Spawning synchrony in *Arenicola marina*: evidence for sex pheromonal control

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SUMMARY

Chemical communication systems controlling reproductive behaviour have been shown in a number of marine polychaetes. This study investigated the use of sex pheromones to coordinate spawning behaviour in gravid lugworms (*Arenicola marina*). Lugworms typically reproduce in the autumn, during low water of spring tides, and often exhibit epidemic spawning. Females release gametes within the burrow whereas males deposit spermatozoa on to the beach surface. The incoming tide dilutes the spermatozoa and transports them to the females' burrows. Sperm is diluted rapidly and sperm concentrations fall below the minimum required for fertilization within a few minutes. The present investigation establishes the existence of chemical signals synchronizing spawning for the first time in an iteroparous polychaete. The process can be divided into two steps, the induction of gamete release by waterborne chemical cues and burrow irrigation behaviour in females—burrow irrigation representing the means by which spermatozoa are carried to the eggs. In both sexes, the release of gametes can be induced by exposure to sea water into which other individuals had previously spawned. Males also respond to odour compounds from other males. The overall effect of the chemical signals results in synchronized, mass spawning of a population.

1. INTRODUCTION

Synchronous spawning of neighbouring individuals in a population has been described in a number of marine invertebrate species. The best known examples are the mass spawning of corals on the Great Barrier Reef (Babcock *et al.* 1986) and the Palolo worm (*Eunice viridis*) in Western Samoa (Caspers 1984). Synchronized release of gametes by individuals within a population has the effect of maximizing the chances of gamete interaction and fertilization. This is thought to be of particular importance for semelparous (monotelic) species where reproduction is a single terminal event and where fertilization is such a critical stage in their life history (Denny & Shibata 1989). These species include the nereid polychaetes, of which a number show 'swarming' of epitokous individuals (Clark 1961). Endocrine and environmental timing of maturation and spawning have aroused a great deal of interest and have been the subject of a recent review (Bentley & Pacey 1992). Environmental factors such as photoperiod, temperature, lunar periodicity and tidal cycles may act to synchronize maturation in broadcast spawners, and these may be combined with behavioural adaptations that reinforce their effect. Spawning hormones and

pheromonal communication are involved in the transduction of this information. Although chemical communication through sex pheromones has been proposed in numerous marine invertebrates, chemical characterization of these waterborne cues has been achieved only rarely (Agosta 1992).

The common lugworm, *Arenicola marina* L., is one of the main macrobenthos species of the intertidal areas of north-western Europe. *A. marina*, unlike nereid polychaetes, is an iteroparous (polytelic) species with a single annual spawning crisis. At most localities the spawning activity of a population of *A. marina* is highly synchronized and patterns of natural spawning have been discussed in detail by Howie (1984). Synchronized, epidemic reproduction is easily identified during low water of spring tides by the appearance of sperm puddles on the surface of the sediment (Bentley & Pacey 1992), and it appears that this has a marked influence on fertilization success (Williams *et al.* 1997).

Spawning of both sexes of lugworm is brought about by hormones that control egg maturation, sperm activation, and the process of gamete release (Howie 1961*a*, 1984; Pacey & Bentley 1992). It now appears that control of oocyte maturation that precedes spawning is a two-step process. A prostomial maturation hormone (Howie 1961*a*, Pacey & Bentley 1992) induces the production of a coelomic maturation factor (CMF) (Watson & Bentley 1997). CMF then causes the oocytes to proceed from prophase to

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metaphase of the first meiotic division. The chemical nature of the female prostomial maturation hormone and CMF remains unknown. Sperm activation and spawning in the male is brought about by the action of a polyunsaturated fatty acid sperm maturation factor, 8,11,14-eicosatrienoic acid (Bentley *et al.* 1990; Pacey & Bentley 1992; Bentley & Hardege 1996).

Although exposed to the same or very similar environmental conditions, two populations of *Arenicola marina*, inhabiting neighbouring beaches at St Andrews (Scotland), have different spawning dates (Bentley, personal observation). This observation, together with evidence that a proportion of individuals maintained in the laboratory under constant temperature and daylength conditions and absence of moonlight, spawn at exactly the same time as their field counterparts (Howie 1963, 1984), suggests that an endogenous rhythm and early Zeitgeber may control the timing of spawning in a given population. Environmental and endocrine factors alone are not always sufficient to ensure simultaneous release of gametes in broadcast spawning individuals, and it has been shown (for example, in nereid polychaetes) that sex pheromones provide an additional fine tuning of reproduction (Boilly-Marer & Lassalle 1978).

There is now strong evidence that in both iteroparous and semelparous polychaete species, well-synchronized reproduction is achieved finally by inter-individual communication through pheromonal means. In this paper we describe the role of such chemical signals in the control of epidemic spawning of *Arenicola marina*.

2. MATERIALS AND METHODS

Sexually mature specimens of *Arenicola marina* were collected by digging from four populations on the east coast of Scotland, at East Sands and Eden Estuary in St Andrews (Fife), at Kingsbarns (Fife) and at Dunbar (Lothian) during the weeks immediately prior to the natural spawning which occurs during late October (East Sands, St Andrews; Kingsbarns), early November (Eden Estuary, St Andrews) and early December (Dunbar). Animals were transported back to the laboratory, sexed by light microscopical observation of gametes and placed individually in polyethylene containers filled with sea water (*ca.* 200 ml). The animals were maintained for up to two weeks in the laboratory at constant 10 °C and ambient photoperiod.

From about six weeks prior to the natural spawning period in the field, gravid *Arenicola marina* were collected weekly from the beach in order to test the state of maturity of the population by endocrine induced spawning, in order to ensure that worms used for behavioural assays were ripe and subsequently receptive to sex pheromonal communication. In male lugworms, spermatozoa are held immotile in clusters known as sperm morulae and are not capable of fertilization until just prior to spawning (Bentley 1985). Final maturation of sperm, sperm activation, and the process of active release of spermatozoa are under the control of a spawning hormone (SMF, sperm maturation factor), the fatty acid 8,11,14-eicosatrienoic acid (Pacey & Bentley 1992). The SMF and a female maturation hormone (Howie 1961*b*; Meijer 1979) are associ-

ated with the prostomium, but only induce a reaction in gravid animals shortly prior to the natural spawning season. Subsequently, 'maturity' of an individual can be evaluated by the response to either the injection of the spawning hormone or an *in vitro* assay developed by Bentley (1985).

In our experiments, gravid males ($n = 10$) were tested for spawning ability by injecting with $13 \mu\text{g g}^{-1}$ body weight of the spawning hormone 8,11,14-eicosatrienoic acid as described by Pacey & Bentley (1992) and the spawning response was recorded. 8,11,14-eicosatrienoic acid was obtained from Sigma Chemical Co. and 1×10^{-2} M stock solutions were prepared in methanol. Aliquots of these stock solutions were diluted 100-fold to give a final concentration of 1×10^{-4} M. Females were injected with prostomium homogenate (one prostomium per female) which causes spawning in fully mature specimens (Howie 1961*a,b*; Pacey & Bentley 1992) and the percentage of responding worms was observed. Only those groups of animals were used in behavioural sex pheromone bioassays where all test males shed sperm within 60 min of fatty acid injection and where more than 50% of all test females released eggs within 6 h of injection with prostomium homogenate.

'Male spawning water' used for assay on ripe females and males was prepared as follows. A freshly spawned male was removed from the sea water (200 ml) into which it had shed spermatozoa following spontaneous spawning. The water was then centrifuged (12 000 g for 10 min). A volume of 100 ml (equivalent to the spawning water from 0.5 male) was then used in each test assay. 'Female culture water' (after 24 h individual culture in 200 ml sea water) and 'female egg water' (200 ml of sea water from a freshly spawned female) were prepared in an identical fashion. Contamination was avoided by maintaining each individual in clean polyethylene containers for the preparation of each sample, which was then handled independently during centrifugation.

Two different types of behavioural bioassays were used in order to examine the effects of chemical signals on *Arenicola marina*. In the first bioassay system, ripe individuals were cultured individually in polyethylene boxes (200 ml sea water, changed daily). Substances under investigation were added with a micropipette. Behavioural changes were monitored continuously for 60 min and then subsequently every 30 min. A behavioural reaction was recorded as positive only if it resulted in gamete release. In each experiment a group of worms was treated with twice filtered sea water (TFSW) as a negative control. In all experiments the same group of animals was used repeatedly (daily) during the experiments unless worms released gametes. In such cases worms were replaced with other individuals collected from the same population at the same time, thus representing the same 'batch' of animals. In the second bioassay design, ripe individuals were maintained for at least 24 h in an aquarium with U-tubes (glass) prior to experimentation. The aquarium was connected to the running sea water system of the laboratory. The tidal cycle was simulated by manipulation of flow rate, and test substances were added either to the sea water inflow or individually to each specimen U-tube. The aquarium was divided into two separate chambers and, therefore, all water flow between the compartments was through the U-tubes. This apparatus was used in order to examine the influence of external stimuli (tide, semiochemicals) on the irrigation activity of lugworms, and to observe the spawning behaviour during natural as well as induced spawning (by injection of spawning hormones). Pumping activity of ripe worms was monitored

by the addition of brilliant blue FCF food colouring to the inflowing sea water (in one compartment of the U-tube tank). The frequency and duration of pumping activity induced by substances under investigation was observed visually and compared with those elicited by controls.

3. RESULTS

Field studies were undertaken at St Andrews East Sands, Eden estuary, and Kingsbarns beach (Fife) and Dunbar (Lothian) in autumn 1993 and 1994. Spawning activity was monitored daily during periods of spring tides by counting sperm pools on the beach and digging of 20 specimens per day followed by determination of maturational stage (gravid/spent). Spawning in all four populations takes place during periods of spring tides, although there are differences in spawning dates between populations. Spawning of the population at East Sands was observed between 27 October and 1 November 1993, the Eden Estuary population spawned between 9 and 13 November 1993, whereas spawning at Kingsbarns beach occurred from 26 October to 2 November 1993. At Dunbar, the population reproduced in early December (2–6 December 1993). Sperm puddles appeared coincident with the outgoing tide about 1–5 min after exposure of the beach. The number of sperm puddles per 100 m² reached a maximum of 50 (± 10) on the 1 November 1993 (East Sands), while in 1994 the spawning activity of the population at Kingsbarns resulted in up to 250 per 100 m². Digging of 20 worms per day revealed that even on the first day of observed male spawning activity on the beach, only a few (2 out of 20, 28 October 1993) gravid females were present. The number of gravid males decreased from 11/20 on 28–30 October 1993 to 7/20 and 2/20 on 1 and 2 November 1993, respectively. In parallel, the number of spent worms increased from 1/20 (20 October 1993) to 8/20 once the females had spawned (28 October 1993) to reach 18/20 at the end of the spawning season (2 November 1993).

When exposed to different cues (figure 1a–c), males showed increased activity (strong movement) but did not release gametes immediately such as has been described for nereids (Zeeck *et al.* 1988). However, figure 1a–c shows that males from all three populations released gametes when exposed to 'spawning water' from females, indicating that a waterborne chemical signal is involved. Interestingly, water from culture boxes of gravid male *Arenicola marina* also induced males to release gametes in a type of 'homosexual' response. Removal of eggs or spermatozoa (centrifugation at 19 000 g for 20 min) demonstrated that in both cases this biological activity is associated with the non-cellular fluid components (supernatant), whilst the 'washed' gametes showed no or only very little activity. The chemical cue(s) seems to have a very short half-life because egg-containing sea water collected from females which had spawned overnight failed to induce any reaction in males (1/20, East Sands, 27 October 1993). The

Figure 1. *Arenicola marina*. Induction of spawning and release of gametes in gravid males collected from three populations in northeast Scotland: (a) East Sands, St Andrews; (b) Eden Estuary, St Andrews; (c) Dunbar, Lothian. Twenty males were assayed per day per population. Individual worms were kept isolated in boxes with 200 ml of sea water. Data represent the number of males actively releasing gametes upon exposure to 'male spawning water', 'female culture water', 'female spawning water' and control (fresh sea water).

release of gametes usually occurred 1–4 h after exposure. The percentage of males responding increased daily to reach a maximum at around the time of the natural spawning. In parallel, the number of males spawning spontaneously (controls) without contact with other individuals increased to 20–30%.

Figure 2a demonstrates that males are able to spawn repeatedly during the spawning season. Nineteen out of 20 males spawned upon exposure to female 'spawning water', all of these males spawned for a second time over the next days (19/19) and 17 of these 19 released gametes for a third time, but in each case subsequent spawning resulted in re-

Figure 2. *Arenicola marina*. Repeated spawning activity in males: 20 gravid males were induced to spawn upon exposure to 'female spawning water', the number of spawning males was recorded and the spermatozoa were counted using a haemocytometer. Nineteen males (95%) shed gametes and these males were then transferred into new boxes with fresh sea water and exposed to fresh 'female spawning water' every morning in order to examine their capability of multiple spawning. Sea water was changed daily (evening), and also when gametes had been released, in these latter cases to estimate the amount of spermatozoa that had been released. (a) Data show the number of males shedding sperm upon exposure to 'female spawning water' for the second and third time in November 1994. (b) Bars represent the mean number of spermatozoa emitted ($\pm$ standard deviation) during the first, second, and third spawnings.

lease of fewer sperm (figure 2b). The ability of male *Arenicola marina* to release sperm repeatedly forms the basis of the observed 'homosexual' response of male lugworms to 'spawning water' cues from other males (figure 1a-c). The quantities of sperm released by individuals may have a bearing on the subsequent dilution and consequent availability of sperm for fertilization of neighbouring females' oocytes, and this experiment demonstrates that the small (8.6 g; s.d. = 4.1 g, $n = 39$) males from the Eden Estuary are capable of releasing almost identical amounts

($8.0 \pm 1.54 \times 10^{11}$) on each of two consecutive daytime tides.

In female *Arenicola marina*, there is an increasing readiness to spawn amongst controls, but also an increase in the response to substances emitted by male *Arenicola marina*. Sea water samples into which males had released gametes ('spawning water') induced females to shed eggs, regardless of whether the spermatozoa had been removed or not, indicating that a waterborne pheromone had induced this reaction in a similar way to the males described above (figure 3a). Injection of the sperm maturation factor (8,11,14-eicosatrienoic acid), recently identified by Bentley *et al.* (1990), induces male *Arenicola marina* to release sperm within 45–60 min (Pacey & Bentley 1992). Exogenous application of 8,11,14-eicosatrienoic acid, and arachidonic acid, did not affect male or female *Arenicola* even when high concentrations ($1\text{--}100 \mu\text{g ml}^{-1}$) were used. Similarly, the neurotransmitters, serotonin, dopamine and GABA, as well as exposure to a sex pheromone of nereid polychaetes, 5-methyl-3-heptanone (Zeeck *et al.* 1988), had no effect on the behaviour of mature lugworms (figure 3a). The sex pheromones are associated with coelomic fluid of gravid animals, and this has similar biological activity as spawning water. Figure 3a also shows that spawning water of 8,11,14-eicosatrienoic acid-injected males induced release of gametes, indicating that the production of the sex pheromones is somewhat correlated with the presence of the gamete maturation hormone.

Upon exposure to male spawning water, female *Arenicola marina* (East Sands, 1993) placed in the U-tube bioassay system started 'pumping' actively. The pumping frequency increased from less than one female out of eight being active during the examination period of 10 min (duration 1–2 min) without stimulus to 6/8 (30 October), 5/8 (31 October) and 5/8 (31 October). Females started this pumping within 2 min of exposure, showing that a waterborne signal indicates the presence of gametes from ripe males (figure 3b). The pumping activity of active females often showed great amplitude and continued for prolonged periods of up to 1 h. During these periods the females also often reversed the direction of the normal pumping, especially when the simulated tide was rising over the surface. In contrast, immature specimens collected from a juvenile site at East Sands, St Andrews (20 October 1993), used as a control showed no response.

4. DISCUSSION

The data presented here provide the first evidence for the role of sex pheromones in epidemic spawning in an iteroparous polychaete, *Arenicola marina*. Waterborne chemical signals coordinate the release of gametes and the burrow irrigation behaviour in females. In *Nephtys hombergi*, both sexes were observed to shed gametes onto the surface of the sand immediately before the incoming tidal waters, and gametes will be mixed in the turbulent shallow water

Figure 3. *Arenicola marina*. Induction of the reproductive behaviour in gravid females. (a) Release of gametes upon exposure to: sea water as negative control; 8,11,14-eicosatrienoic acid (10 μ M); 11,14,17-eicosatrienoic acid (10 μ M); 4. Serotonin (10 μ M); Gamma-amino-butyric acid (GABA, 10 μ M); Dopamine (10 μ M); 5-methyl-3-heptanone (1 μ M); 'male spawning water', 8,11,14-induced males ('male spawning water' from males which had previously been induced to release gametes by injection of 13 μ g g⁻¹ body weight 8,11,14-eicosatrienoic acid); and 1 ml of coelomic fluid of gravid males (supernatant of centrifugation at 12 000 g). Ten females were used in each treatment (with three replicates). Data shown represent the mean ($\pm$ standard deviation) number of females releasing gametes within 6 h after exposure. (b) Induction of burrow irrigation behaviour ('pumping') upon exposure to: hw = high water; low tide = low water, incoming tide = low water for 2 h followed by increase of water level; and hw + 'male spawning water'. Eight females were used in each treatment (with three replicates). Data shown represent the mean ($\pm$ standard deviation) number of females showing pumping activity within 10 min of exposure.

(Bentley *et al.* 1984). In *Arenicola marina*, only males eject sperm onto the surface, eggs are kept within the burrow of the female (see Bentley & Pacey 1992) and consequently, a high fertilization rate in the field depends on the transport of spermatozoa into the burrows of the females with the process being correlated with the incoming tide during the spring tides. Field

studies at East Sands, St Andrews, have shown that the dilution of spermatozoa by the incoming tide is so rapid that fertilization success requires an active transport mechanism to be provided for sperm by the females (Williams *et al.* 1997). Due to a chemical signal associated with male spawning activity, this is achieved by pumping sea water through the burrows (figure 3b). This pumping activity is different to the intermittent tube irrigation behaviour described by Wells (1945, 1949, 1966) as normal cyclic pattern (NCP) during routine daily activity. A characteristic feature of the NCP is the repetition of pumping (which lasts for a few minutes) at regular intervals, generally about every 40 min (Wells 1953; Riisgard *et al.* 1996). In contrast, females exposed to pheromonal cues often exhibited pumping for up to 1 h, sometimes changed direction of pumping followed by prolonged periods of inactivity of up to 24 h. During this time no water movements were recorded, but afterwards the worms again became active. Control animals kept in the glass tube apparatus were generally very inactive and sometimes suspended their pumping during the experimental period, as described by Wells (1966) as typical behaviour of lugworms kept in glass tubes without feeding. Future investigations will also reveal whether female *Arenicola marina* exploit the full capacity of its pump during this process. Riisgard *et al.* (1996) described the potential maximum pump pressure to be 30–150 times higher than used in the 'standard' *Arenicola marina* during the NCP.

At the time of the natural spawning season, the release of gametes in males can be induced not only by chemicals associated with spawning activity of females (egg-water), but also excretions from other spawning males and odour compounds from gravid, non-spawning females (figure 1a–c). As shown in figure 2a, b, the response of gravid males to other spawning males is based on their capability of multiple spawning over a period of up to three days that had not been described previously (Howie 1984). The chemical communication is likely to result in a 'snowball effect' leading to mass spawning of a population. This is likely to maximize the number of different sexual partners with which the gametes of an individual lugworm will be combined, and has been described in polychaetes as a possible strategy for optimizing fertilization success through synchronization (Olive 1992). The chemical nature of these substances remains unknown but our experiments indicate that the production and/or release of these cues is associated with the gamete maturation hormones. In males, injection of the sperm maturation factor 8,11,14-eicosatrienoic acid (Bentley *et al.* 1990) results in spawning of activated spermatozoa and the release of spawning-associated odour compounds that induce females to spawn (figure 3a). A proportion of individuals maintained in the laboratory under constant temperature and daylength conditions, and in the absence of moonlight, spawn at exactly the same time as their field counterparts (Howie 1963). Thus, endocrine factors alone may

Figure 4. A model for the interaction between environmental factors (Zeitgeber), sex pheromones and gamete maturation hormones in the control of spawning in *Arenicola marina*. The pathway marked (?) is one where an endocrine or neural link has yet to be established.

trigger the spawning activity of individuals and result in the increase of spontaneous spawning in our experiments (figure 1a–c). A schematic description of the environmental and endocrine factors influencing the maturation and spawning in *A. marina*, based on field observations, previous studies on the reproductive behaviour (see Bentley & Pacey (1992) for a review) and the behavioural experiments (figures 1–3), is shown in figure 4.

In marine polychaetes, the existence of sex pheromones has been postulated in a number of species. These pheromones may be involved in controlling the induction of gamete release and the stimulation of specific reproductive behaviours, such as the nuptial dance of nereids. In *Platynereis dumerilii*, the nuptial dance is triggered by a volatile ketone, 5-methyl-3-heptanone (Zeeck *et al.* 1988), which showed no activity on *Arenicola marina*. This is followed by the release of gametes of both partners due to further pheromonal signals (Zeeck *et al.* 1992). The female-produced pheromone was recently identified as uric acid (Zeeck *et al.* 1996). Chemical analysis of the *A. marina* sex pheromones is under way and preliminary data suggest that polar, water soluble substances of low molecular weight (less than 1000 Da) act as gamete release pheromones, and that a very labile substance, probably a volatile compound (Hardege *et al.* 1996), is involved in causing the ‘pumping’ behaviour of females. This pattern of chemical communication would then be similar to the situation in nereid polychaetes where, in addition to the volatile ketone two sex specific, polar and water soluble substances are the ‘gamete release’ pheromones (Hardege *et al.* 1994; Zeeck *et al.* 1996).

On the basis of the results described here, and the extensive knowledge of the reproductive biology of *Arenicola marina* (see Bentley & Pacey (1992) for a

review), we suggest that this species provides an ideal model for the study of the chemical nature of sex hormones and aquatic sex pheromones, their interactions, and their role in maturation and spawning. A distinct reproductive behaviour, the ready availability of the species for study and the possibility of manipulation of maturity with the fatty acid hormone, provide a realistic opportunity to increase our knowledge of semiochemicals in marine organisms.

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