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CHEMICAL SIGNAL MEDIATED PREMATING REPRODUCTIVE ISOLATION IN A MARINE POLYCHAETE, *Neanthes acuminata* (*arenaceodentata*)

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Abstract—*Neanthes acuminata* Ehlers (1868) is a monogamous coastal polychaete with male parental care and a high level of sexual selection. We measured the level of prezygotic isolation among allopatric populations of *N. acuminata*; from the East and West Coast of the USA, a population from Hawaii, and a laboratory culture originating from Los Angeles, CA. All populations were found to preferably mate with members of their own population. Individuals from populations from Atlantic vs. Pacific Ocean failed to pair and to mate, either during the 10 min or 48 hr experiments. Instead, individuals showed high levels of aggressive behavior. Experiments measuring the levels of interpopulation aggression, established that individuals can recognize and discriminate among different populations of *N. acuminata* on the basis of olfactory cues. Aggressive behavior was induced by exposure of animals to seawater "conditioned" by individuals from the other populations, thus demonstrating the role of olfaction in the detection of "home" populations. The aggressive display was stronger upon exposure to seawater conditioned with "unrelated" populations and especially between Pacific and Atlantic populations.

Key Words—*Neanthes acuminata*, premating isolation, sex pheromones, population variations in chemical cues.

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INTRODUCTION

The reproductive behavior of nereidid polychaetes has been studied for many years, and most species are known to spawn as heteronereids following a metamorphosis into this sexually mature form. In the broadcast spawning species, reproduction takes place in the free seawater column when performing a typical swimming behavior, the "nuptial dance," which culminates in the release of gametes by the sexual partners (Hardege, 1999). The reproduction of an individual is timed via environmental triggers including temperature, day length, lunar cycle, and additional cues such as daytime and weather conditions (Hardege et al., 1990, 1994; Bentley and Pacey, 1992) to coincide with the majority of worms in the same population. For monotelic species that die after reproduction, such coordination is essential to ensure successful fertilization (Denny and Shibata, 1989). The spawning process itself is controlled by sex pheromones released from sexually mature individuals of the opposite sex (Boilly-Marer and Lassalle, 1980), a number of which have been identified (Zeeck et al., 1996, 1998; Hardege, 1999).

Nereis (*Neanthes*) *arenaceodentata* (Moore, 1903), also known under the synonyms *Nereis* (*Neanthes*) *caudata* (Delle Chiaje, 1841) and *Neanthes* (*Nereis*) *acuminata* (Ehlers, 1868), is widely distributed along coastlines of North America, Europe, Africa, and the Indo-West Pacific (Pettibone, 1963; Day, 1973). This population complex is characterized by numerous paragnaths on both rings and hooked falcigerous seta on the proboscis. The "original" species was described as *Neanthes caudata* from European waters. Pettibone (1963) corrected this and named it *Neanthes arenaceodentata* based on the material from New England. Day (1973) renamed a North Carolina population as *Neanthes acuminata*. Reish established a laboratory population in 1964 from six specimens collected at Los Angeles Harbor, and referred to it as *N. arenaceodentata*. Weinberg et al. (1990) studied the chromosome number of different populations in this species complex. *N. acuminata* from New England has $2N = 22$, *N. arenaceodentata* from Reish's laboratory culture has $2N = 18$; collections from the mouth of San Gabriel river and Newport Bay has $2N = 18$, but the centromere of one chromosome is in a different location, and a Hawaii population has $2N = 28$. We are using the name *N. acuminata* to designate the New England populations and use *N. arenaceodentata* for California populations with specific site designation.

For over 40 yr, the species has been used as a laboratory experimental animal for ecotoxicology studies because of the ease of culture and its well-studied reproductive behavior and early development (Reish and Stevens, 1969; Reish, 1985). Among the nereidids, it is an unusual species because of its premating pairing behavior, male parental care, and the direct development of the young (Reish, 1957; Reish and Alosi, 1968). Typical behavior in encounters

between individuals, especially between those of the same sex, often involves aggressive displays, and fights that can cause cannibalism and mortality (Starczak, 1984). In contrast, intersexual encounters of ripe individuals show little degree of aggression (Reish, 1957), with the partners passing each other, and secreting mucus to form a burrow/tube inside which they remain for 1–3 wk. During reproduction, the male will increase movement (rhythmically beating of tail) within the tube and release a small amount of a white, mucus-like cloud that resembles the gamete release behavior of broadcast spawning nereidids (Weinberg and Starczak, personal communication). This is followed by the release of eggs by the female that are fertilized by the male, releasing a whitish cloud of gametes. Once the female has laid her eggs into the mucus-lined tube, she dies outside of the tube within a few hours. The male then incubates the young; the exact nature of his care is unknown but it was observed that males rhythmically beat the length of the body while positioned in the mucus tube adjacent to the egg mass. The larvae leave the parental tube after a period of approximately 21–25 d (under laboratory conditions) and construct their own individual tubes. The adult male may then find a second mate and may continue to mate. In total, the life cycle takes approximately 12–16 wk to complete in the laboratory (Reish, 1985).

When attempting to pair individuals from populations of *Neanthes acuminata* collected from various sites off the Atlantic and the Pacific coast of the US, Weinberg et al. (1990) found a significant level of interpopulation aggression that in some cases prevented the worms from pairing. While two populations from the Los Angeles area (San Gabriel River and Newport Beach) showed little aggression toward each other, there was some initial aggression between two Atlantic coast populations (Falmouth, MA, and Stonybridge, CT), but eventually these animals did pair during an observational period of 36 hr. In contrast, all attempts to pair Atlantic populations with Pacific populations failed, and the worms showed high levels of aggression and a substantial number of casualties because of the intense fighting. Interestingly, Weinberg et al. (1992) found that both Pacific populations failed to pair and mate with individuals taken from the above-mentioned laboratory-cultured collected at Los Angeles Harbor by Reish in 1964. Although these three sampling sites are separated by less than 25 miles, no successful offspring could be reared, and aggression levels were high. These results suggested evidence for a rapid species isolation following a founder event (Weinberg et al., 1992), a theory that was since rejected by Rodriguez-Trelles et al. (1996). Based on allozyme electrophoretic studies, the authors found differences between these populations that were as large as those to the Atlantic populations, and concluded that at the time when the Los Angeles Harbor (laboratory culture) population was collected it was already genetically distinct from the San Gabriel and Newport populations.

None of these studies provided an explanation of the possible causes of interpopulation aggression that prevent mating and may function as mechanisms in reproductive isolation. Weinberg et al. (1990), discussing the interpopulation aggression, suggested that one of the mechanisms by which individuals may be able to recognize kin could be the use of population-dependent chemical signals, which he described as "differences in their sex pheromones." Sex pheromones have been described in a number of nereidid polychaetes (see Hardege, 1999 for review), but little is known about pheromone variability among populations in marine invertebrates. "Kin recognition" and "detection of self" via chemical signals has been described in a number of species (see Cardé, 1986 for review) including lizards (Cooper et al., 1999; Bull et al., 2000), bumblebees (Ayasse et al., 1999), and salamanders (Rollmann et al., 2000). In the aquatic environment, little is known about the chemistry involved, and few studies have addressed this topic. Stanhope et al. (1992) describes behavioral assay-based evidence for a habitat-modified, race-specific sex pheromone in amphipods (*Eogammarus confervicolus*) based on genetic differences in the algal diet of the gammarids. Here, we demonstrate that premating isolation in *N. acuminata* populations is based on chemical cues rather than visual or tactile ones. "Conditioned seawater" taken from the various populations induces "fighting behavior" indicating that the species is capable of chemically based kin (population) recognition.

METHODS AND MATERIALS

Experimental Animals. Experimental animals represent cultures originating from collections by Dr. J. Weinberg from the intertidal sands at the mouth of the San Gabriel River, Long Beach, CA, USA, from the upper bay at Newport Beach, CA, from Alwife Cove, New London, CT, USA, West Falmouth Harbor, MA, USA, Hawaii, and the laboratory culture of Dr. D. Reish. The latter culture ("Reish culture") was established in 1964 from six animals collected at Los Angeles Harbor, allowed to grow into a population of thousands of individuals, and is maintained until today at California State University, Long Beach. A subculture of the "Reish culture" was established at Woods Hole, MA, by Weinberg in 1986. Additional cultures were established by Weinberg from field collections at West Falmouth Harbor, MA, Hawaii, San Gabriel River, Long Beach, CA, from the upper bay at Newport Beach, CA, and from Alwife Cove, New London, CT in the early 1990s. For the present study, approximately 300 individuals per population were obtained from Weinberg and cultured in the laboratory starting in 1995. Collection of worms in the field was undertaken in June between 1997 and 2002 at West Falmouth Harbor, MA, and Alwife Cove (New London), CT.

Laboratory Animal Culture. Worms were kept in natural seawater at a salinity of 32‰ and a water temperature of 20°C. Seawater was changed on a weekly basis, and the temperature and salinity were checked twice a week. The nereids were initially fed four times a week with fish food flakes (Tetra Min™) as the primary source of food, and later with low protein rabbit food and freeze-dried or frozen algae (*Enteromorpha* ssp.). These were soaked in seawater before feeding. The small particles were pipetted and dispersed through the water. The term "sexually mature" is used as a loose definition to distinguish "adult" nereids from the immature individuals. It is not a direct indication of the specimen's level of sexual receptiveness, although this is intrinsically linked. Females were identified as being mature on the basis of visible presence of eggs in the coelom. Occasionally, males could be distinguished by the whitening of coelom, due to the presence of sperm. An increase in size from the juvenile stages was the most obvious indication of maturity. Behaviorally, the mature males were also easily recognized, as they would often attempt to pair and mate. Only those worms that had been previously paired with an individual from their own population were used in behavioral experiments. This procedure was chosen to guarantee that all worms were capable of pairing given contact with the "right" partner. "Conditioned seawater" samples were produced through incubating 100 ml sterile filtered seawater (0.2 μm) with five mature males for 24 hr.

Behavioral Assays. For prezygotic isolation experiments (intra- and interpopulation aggression), individuals were taken from culture and were defined as sexually mature on the basis of physical morphology as described above, and then isolated in small glass beakers (50 ml) for 24 hr. Two individuals (one of either sex) were placed in a Petri dish with seawater and left for a 10-min period to determine if they would pair. Pairing was defined as "both individuals laying alongside one another with no signs of aggression toward or movement away from the other individual" as stated by Weinberg et al. (1990). The pairing as defined above indicates a pair bond between two individuals, which would eventually lead to mating. All experiments were carried out over a 6-wk period to minimize any differences in aggression through seasonal effects, and all measurements were taken between 12:00 and 17:00 hr to reduce diurnal variations in aggression that may occur. A Petri dish (diam 6.6 cm) was filled three quarters with seawater, and two individuals were placed into the dish, one immediately after the other. Timing was initiated at the first point of contact (proximity of less than 0.5 cm, usually followed by behavioral change such as extending jaws within seconds, but not always followed by physical contact) between the two specimens. "Conditioned water" samples used to investigate whether the observed kin recognition behavior is based on chemical cues were obtained by placing 25 individuals of a given population in 50 ml sterile filtered (0.2 μm) seawater for 2 hr. Samples were

applied to individual worms with glass pipettes (0.1 ml) at 1 cm distance in front of the individuals, and this was defined as "contact" to "odor samples." "Point Sampling" (action at a particular point in time) was used at 30-sec intervals to compare the action of the nereids with the aggression score. Sampling continued for 10 min, and all scores were tallied. Scoring of aggression was modified slightly from Reish and Alosi (1968, modifications are shown in italics): Score 0—no aggressive behavior; Score 1—defensive or avoids contact with other specimen; palpi may be flared; *or aggressive displays may be shown while the two nereids are distanced within 1 cm from one another*; Score 2—fighting position assumed; palpi flared and jaws may be extended; Score 3—aggressive, with violent attacks upon other specimen; jaws extended and biting, *following a level 3 aggression further aggressive displays may be shown while the two nereids are distanced no more than 1 cm from one another and worms actively avoid close contact*.

Statistical analysis of variance within and differences among samples was undertaken using a median test and a Kruskal–Wallis test (ANOVA). A rigorous Wald–Walfowitz test (ANOVA) was used for testing significant differences between "exposure to conditioned seawater" and direct encounters.

RESULTS

Pairing of sexually mature individuals from the five populations show significant levels of premating aggression among all specimens, except when paired with individuals from their "own" population (Figure 1a, e.g., San Gabriel males vs. other populations: $\chi^2 = 24.996$, $FG = 4$, $P < 0.001$ median test, $P < 0.001$). Specimens used for the experiment were taken from tanks with populations kept in the laboratory for at least 3 mo and in some cases ("Reish culture") nearly 40 yr. Although San Gabriel River and Newport Beach populations are separated by less than 15 miles, significant levels of premating isolation ($\chi^2 = 15.7$, $P = 0.013$ median test, $P = 0.011$) were observed with aggressive encounters reaching behavioral score 3.

Exposure of mature specimens to seawater "conditioned" by individuals from other populations also induced a behavioral response in the worms, which in some cases resulted in attacks on the glass pipette used. Similarly to worm–worm encounters, aggression levels were higher between populations than within the populations (e.g., San Gabriel male conditioned seawater: $\chi^2 = 26.499$, $FG = 4$, $P < 0.001$, median test, $P < 0.001$; Figure 1b). The chemically induced (population odor) premating isolation levels were generally lower than during worm–worm direct contact encounters, but not often statistically significant. For example, in the San Gabriel worms "conditioned seawater" was only significantly less effective than direct contact using samples from the

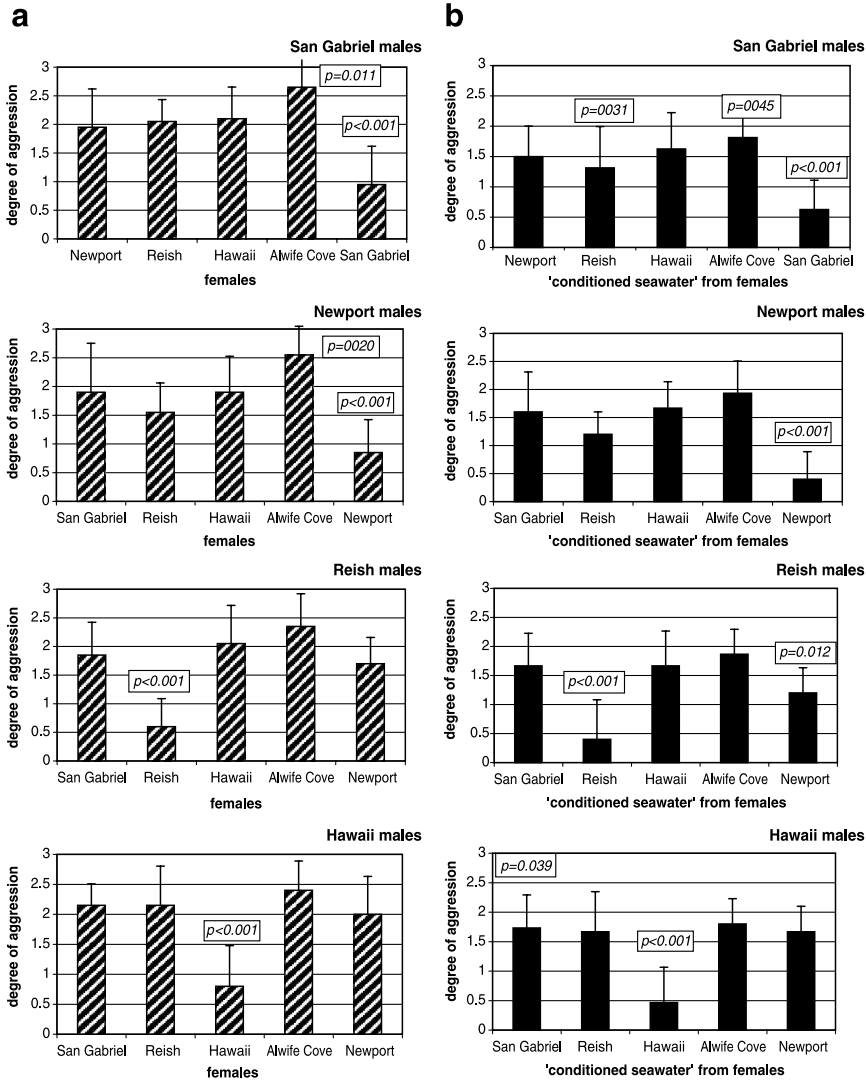

FIG. 1. Behavioral assay on pairing behavior towards various populations using: (a) exposure of individual mature males to females and (b) to "conditioned seawater" representing the (female) odor of the various populations. The *P* values, presented in boxed text, show significant differences using Wald–Walfowitz tests (ANOVA) for testing significant differences between "exposure to conditioned seawater" and direct encounters (b vs. a), and a Kruskal–Wallis test (ANOVA) to examine differences within an experiment. Number of pairing experiment repeats vary between different combinations ($N = 19-22$ repeats) due to availability of worms, error bars represent mean/SD.

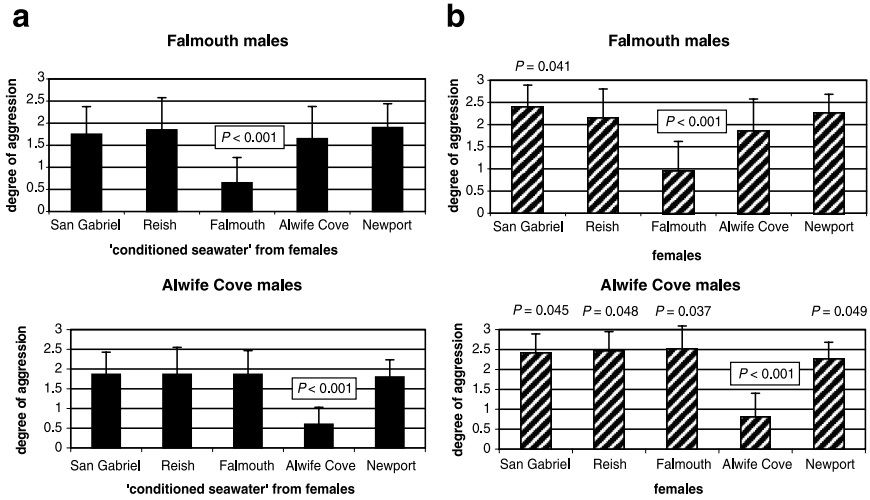

FIG. 2. Behavioral assay on pairing behavior of mature male individuals from Alwife Cove (Connecticut) and Falmouth harbor (Massachusetts) collected in the field 2 wk prior to the assays towards various populations using: (a) exposure to males, and (b) exposure to "conditioned seawater" representing the (male) odor of the various populations. The *P* values, presented in boxed text, show significant differences using Wald–Walfowitz tests (ANOVA) for testing significant differences between "exposure to conditioned seawater" and direct encounters, and a Kruskal–Wallis test (ANOVA) to examine differences within an experiment. Number of pairing experiments: $N = 21$ per data set, error bars represent mean/SD.

Alwife Cove and Reish culture samples (Reish vs. Reish H_2O: $P = 0.03$, Alwife Cove vs. Alwife Cove H_2O: $P = 0.045$; Figure 1b).

Figure 2 shows data obtained from specimens collected in the field (Falmouth, Alwife Cove) and assayed within less than 2 wk after sampling. As with all assays with culture-reared worms, the field collected population from Falmouth and from Alwife Cove showed aggressive displays toward all other populations expect to individuals of their own population ($\chi^2 = 22.467$, $FG = 4$, $P < 0.001$ median test, $P < 0.001$; Figure 2b), and the aggressive display was also inducible using "conditioned seawater" (Figure 2a).

DISCUSSION

Extreme levels of inbreeding are found in a number of species, particularly in species that are of "domestic use" and as such artificially selected and reared for consistency. In rodents, such inbreeding is thought to compromise an

individual's ability to discriminate among individuals (Nevison et al., 2000). Individual recognition and kinship play an important role in social organisms and influence competition and mate choice. In mice, as with the majority of animals, mate choice is influenced by chemical signals, pheromones. Hurst et al. (2001) demonstrated that wild house mice (*Mus domesticus*) use mouse urinary proteins (MUPs) that bind and release small volatile pheromones to mediate individual recognition. Wild mice show a large degree of diversity in the expression of MUPs that might be as great for the major histocompatibility complex (MHC) that has been suggested as the main source of the immense odor complexity in rodents, fish, and possibly in humans (Yamazaki et al., 1999). Consequently, "odor individuality" in mice and other organisms that use urine marking may be based on specific release rates of such volatile compounds rather than differences in MHC. Currently, we do not have a well-characterized aquatic invertebrate system to study this question, but the observed population differences in *Neanthes acuminata* may enable us to address this in the future. This will require studies of the chemistry involved as well as the biodiversity of MHC complexes.

The use of chemical signals in discrimination between self-produced, own population, and those produced by conspecifics has been studied in gregarious lizards, *Cordylus cordylus* (Cooper et al., 1999) and *Egerinia stockesii* (Bull et al., 2000), a number of insects (Gemeno et al., 2001; Evenden et al., 2002), and rodents (Hurst et al., 2001). In the aquatic environment, a number of studies have shown that closely related species can distinguish other species by the use of odor (e.g., stomatopods, *Gonodactylus zacae* and *G. bahiahondensis*; Caldwell, 1982), but little is known about the chemical basis of such phenomena. Kin recognition studies have focused on amphipods, *Eogammarus confevicolus*, where race-specific sex pheromones were postulated by Stanhope et al. (1992), and copepods, *Tigriopus californicus* (Palmer and Edmands, 2000).

Our experiments using *Neanthes acuminata* confirm that marine invertebrates can use odor for kin recognition. Aggression levels within a population were lower than between populations (Figure 1a), and the high degree of fighting behavior suggests that this could lead to premating reproductive isolation among the different populations. Exposure of male individuals to "conditioned seawater"—meaning incubation water in which females from various other populations were kept (see Methods and materials)—induces "aggressive display" demonstrating the importance of "chemical cues" in this behavior (Figure 1b). Odors induced aggression levels between worms from the East and West Coast of the USA are significant and confirm the allozyme electrophoretic studies by Rodriguez-Trelles et al. (1996), and the chromosome complements undertaken by Pesch et al. (1988), suggesting that it is unlikely that successful offspring can occur. Nevertheless, in all "conditioned seawater" experiments, the aggression levels are lower than in direct confrontation be-

tween worms. Although these differences are only in some cases statistically significant (see Figures 1b and 2), the ubiquitously lower level of chemical odor induced aggression (many P values between 0.06 and 0.14) indicates that the level of such interactions may not be solely chemical. Behavioral responses, such as avoidance, fleeing, and aggression display, cause additional stress, which increases the level of fights between "unfamiliar" individuals while exerting little to no effect upon "familiar, own population" pairs. This is known from crayfish (Breithaupt and Eger, 2002) and American lobster, *Homarus americanus* (Bushmann and Atema, 2000; Breithaupt and Atema, 2000), both of which use chemical signals for dominance fights to establish social hierarchies. Specimens collected in the field (Falmouth, Alwife Cove) and assayed within less than 3 wk after sampling (Figure 2b) showed aggression levels not significantly different from those obtained from the cultured worms (Figure 1a, b), indicating that culture in the laboratory did not influence the behavioral responses.

Aggression levels among individuals from the three Los Angeles area populations are significantly higher than within these populations, suggesting that all three southern Californian populations can discriminate each other, are independent, and have different odor profiles. Further genetic evidence is required to test whether these odor differences have led to the existence of at least three races within the L.A. area or even "represent a case for rapid speciation" in the laboratory, as hypothesized by Weinberg et al. (1992), but disputed by Rodriguez-Trelles et al. (1996). In addition to population genetics studies, future research will focus on high-performance liquid chromatography (HPLC) analysis of odor profiles from the various populations by using "conditioned seawater." Today, only a few examples exist where odor profiles of aquatic organisms have been studied. These includes lobster, where urine derived proteins may function in individual recognition (Karavanich and Atema, 1998; McLaughlin et al., 1999), and salmon, where odor profiles are implicated in "homing" (Solomon, 1973; Brannon and Quinn, 1990).

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