposex site, there was a clear dimorphism in this group with the majority of snails responding female pheromone categories. Statistics in text.
contamination (Gibbs et al., 1987; Matthiessen and Gibbs, 1998). Although at this point we do not know if there is a direct or indirect link between imposex and disruption of sexual behavior, a more detailed study using more sophisticated measures of imposex is planned.

If mud snails can be used to predict environmental risks for mollusks in general, then detailed understanding of the relation between parameters that are easily measured such as morphological imposex and much less easily measured parameters such as alteration in cytochrome p450 levels (Oberdörster et al., 1998) neuropeptides (Oberdörster and McClellan-Green, 2000), aromatase (Oberdörster and McClellan-Green, 2002), changes in fatty acid esters of testosterone (Gooding et al., 2003) and sexual dysfunction should be important knowledge for environmental toxicology assessment. If other mollusks from contaminated areas lose the ability to respond to pheromones that synchronize gamete release, this could contribute to the observed dramatic decline globally in commercially important bivalve populations.

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