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Aspects of gametogenesis, oocyte morphology and maturation of the lugworm *Arenicola marina* (Annelida: Polychaeta) in relation to commercialised procedures to extend the breeding season

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ABSTRACT

Environmental manipulation is a common method of extending the spawning season of aquaculture species including the polychaete worm *Arenicola marina*. Temperature synchronises autumn spawning populations and so its manipulation was used to advance and delay spawning. Females were exposed to a minimum period of 3 weeks at 5 °C in conjunction with the injection of prostomial homogenate to induce spawning up to 4 weeks prior to the natural spawning date. We also maintained individuals at 15–17 °C starting 4 weeks prior to, and then continuing after the natural spawning date, delaying spawning for up to 4 months. Both sexes can be manipulated, but males suffered higher mortalities and a greater rate of spontaneous spawning within the tanks. In 'advanced' females, mean oocyte diameters (measured in September, one month prior to spawning) were significantly larger and more homogenous compared to ambient individuals, whilst 'delayed' females produced a second cohort of oocytes approximately 8 weeks into the treatment. Delaying and advancing spawning induced significant changes in the ultrastructural morphology of prophase and metaphase oocytes, and delayed prophase oocytes showed a significant increase in the number with cracks on the surface of the vitelline membrane. Although, SDS-PAGE and Western blots confirmed that Maturation Promoting Factor (MPF) activity was not different from ambient controls, there were significant changes in MPF activity levels (measured by histone kinase activity) in manipulated oocytes. *A. marina* has the plasticity for spawning to be delayed and advanced by a number of months; this is essential for the continued development of aquaculture of this species. However, the maturational ability of the oocytes is compromised and this may have significant implications for production and quality of the offspring from manipulated individuals.

1. Introduction

The gonochoristic, annual iteroparous polychaete *Arenicola marina* (L.) is an integral part of the fauna of Northern Europe (Jacobsen, 1967). Commonly known as the lugworm, this species is collected for the bait industry by manual turning of the sediment and the impacts of this activity have received considerable attention (see Fowler, 1999 for review). In recent years there has been significant interest in its aquaculture to supply the bait trade and using procedures of Olive et al. (2003: WO 03/007701), commercial production is now routine. It is also used for ecotoxicological testing and its culture for biomedical applications (e.g. harvesting its respiratory pigments) has received significant interest from biotechnology companies (e.g. Rousselot et al., 2006).

A key component for successful culture is the ability to manipulate a species' reproduction, and for seasonal breeders it is year-round juvenile production that is the ultimate goal. Breeding in *A. marina* is highly synchronised, with the majority of UK populations exhibiting epidemic spawning over a few days during autumn. This is maintained through the transduction of a hierarchy of environmental cues by the endocrine system (Watson et al., 1998, 2000). The endocrine control of oocyte maturation and spawning in *A. marina* has been extensively studied. Briefly, ovulation occurs between February and April and is followed by a rapid oocyte growth phase, lasting until the end of August when oocytes are nearly fully-grown (approx. 180 μm in diameter), yet sub-mature. At this point, they arrest (in prophase I of meiosis) and await activation through a two-step hormonal process; a prostomial maturation hormone (PMH) and a coelomic maturation factor (CMF), which allows the oocytes to progress to metaphase I (oocyte maturation). They are then spawned, but remain in meiotic arrest until reactivation by fertilization (Watson and Bentley, 1997).

The extension of spawning in many aquacultured species has been achieved through the manipulation of both endocrine systems and environmental cues. For female *A. marina*, endocrine manipulation (the injection of prostomial homogenate) can induce spawning, but this is only successful up to 3–4 weeks before the natural spawning

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date (Watson, 1996). Males can also be induced to spawn by the injection of prostomium or the putative spawning hormone 8,11,14 eicosatrienoic acid (Pacey and Bentley, 1992a).

Environmental temperature has been shown to be one of the easiest cues to manipulate for *A. marina*. Preliminary investigations by Howie (1959) found that spawning ceases when minimum air temperatures were approximately 11 °C, and Farke and Berghuis (1979) showed that the timing of spawning can be controlled by simple temperature manipulation. Individuals maintained in boxes of sand could be held past the natural spawning date for up to 1 month by holding the temperature at 15 °C. Subsequent exposure to a temperature of 8 °C for 26 days induced spawning. By combining this published work with that on the endocrine manipulation (e.g. Howie, 1959; Pacey and Bentley, 1992a; Watson and Bentley, 1998; Watson et al., 1998; Betteley and Bentley, 2001), larvae can be produced under commercial conditions throughout the entire year for *A. marina* and *A. defodiens* (Olive and Craig, 2005; WO 03/043994 A1; Craig pers. comm.). However, a rigorous scientific assessment combined with controlled conditions has not been employed to answer the key questions about the effects of the manipulation process on gamete quality.

The extension of the breeding season using environmental manipulation is often detrimental to final reproductive success, for example lower larval survival rates have been observed from those individuals spawning outside the natural spawning time in the commercial production system (Olive and Craig, 2005: WO 03/043994 A1). Fertilization and the production of viable offspring are dependent upon many cellular and molecular processes and one of the most critical is the completion of oocyte maturation. Pivotal to the transition of the oocyte from prophase to metaphase (M-phase) is the M-phase promoting factor (MPF). MPF is responsible for regulating entry into M-phase in all eukaryotic cells. It is a heterodimeric protein formed from a catalytic subunit $p34^{cdc2}$, also known as the cyclin-dependent kinase (Cdk1) and a regulatory subunit, cyclin B (Arion et al., 1988; Gautier et al., 1988; Labbé et al., 1989). Recent work by Chausson et al. (2004) has begun to elucidate the process in *A. marina*. In prophase, Cdk1 is present both as an inactive, Threonine 161 phosphorylated monomer and as an inactive Tyrosine 15 and Threonine 14 phosphorylated heterodimer with cyclin B (pre-MPF). In metaphase oocytes only the cdk1/cyclin B heterodimer is active. This is achieved through the dephosphorylation of both the Tyrosine 15 and Threonine 14 of the cyclin B-associated Cdk1, with the reciprocal phosphorylation of the Serine residue on cyclin B. Threonine 161 remains phosphorylated during the activation process.

In this paper we have delayed or advanced spawning using temperature manipulation in a number of different UK lugworm populations under laboratory conditions. Subsequently, using a number of techniques, we have investigated the effects on cellular and molecular processes involved in oogenesis and oocyte maturation. Oocyte diameter measurements at different times were taken to investigate the gametogenic processes. Transmission electron microscopy (TEM) was used to detect changes at the ultrastructural level, whilst other surface structural (membrane) changes were investigated using scanning electron microscopy (SEM). Finally, SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting established the meiotic stage of the oocyte before and after spawning with regards to MPF activation. Subsequent quantification of MPF under different temperature treatments was assessed through the Histone H1 Kinase assay as detailed in Chausson et al. (2004).

2. Materials and methods

2.1. Specimen collection

Adult *A. marina* were collected at low tide from a number of UK sites (Table 1) from October through to December between 1999 and 2003 as detailed in Watson et al. (1998). Three systems were used for the temperature manipulation experiments depending on the laboratory in which the experiments were performed (summarised in Table 1). These were based on earlier investigations (Howie, 1959, 1961a,b; Farke and Berghuis, 1979; Pacey and Bentley, 1992a; Watson et al., 1998; Betteley and Bentley, 2001) as well as unpublished data from P.S. Cadman (pers. comm.). These have subsequently been used for commercial production by Olive and Craig (2005: WO 03/043994 A1).

Table 1
The location of UK populations of *A. marina* used in this study and the natural spawning date (based on pers. obsers. by the authors) and the method of maintenance during manipulation (see text for details)

Location	Latitude/ longitude	Time of spawning	Manipulation method and system used
Hauxley Nature Reserve, Northumberland, England	55:19:10 N 1:33:45 W	Early November	Advanced and delayed Static and recirculating
West Sands, St Andrews, Scotland	56:20:52 N 2:48:10 W	Early-Mid November	Delayed Static and recirculating
Red Wharf Bay, Anglesey, Wales	53:18:17 N 4:12:51 W	Mid November	Delayed Flow-through
John Muir Country Park, Dunbar, Scotland	56:00:22 N 2:34:14 W	Mid-Late December	Delayed Static and recirculating

2.2. Static and recirculating systems: ambient conditions

Worms described as 'ambient' individuals were collected around the time of the natural spawning date for each population. Upon return to the laboratory, individuals from each population were isolated and kept in large polypropylene containers (30 H×22 D×38 L cm) in an ambient temperature recirculating system. As the spawning date approached, the worms were removed from the communal tanks and placed in individual containers with 200 ml of filtered seawater (changed every 2 d) in each, in a 10 °C temperature controlled room (ambient photoperiod).

2.3. Static system: advanced spawning

A period of 21 days at approximately 5 °C was used to induce early spawning in *A. marina.* On a number of occasions up to 8 weeks in advance of the natural spawning date, worms were collected from Hauxley, NE England. They were sexed and placed in aerated filtered seawater in polypropylene containers (30×22×38 cm) each containing 15–30 individuals. They were left for 24 h under ambient temperature conditions before being placed in to containers containing sand at 5 °C. After 21 days at 5 °C, individuals were removed from the sand and placed into separate containers with approximately 200 ml of seawater at 5 °C and the water was changed on alternate days.

2.4. Recirculating system: delayed spawning

Four weeks prior to the natural spawning date individuals were placed in polypropylene containers with sand mixed with seaweed (*Ulva* spp.) in a seawater recirculating system set at 17 °C. Similar densities to those described above were used and individuals were then maintained at this temperature for a number of weeks. At specific times after the natural spawning date of the population, 10 individuals were transferred into separate containers with approximately 200 ml of filtered seawater at 17 °C. 'Cold-treated delayed' spawning individuals from Dunbar followed the same exposure to 17 °C as 'delayed' spawning individuals, but they were subsequently exposed to 21 days at 5 °C. This 5 °C exposure was after two days exposure at 10 °C to reduce any temperature shock.

2.5. Flow-through system: delayed spawning

Individuals were collected from Red Wharf Bay, N. Wales at the beginning of October one month prior to the natural spawning date.