Reproduction of *Marphysa sanguinea* Annelida, Polychaeta (Eunicidae), at Mount Edgecombe, Plymouth, near the type locality in Southwest England

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Abstract

The reproductive cycle of *Marphysa sanguinea* is described for a population at Mount Edgecombe, Plymouth, near the type location in Southwest England, using a data set obtained previously (October 1999 to September 2000). The species is iteroparous without schizogamy, spawning prior to October 1999 and during a short breeding season in 2000 from end August through September. The sexes are separate with a sex ratio of 1:1. Mature oocytes and spawned eggs are $215\,\mu m$ in diameter and spermatozoa of the ectaqua sperm type. Mature gametes of both sexes are discharged through paired coelomoducts, and the diploid chromosome number is 28. Proliferation of new coelomic gametes from paired gonads began within a month of spawning and continued for 8–9 months but ovulation was suppressed in June and July. Attempts to undertake fertilisation using spawned oocytes and active spermatozoa were unsuccessful. The size of discharged oocytes suggests a short pelagic larval duration of a few days. This is the first publication about the reproduction of this species, and our results suggest that *M. sanguinea* is restricted to intertidal areas in SW England, NW France and southern North Sea. The highly synchronised pattern of reproduction observed is not compatible with a quasi-cosmopolitan species range indicating that this species has been mistakenly reported from around the world. Future studies of the genus should combine rigorous taxonomy with observations of reproduction to facilitate comparison among *Marphysa spp*.

Introduction

Marphysa sanguinea (Montagu, 1813) was first described from Cornwall (England) as *Nereis sanguinea* and it is the type species of the genus *Marphysa* Quatrefages, 1866. However, because of the brief nature of the initial description and absence of known type material, many different species have subsequently been erroneously assigned to the type species (Lavesque *et al.*, 2019), thereby giving the spurious impression that this species had a broad worldwide distribution. A number of records of this species have now been redescribed as new species (see Glasby *et al.*, 2019; Hutchings and Karageorgopoulos, 2003; Lavesque *et al.*, 2017; Hutchings *et al.*, 2020) but the redescription of the species casually assigned to *M. sanguinea* is ongoing (Liu *et al.*, 2017) and other species, no doubt, still await description (Karageorgopoulos, 2003; Lavesque *et al.*, 2017). Far from having a quasi-cosmopolitan distribution, *M. sanguinea* is now thought to be restricted to Southern North Sea (Hutchings *et al.*, 2012), and to the south-west Atlantic coast of France at Arcachon (Lavesque *et al.*, 2019; Martin *et al.*, 2020).

Differences in the ecology and physiology of some of the species erroneously assigned to *M. sanguinea* are known, being considered variously to exhibit deposit feeding (Onozato *et al.*, 2010; Nishigaki *et al.*, 2013) and herbivory in *Marphysa formosa* Steiner & Amaral, 2000 (Pardo and Amaral, 2006) in addition to carnivory which is considered typical (Jumars *et al.*, 2015). Such ecological and physiological variability is highly likely to have resulted in scientific confusion, as putative members of this so-called cosmopolitan species have been used in investigations of their biochemistry, biology, physiology, genetics and genomics (see references in Lavesque *et al.*, 2019). For example, a study dealing with the development of the gonads and reproductive cycle of a putative *M. sanguinea* population was based on specimens collected in China (Yu *et al.*, 2005), but it is very unlikely that those individuals indeed belonged to this species. There has been no description of the reproduction nor reproductive cycle of the type species hitherto and we address this problem using a data set collected earlier (Karageorgopoulos, 2003).

Karageorgopoulos (2003) investigated the aspects of the biology of *M. sanguinea* (collected near the type location for the species) and compared these traits to some other eunicids in order to gain a better understanding of their potential for aquaculture. This was considered important because of the emergence of 'bait fisheries' for species formerly assigned to *M.*

sanguinea, for instance *M. mullawa* Hutchings & Karageorgopoulos, 2003, in Queensland (Australia) (Glasby and Hutchings, 2010) and *M. victori* Lavesque *et al.*, 2017, in Arcachon Bay (France) (Lavesque *et al.*, 2017). Karageorgopoulos (2003) concluded that *M. sanguinea* was not readily adaptable for aquaculture because of their sensitivity and trauma on collection, and the difficulty he experienced in inducing spawning and fertilisation. It was therefore not possible to complete the life cycle (from egg to egg) under culture conditions, which is a requirement for truly sustainable aquaculture. The data set obtained included details of gametogenesis, sexual maturation and spawning, and the temporal organisation of the reproductive cycle. Given that substantial variation has been revealed in the patterns of reproduction of species formerly, or still erroneously, given the designation *M. sanguinea*, we believe that this data set, though obtained some 20 years ago in 1999 and 2000, provides an opportunity to describe in detail the reproductive cycle of this species without unnecessary environmental or faunistic damage. This will facilitate comparison with other species of *Marphysa* as the re-evaluation of the genus progresses and will be of value to taxonomists and ecologists alike. The locality where the samples were obtained is now subject to protection, and large-scale, repeated sampling is unlikely to be approved.

Most members of the Eunicida are gonochoristic and iteroparous. Some species of *Palola* Gray in Stair, 1847, exhibit a form of schizogamy in which terminal segments detach at the time of reproduction and take part in a highly synchronised spawning event, subject to tight biorhythmic regulation (Caspers, 1984) and, by analogy with other polychaetes, is likely to be under the ultimate control of the genomic canonical clock (Tauber *et al.*, 2004; Olive *et al.*, 2005). Highly synchronised spawning is also likely to involve release of pheromones and other chemical signals (Watson *et al.*, 2003), but no information is available for eunicids. Schizogamy has not been described for any species of *Marphysa* examined so far.

In analysing in detail this data set our aims are to (i) describe the progress of gametogenesis that culminates in the production of sexually mature animals at the appropriate time, (ii) establish the time of breeding in this species at the type locality, (iii) set our observations in the context of environmental changes occurring at the type locality, (iv) assess possible impacts of changing climatic conditions and (v) facilitate comparison with other members of the genus including species that may have been formerly misidentified as *M. sanguinea* to provide for a more comprehensive understanding of the genus and its reproductive strategies.

Materials and methods

Collection and examination of coelomic contents

Samples of *M. sanguinea* were collected from rocks in the littoral zone extending for approximately 300 m along the exposed shore line and for 100 m down the shore, at Mount Edgecombe (50° 21′10″N, 04°09′30″W), Plymouth Sound, UK. The shoreline was dominated by an outcrop of sandstone and shale extending to the sublittoral.

Marphysa sanguinea was found in rocks from approximately 0.5 m above chart datum to 0.6 m below chart datum, and possibly further into the sublittoral. The maximum population density was around 10 individuals m^{-2} and this species was the most common macro-polychaete found in the substratum. The specimens of *M. sanguinea* occupied permanent, blind ending burrows with a single entrance that seem to have been constructed within natural fissures and crevices in the rock. The galleries were lined with mucus and could reach 0.5–1 m in length. The polynoid polychaete *Malmgrenia marphysae* (McIntosh, 1876) was found in about 5% of the burrows.

Sampling expeditions were carried out monthly, at spring tides, between October 1999 and September 2000. Specimens could only be obtained by detaching parts of the sandstone/ shale substratum to reveal the animals in their mucus-lined burrows. The number of specimens collected was therefore restricted to the minimum thought necessary to establish the seasonal cycle and was restricted to one complete cycle only, to minimise the environmental impact. Table 1 shows the sampling dates, the numbers of specimens taken, their sex and the position of each sampling site relative to chart datum. At a height of 0.4 m above chart datum, the sampling time was restricted to approximately 2 h.

Specimens were first placed in an insulated cool box, then kept overnight in a recirculating aquarium (Plymouth Marine Laboratory) and transported to Newcastle in a cool box the next day. The specimens were then placed in separate, shallow polystyrene dishes, with 100 ml of 0.2 μm-filtered seawater and kept at constant 10°C, in darkness and examined within two weeks of collection. The sex of each specimen was established by light microscopic examination of a small sample of coelomic fluid; for gravid worms it was also possible to determine the gender from their colour, since the green eggs or milky sperm are visible through the body wall. Coelomic fluid containing gametes was obtained using modified glass pipettes, their tips drawn to produce a finely tapered glass syringe, which are easily inserted through the muscular body wall. Live specimens were lightly held with wet paper towels and the glass syringe was inserted into the body cavity near the parapodia to extract small volumes of coelomic fluid with minimum negative pressure; each worm had coelomic fluid removed from at least three different segments

Table 1. Numbers of *Marphysa sanguinea* collected from Mount Edgecombe, Plymouth Tidal elevations are in meters above chart *datum*

Date	Tidal elevation	Females	Males	Immature
25/10/99	0.6	10	13	7
24/11/99	0.5	16	11	11
24/12/99	0.5	No sample		
23/01/00	0.4	14	16	12
21/02/00	0.3	13	13	9
20/03/00	0.3	14	17	11
19/04/00	0.4	13	9	9
05/05/00	0.5	13	16	13
04/06/00	0.5	13	13	14
03/07/00	0.5	11	9	12
01/08/00	0.5	8	14	9
31/08/00	0.3	15	11	17
28/09/00	0.4	15	15	8

from the middle of the body and the samples were pooled prior to analysis. Animals survived this procedure and could be maintained in the aquarium for up to two weeks.

Upon extraction, a small amount of pooled coelomic fluid was diluted with one drop of filtered seawater onto a microscope slide and observed with a microscope equipped with a video camera linked to a PC. Two to four random microscope fields of view, containing oocytes, were digitally captured for each female. The diameter of 50 oocytes or, when multiple oocyte cohorts were present, 100 oocytes were measured. The accumulated data were stored and further analysed in MINITAB 5.0 and MICROSOFT EXCEL 2000.

The coelomic fluid extracted from males was either devoid of developing germ cells, or contained predominantly one of the following stages of male germ cell development: (i) *small platelets* ($\leq$40 μm), (ii) *larger platelets*, i.e. groups of translucent male germ cells associated in an irregular ovoid disc up to 200 μm longer axis, (iii) *short tails*, i.e. platelets with spermatozoa with visible short tails, (iv) *morulae*, in which there were abundant long tails, one for each of the numerous spermatozoids, and (v) *dissociated sperm*, in which the morulae had broken down to release free spermatozoa.

Visualisation of meiotic chromosomes in females

Stock solutions of Hoechst 33342 (1 mg ml^{-1}) (Sigma, Gillingham, Dorset, UK) were prepared in distilled water and stored at −20°C until required. From the latter, more dilute solutions (10 μg ml^{-1}) were made in 0.2 μm-filtered seawater and these were added to seawater containing eggs, to a final dye concentration of 1 μg ml^{-1}. The oocytes were incubated in the dye for 20 min at room temperature and their chromosomes were examined with fluorescence microscopy. The Hoechst-incubated oocytes were mounted on microscope slides with coverslips and viewed with a Leitz Dialux 20 microscope, fitted with a HBO 50 W/AC mercury short arc lamp, with filter block A (exciting at 340–380 nm and suppressing at 430 nm). Photographs of the chromosomes were taken on the WILD Photoautomat MPS25 stills camera mounted on the Leitz Dialux 20 microscope approximately half an hour after the slide preparations were made. This lapse in time allowed for the compression of the eggs by the coverslip, which in turn freed the germinal vesicle from the obscuring yolk and produced lucid images of the chromosomes.

Sexual maturation of gravid male specimens

To assess sperm activation, one drop of 'dry sperm' (i.e. as removed from the coelom) was diluted in 10 ml of 0.2 μm-filtered seawater and a small volume of that was immediately viewed under the microscope for signs of sperm movement.

Histology

Approximately ten consecutive segments, from the mid region of the body of five worms, were isolated by transverse section using a scalpel and immediately fixed in Bouin's solution for 48 h, then washed in tap water and dehydrated. After clearing in toluene and paraplast infiltration, ribbons of serial transverse sections (7 μm thick) were cut on a rotary microtome. Short lengths of the ribbons were flattened onto albumen smeared slides using water and a hot plate, dewaxed in toluene, rehydrated, stained with haematoxylin and eosin and permanent preparations prepared following standard histological procedures (Humason, 1962).

Statistical tests

All statistical tests were performed on MINITAB 5.0. A χ^2 test was utilised to determine if sexes deviated from 1:1 ratio. The relationship between oocyte diameter and chromosome stage was established with a one-way analysis of variance (ANOVA). Five to ten animals were selected, having egg diameters that when combined would include the complete oocyte size range and oocyte size frequency distributions obtained. For these specimens the diameter of each stage of meiotic prophase (as determined by their chromosomes) was measured. The diameter size distribution was tested for normality (Anderson–Darling test, see Scholz and Stephens, 1987) and homogeneity of variance (Bartlett's test following Sokal and Rohlf, 1987), in the absence of which the data were transformed (squared). Subsequent pairwise differences between the diameters of primary oocytes at different stages of meiotic prophase I were detected with Tukey's test.

Results

Sexual condition

The sexes in *M. sanguinea* are separate, as none of the specimens examined (see Table 1) shared both male and female gametes. The numbers of males and females did not significantly depart from a 1:1 ratio ($\chi^2 = 0.013$, df = 1, $P < 0.05$).

In females, there are two discrete ovaries (left and right) in almost all segments (Figure 1A). These prominent organs, alongside the ventral, longitudinal muscle blocks, are associated with a large, lateral blood vessel, which it completely envelops. The ovaries consist of a proliferative epithelium close to the blood vessel with oogonia and an array of differently sized primary oocytes. The primary oocytes remain in the ovaries until they attain a diameter of 35–40 μm, upon which they are released into the coelom. Once in the coelom, the oocyte progress through the prophase of meiosis I and undergo solitary vitellogenesis (i.e. without follicle cells or nurse cells) until they eventually reach a measured size of 215 ± 6 μm.

In males, paired testes are similarly found in segments throughout the main body region. Testes have an overall similar appearance to the 'bunch of grapes' appearance of the ovaries. Spermatogonia are released into the coelom, where mitotic proliferation generates groups of associated undifferentiated male germ cells, designated 'platelets'. Subsequently meiosis is completed and spermiogenesis takes place, culminating first in the development of 'morulae' in which developed sperm with long tails remain associated together, and which eventually break down to release differentiated spermatozoa which accumulate in very large numbers in the coelom. Platelets, morulae and finally dissociated sperm were observed by light microscopy and scanning and transmission electron microscopy (Karageorgopoulos, 2003), which concluded that a distinct cytophore was absent.

Prophase and metaphase of meiosis I in coelomic primary oocytes and chromosome number

The Hoechst 33342 stain proved to be a very reliable and relatively simple method of visualising the chromosomes of oocytes. Figure 1B–E depicts the chromosomes observed in *M. sanguinea* primary oocytes while in the coelom and Figure 1F shows them after their discharge from females in the laboratory. Primary oocytes in the ovaries and small oocytes in the coelom were interpreted as being in leptotene of prophase I, having a characteristic appearance (Figure 1B) with a disproportionately large nucleus, containing numerous strands of heterochromatin. Larger oocytes reach zygotene of meiosis I and the genetic material condensed sufficiently for individual chromosomes to be visible
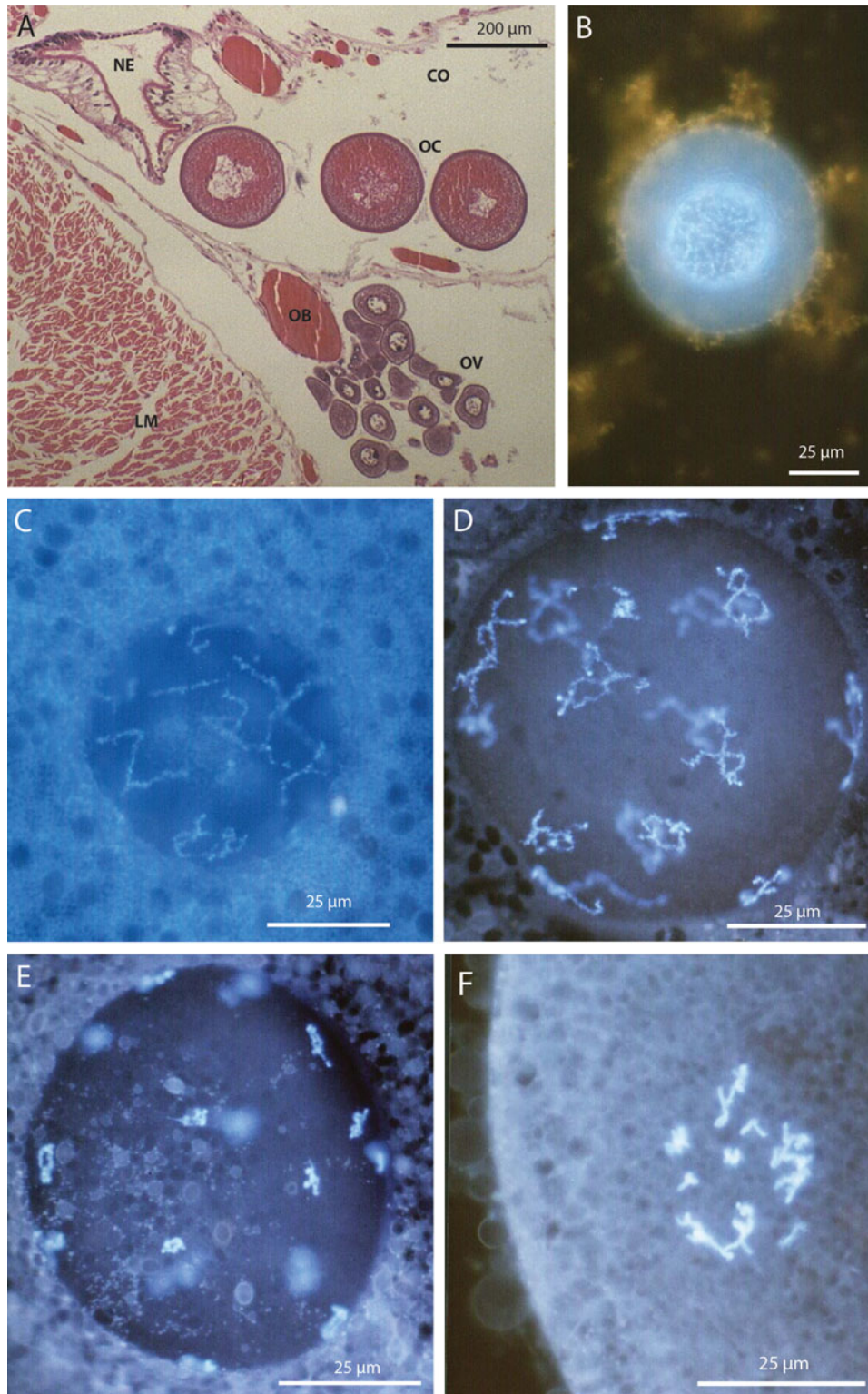
Figure 1. (A) Photomicrograph of a transverse section of the ventral, left quarter of the body cavity of *Marphysa sanguinea*. Co, coelom; LM, longitudinal muscles; Ne, nephrostome; OB, ovarian blood vessel; Oc, oocyte; Ov, ovary with primary oocytes; scale bar, 200 μm. (B–F) Stages of prophase and metaphase of meiosis I for *M. sanguinea* as visualised by fluorescence microscopy of Hoechst 33342 stained oocytes; (B–E) stained primary oocytes from the coelom; (F) chromosomes of spawned oocytes. (B) Leptotene; (C) zygotene; (D) pachytene–diplotene; (E) diakinesis; (F) metaphase. Scale bars in figures B–F, 25 μm.

(Figure 1C). Following zygotene, the nucleolus disappears, and the nucleus enlarges to form the germinal vesicle as is characteristic of oocytes during vitellogenesis. Eventually the chromosomes condense further, and homologues come together to form tetrads that are displaced peripherally in the germinal vesicle, this being characteristic of primary oocytes that have reached the pachytene–diplotene stage (Figure 1D). Chiasmata between non-sister chromatids of homologous chromosomes can be observed (Figure 1D). The termination of meiotic prophase I in the coelom culminates with the onset of diakinesis, during which the bivalents move to the metaphase plate and their number can easily be determined (Figure 1E). A maximum of 14 tetrads could be observed, which corresponds to the haploid chromosome number indicating that the diploid nucleus of this species has 28

chromosomes. The chromosomes are by this time greatly condensed and the maturation of the oocytes becomes arrested at this stage.

The opportunity was taken to observe the chromosomes of recently spawned oocytes (Figure 1F). The nuclear membrane has disappeared (i.e. germinal vesicle broken down) and the chromosomes have repositioned on the metaphase plate. Some chromosomes have the characteristic 'v' shape as the spindle fibres are beginning to separate them towards opposite poles of the cell indicating that re-initiation of meiosis has occurred and the oocytes, now in metaphase of meiosis I, were likely to have been ready for fertilisation. However, on no occasion was successful fertilisation of such eggs achieved, despite numerous attempts.

Annual reproductive cycle

For much of the year, from October to June, individual females were found to contain both growing oocytes with a diameter up to a maximum of 140 μm and small oocytes newly released from the ovary. During this period substantial variation was observed between individuals collected on the same occasion. The frequency distributions obtained from individuals could be variously highly skewed, bimodal or polymodal, suggesting that continued intermittent ovulation of batches of oocytes continued while vitellogenesis was ongoing.

In order to provide a picture of the overall cycle of oogenesis at a population level, pooled oocyte frequency distributions for the sampled population were determined for each time point (Figure 2; Supplementary data). The mean of the pooled oocyte diameter distributions followed an almost linear increase, during the period of vitellogenesis from September to June, when a size plateau of about 180 μm was attained. It is not, however, suggested that the progressive increase in diameter of the pooled oocyte samples represents the growth of individual oocytes. During the protracted period of gamete proliferation and vitellogenesis, the largest oocytes in the females were held at the threshold diameter of 180 μm, which is substantially less than the diameter of the fully mature oocytes. During this time the pooled oocyte frequency distributions became much more uniform in size (see Supplementary data) and the standard deviation of the pooled mean became progressively reduced, as seen in Figure 2.

The standard deviation of the pooled oocyte distributions is therefore an important statistic for interpreting the annual cycle. The number of individuals examined was similar for each sample (see Table 1) and change in the standard deviation is not likely to be due to sampling error. The standard deviation reduced sharply from June onwards to the time of maximum maturity shortly before spawning (see also Supplementary data). These changes strongly suggest that ovulation is suppressed from about May onwards allowing late released oocytes, as it were, to 'catch up' with ones that were ovulated earlier in the cycle. The oocytes escape the sub-mature size threshold of about 180 μm, which represented the upper bound of oocyte diameter during the period from October to May at this time (Figure 2; see also Supplementary data), and by August, before spawning, virtually all coelomic oocytes had increased in size to a diameter of approximately 215 μm at the time of spawning. Spawning occurred during the months of August and September 2000, but the exact dates of spawning could not be determined with greater precision because of the sampling regime adopted and the intertidal nature of the habitat.

The data summarised in Figure 2 are pooled population means for all the animals collected on each occasion except for the samples obtained on 4th of June 2000, when a few very much smaller animals (relaxed length much less than 10 cm) were sampled. These small individuals contained only small recently ovulated oocytes, whereas all the larger members of the population had much larger oocytes approaching the temporary threshold diameter of about 180 μm. Since there were obviously two types of individuals collected on that one occasion, they are shown separately in Figure 2. The small individuals may represent young recruits to the adult population perhaps able to spawn in their first year, or, such individuals may have retained their oocytes and would have progressed to spawn during the following year. Further information and interpretation are presented in the Supplementary data.

All the specimens of female *M. sanguinea* collected at the beginning of August 2000 were gravid and mature. The diameter of the uniformly sized coelomic oocytes had increased to a mean diameter of 215.4 ± 7.2 μm. The situation at the end of August was essentially the same (mean: 215.0 ± 5.8 μm) (see also Supplementary data). However, between 31/08/2000 and 28/09/2000, all the remaining female worms spawned and soon afterwards all the specimens collected contained batches of small eggs indicating that a new reproductive cycle had begun.

There is a strong relationship between the size of primary oocytes in the coelom and the stage of meiosis as revealed by fluorescence microscopy. The mean oocyte diameters (and standard deviations) at each stage are: leptotene 97.14 ± 33.79 μm, zygotene 199.19 ± 12.40 μm, pachytene 216.85 ± 6.51 μm and diakinesis 214.99 ± 5.54 μm. A one-way ANOVA detected significant differences between the diameter of oocytes at different stages of meiosis I (df = 3, $F = 1.1 \times 10^4$, $P < 0.0001$). Table 2 contains Tukey's pair-wise comparisons and as indicated, each meiosis I stage occurs in oocytes with significantly different sizes. Important cytological changes therefore occur as the oocytes grow during the months prior to spawning.

Table 3 shows for females the percentage (rounded to the nearest whole percentage point) of coelomic oocytes containing each of the defined stages of meiotic prophase (see Figure 1). In November, all the coelomic oocytes observed were at the leptotene stage of meiosis, from January onwards, cells classified as being in zygotene began to appear, and in July, all of the observed oocytes were at this stage. Oocytes in diplotene and then diakinesis appeared during August. Spent animals with no discernible coelomic oocytes became apparent in the population during late August and September.

The final cytological changes associated with maturation of the gametes occur in essentially the same time period in males. Table 4 shows the percentage of males containing predominantly each of the defined stages of spermatogenesis: small platelets, larger platelets, morulae with short tails, fully developed morulae and dissociated sperm at each of the monthly sample points.

Gamete maturation and spawning

Observations on the reproductive cycle were begun in October 1999, and continued through September 2000. Spawning was observed to have occurred in late August 2000, prior to which all females contained a single cohort of large oocytes close to the maximum diameter observed (Figure 2 and Supplementary data). Spent animals without coelomic gametes were first observed in the sample at the end of August. A year earlier in the sample obtained on 24 October 1999 when these observations were initiated, 80% of the females sampled contain only small eggs at leptotene. The remaining 20% had not begun to release their primary oocytes in the coelom.

Using these data covering an entire reproductive cycle, we conclude that it takes 8–9 months for the first released oocytes to progress through leptotene and zygotene to pachytene and beyond. However, for late released oocytes, the oocyte growth period will be much shorter, some oocytes progressing from first release
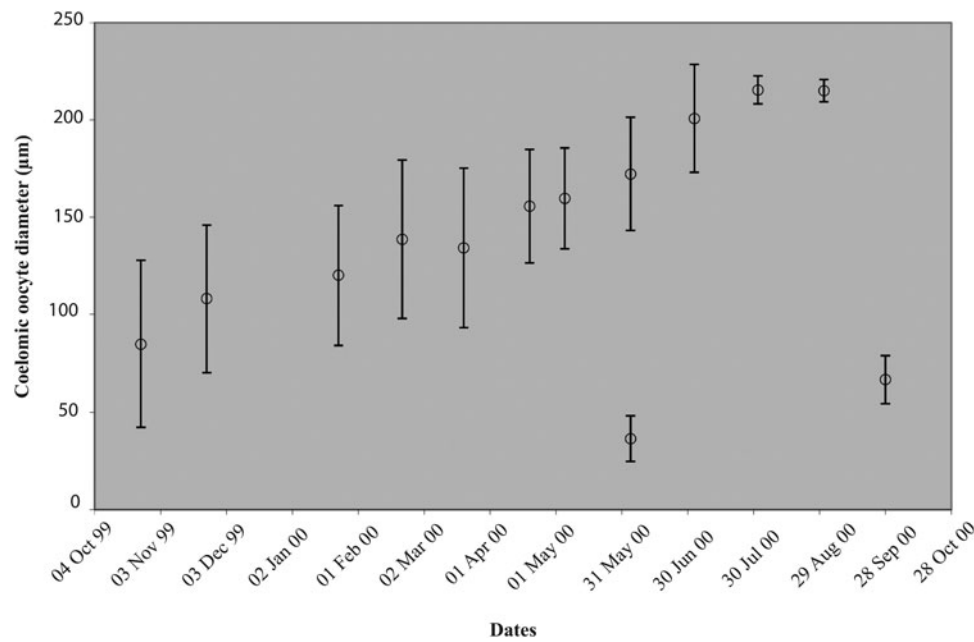
Figure 2. Annual reproductive cycle of female *Marphysa sanguinea* at Edgecombe Bay, Plymouth, UK. Pooled mean coelomic oocyte diameter for all females collected is shown for each monthly sample, or, when two obviously different categories of female were present (4 June 2000), the oocyte diameter for the two were calculated and the mean diameters shown separately. The vertical bars represent ±1 standard deviation.

into the coelom to maturity in 2–3 months. Eggs at pachytene–diplotene and diakinesis were observed only in samples taken on 1 August and 31 August 2000. This represented the culmination of the 1999/2000 proliferative phase of the ovaries.

Paired nephridia/coelomoducts (see Figure 1A) were present in each segment in which gametes develop, and, at the time of spawning, they served for the emission of the gametes. The spawning process was observed directly for only five females in the laboratory, and these provided the material for examination of oocyte nuclei initiating metaphase I of meiosis, as illustrated in Figure 1F. It was not possible to induce spawning at will; several attempts to fertilise fully developed eggs taken from the coelom with spermatozoa showing movement after dispersal in seawater were unsuccessful.

Discussion

Sexual conditions and gametogenesis

The gametogenic cycle *M. sanguinea* conforms exactly to the paradigm of a large-bodied iteroparous (polytelic) polychaete, semelparity in large-bodied polychaetes only being observed in the Nereididae (Olive and Clark, 1978). There is no morphological evidence for schizogamy (Schroeder and Hermans,

Table 2. Comparison of diameter among oocytes at different stages of meiosis I Tukey's pair-wise comparisons

	Leptotene	Zygotene	Pachytene
Zygotene	−29,824		
	−28,683*		
Pachytene	−37,184	−7933	
	−35,794*	−6537*	
Diakinesis	−36,241	−6991	119
	−35,108*	−5851*	1509*

Asterisks denote significant differences.

1975), as found in some *Palola* Gray in Schar, 1847 (see Hofmann, 1974; Caspers, 1984), and we conclude that this feature is absent.

The ovary organisation of *M. sanguinea* is similar to other members of the genus that have been examined, e.g. *Marphysa* cf *sanguinea* from Italy (Prevedelli, 1989; Cassai and Prevedelli, 1998) and *Marphysa mullawa* Hutchings & Karageorgopoulos, 2003 from Australia (Hopper, 1994). However, when compared to the ovaries of other species of the Eunicid complex, important differences become apparent. For example, the ovaries of *Palola siciliensis* (Grube, 1840) contain a single cohort of oogonia that simultaneously progress through oogenesis (Hofmann, 1974, 1975). In contrast, the ovaries of *M. sanguinea* have a stratified arrangement of oogonia and primary oocytes, in a variety of sizes (Figure 1A and Supplementary data). More interestingly, the proliferative and pre-vitellogenic oocyte growth in these organs appears to be continuous throughout most of the year. Even though considerable variation exists between individuals, new cohorts of primary oocytes are regularly released into the coelom of the animals (ovulation) during most of the year, except during the two months prior to spawning, when ovulation is suppressed (Figure 2 and Supplementary data). Even in the two months prior to spawning, the ovaries remain ready to release a new cohort of eggs, and this begins very soon after spawning, as the ovaries do not regress or enter a quiescent stage. In this respect, *M. sanguinea* differs from several other iteroparous polychaete species (Bentley and Pacey, 1992). In *M. sanguinea*, the ovaries are permanently present in adult worms and always contain oogonia and small oocytes.

The ovary of *M. sanguinea* behaves as a cell renewal system (Olive, 1972a, 1972b), subject to a constraint preventing ovulation for the two months prior to the breeding season; this ensures that oocytes that would otherwise be produced just before the breeding season are not 'wasted' at breeding and is functionally related to breeding in which age-specific fecundity, an important component of fitness, is determined by body size and oocyte volume (Olive *et al.*, 2000, 2001). Such a system with cell renewal properties in polychaete ovaries was first demonstrated for *Cirratulus cirratus* (Müller, 1776) by Olive (1971), though in that case,

Table 3. Percentage of females with oocytes at each meiotic stage (see Figure 1) (rounded to nearest whole percentage point)

Date	Leptotene	Zygotene	Diplotene	Diakinesis	Spent (no coelomic oocytes)
25/Oct/2019	80				20
24/Nov/2019	100				
23/Jan/2000	83	17			
21/Feb/2000	61	39			
20/Mar/2000	66	34			
19/Apr/2000	43	57			
5/May/2000	57	63			
4/Jun/2000	43	57			
3/July/2000		100			
1/Aug/2000		22	46	22	
31/Aug/2000		3	67	20	
23/Sep/2000				54	46

unlike in *M. sanguinea*, mature *C. cirratus* females, in which oocyte proliferation was suppressed, were found throughout the year. There was therefore a lack of inter-individual synchrony in *C. cirratus* (Olive, 1970). Such a situation is highly unusual, and in most large-bodied polychaetes that breed in non-tropical, temperate, boreal or polar regions, a degree of seasonal synchronisation is observed. The functioning of the ovarian cell renewal system is, in such cases, an important element in seasonal adaptation (Olive *et al.*, 2000).

In *M. sanguinea*, the new cycle of oocyte proliferation and ovulation begins immediately after spawning, this is also observed in the lugworm *Arenicola marina* (Linnaeus, 1758), which exhibits a similar timing of reproduction to that of *M. sanguinea*, with spawning occurring in September, but the ovaries and testes regress after spawning. In such cases environmental inputs may be required to initiate a new cycle of gamete production (Garwood and Olive, 1982). Such inputs are also important proximate controls in the annual breeding cycle (Olive and Clark, 1978; Olive, 1983a; Eckelbarger and Hodgson, 2021).

Vitellogenesis in *M. sanguinea* is clearly of the extraovarian type and it commences upon the release of the small oocytes into the coelom. In the absence of any abundant or prominent coelomocytes, nurse cells or other accessory cells in the coelom, it is reasonable to conclude that oocytes undergo solitary vitellogenesis (Eckelbarger and Hodgson, 2021), as observed for some other *Marphysa* species (Prevedelli, 1989; Prevedelli *et al.*, 2007). Presumably, the oocytes obtain large molecular weight yolk precursors from the coelom, by receptor-mediated endocytosis (i.e. heterosynthesis) or synthesise vitellin in the ooplasm from exogenous, low-molecular weight precursors (i.e. autosynthesis) (Eckelbarger and Hodgson, 2021). It is interesting to note that in genera of several eunicid families (notably *Diopatra*, *Hyalinoecia*, *Onuphis* and *Ophryotrocha*) nurse cells with a presumptive trophic role are associated with each developing oocyte (Anderson and Huebner, 1968; Emanuelsson, 1969; Pfannenstiel, 1978; Paxton, 1979, 1986; Brubacher and Huebner, 2009; Eckelbarger and Hodgson, 2021), but there are no nurse cells or follicle cells in the *M. sanguinea* oocyte production system.

In male *M. sanguinea*, as in the majority of other polychaetes (Olive, 1983b), the several cycles of mitotic proliferation and the completion of meiosis and spermiogenesis takes place in the coelom, rather than the testes. Spermatogonia are released from the testes into the coelomic cavity where, after several mitotic divisions, platelets with up to 300–400 spermatids are formed. This process commences soon after spawning and the interval between first release into the coelom for some cell clusters is 7–9 months, but much less for clusters released from the testis later in the annual cycle. Synchronisation occurs between and within males only 1–2 months prior to spawning, when dissociated sperm (that becomes active when in contact with seawater) are present in all males.

In polychaetes, it has been repeatedly demonstrated that sperm morphology is more closely related to the functional biology of reproduction than to phylogenetic affinity (Rouse, 1999). The sperm of *M. sanguinea* is of the ect-aquasperm type and scanning electron microscopy reveals that the mature sperm has a spherical head with a small terminal acrosome (Karageorgopoulos, 2003). The ect-aquasperm type is correlated with reproductive traits such as external fertilisation of relatively small eggs and indirect development (Olive, 1983b; Jamieson and Rouse, 1989).

Annual breeding cycle and timing of reproduction

The population of *M. sanguinea* at Mount Edgecombe, Plymouth Sound had completed spawning when first examined (October, 1999) and started spawning again in the last few days of August 2000 and, by the end of September 2000, all the animals had spawned. However, due to the monthly interval between consecutive sampling trips, it was not possible to pin-point the exact duration of the spawning season. Spawned oocytes are in metaphase (Figure 1F) and unspawned eggs remained in prophase indefinitely. The ability of the nephrostome to distinguish between prophase and metaphase oocytes, which this implies, was first demonstrated in *A. marina* (Linnaeus, 1758) by Howie (1961), and it appears that in *M. sanguinea*, re-initiation of meiosis and spawning is similarly linked in a causal relationship, but any endocrine or exocrine factors are unknown.

Environmental correlates: proximate and ultimate determinants

The observation of highly synchronised reproduction in *M. sanguinea* is not compatible with the former concept of it being a so-called 'cosmopolitan species', i.e. with a very wide geographical range (Hutchings and Kupriyanova, 2018). Disparate populations with highly synchronised reproduction and epidemic spawning would be expected to have highly specified responses to local conditions, with direct impacts on fitness (Lewis *et al.*, 2002) and in fact to be genetically isolated. Dispersed larvae from populations adapted to different local conditions would not be expected to

Table 4. Percentage of males with gametes at each stage of spermatogenesis (rounded to nearest whole percentage point)

Date	Small platelets	Large platelets	Short tails	Morulae	Dissociated sperm	Spent
25/Oct/2019	78					22
24/Nov/2019	100					
23/Jan/2000	100					
21/Feb/2000	86	14				
20/Mar/2000	69	31				
19/Apr/2000	54	46				
5/May/2000	21	43	36			
4/Jun/2000	19	32	49			
3/July/2000	17	44	32	7		
1/Aug/2000	22	23	45	10		
31/Aug/2000	9			72	19	
23/Sep/2000	75					25

have the same phase relationships in terms of timing of reproduction as the local population, and would not therefore mature at the same time to breed with them, giving them extremely low fitness.

In recent years, advances have been made in understanding the genomic and chemical signalling of sexual maturation and the linkage to environmental signals this provides, most notably in Nereididae (Andreatta *et al.*, 2020). While it is highly likely that signalling systems exist to facilitate the highly synchronised reproduction of large Eunicidae, no suitable model systems for investigation of such phenomena have emerged. The larvae of *Marphysa* spp. are not abundant in the zooplankton (Bhaud and Cazaux, 1987; Young *et al.*, 2002), which would be compatible with a relatively short (less than one month duration) pelagic phase, and the maximum oocyte diameter of about 215 μm similarly suggests that in *M. sanguinea*, there is a short-lived free-living larva, not adapted to widespread dispersal.

Our data show that spawning had been completed and a new cycle of germ cell proliferation had begun by October 1999, and that spawning occurred only during late August, and early September, in 2000. Inevitably a short spawning period such as this has a fixed phase relationship with two dominant annual environmental cycles, seawater temperature and photoperiod. The phase relationships between reproductive events and these two cycles are illustrated in Figure 3. The timing of spawning is such that it occurs towards the end of a period of about three months, when ambient seawater temperatures in Plymouth Sound typically remain near the maximum (~17°C) (Maddock and Swann, 1977). This period is also one when daylength is decreasing at near the maximum rate, from the longest day (at Plymouth LD 16.5:7.5) at the summer solstice towards LD 12:12 at the autumn equinox in September.

There are differences between these cycles in terms of their suitability to entrain biological cycles. The annual cycle of seawater temperature is an inherently 'noisy' signal, as well attested for the Plymouth region (Maddoc and Swann, 1977), to which variability, anthropogenic influences may be causing additional instability and short-term change. In contrast, the LD cycle, like the other geophysical cycles (lunar, semilunar, tidal and diel) are invariant over ecologically significant time scales (decades to millennia). They change only at an extremely slow rate over geological time scales due to lunar-tidal friction, which slows the rotation of the earth (increasing the duration of the solar day), and similarly increasing lunar distance (see references in Olive

et al., 2005). Genomic autoregulatory clock systems manifestly adapt to these changes (Tauber *et al.*, 2004).

Marphysa sanguinea, as an intertidal animal, will experience strong tidal, diel, semilunar and lunar environmental cycles. These are likely of biological significance to this species, because the time of optimal foraging is likely to be when it is dark (no predating birds) and when the tide is retreating from the burrow (no large fish) but the prey organisms of *M. sanguinea* may still be exposed at the surface, having not yet retreated to their own intertidal burrows or sheltered habitats. The large intertidal polychaete *Alitta virens* exhibits such a combination of circadian and semi-diurnal tidal and lunar rhythms to predict this time as it tracks the subjective night (Last *et al.*, 2009). It is not unreasonable to suppose that in *M. sanguinea* a similar repertoire of entrained biological rhythms may exist, as has been demonstrated in other large polychaetes with a large supra-oesophageal ganglion and well-developed sensory system, but more amenable to experimental investigation (Watson *et al.*, 2000a; Last and Olive, 2004; Olive *et al.*, 2005); notably in *Platynereis dumerilli* (Audouin and Milne Edwards, 1883), which has become the standard polychaete genomic model (Zantke *et al.*, 2014). Such biological rhythms are also observed in diverse geographic regions (Last *et al.*, 2020).

Implications in relation to climatic changes

Our observations were made more than 20 years ago and it is possible that in addition to natural variation, long-term changes in sea surface temperature may be occurring, which may be expected to have fitness impacts (Lawrence and Soame, 2004; Shanks *et al.*, 2020). In this context, it is germane to distinguish between the 'ultimate' and 'proximate' causes of a highly synchronised pattern of reproduction (Olive, 1992; Reitzel *et al.*, 2004; Gourault *et al.*, 2019). Observations on the timing of reproduction in relation to external cycles do not *per se* establish what are the 'ultimate' or 'proximate' factors influencing the timing of the reproductive cycle, but we consider it highly unlikely that the temperature cycle alone is the predominant proximate factor because of the inherent variability of this signal. However, if there are ecologically significant temperature thresholds, or stages of development susceptible to environmental temperature, the phase relationship between these and the reproductive cycle may change, with consequent impacts on fitness.

The time of reproduction we describe delivers the lecithotrophic larvae into seawater close to the local maximum at, or
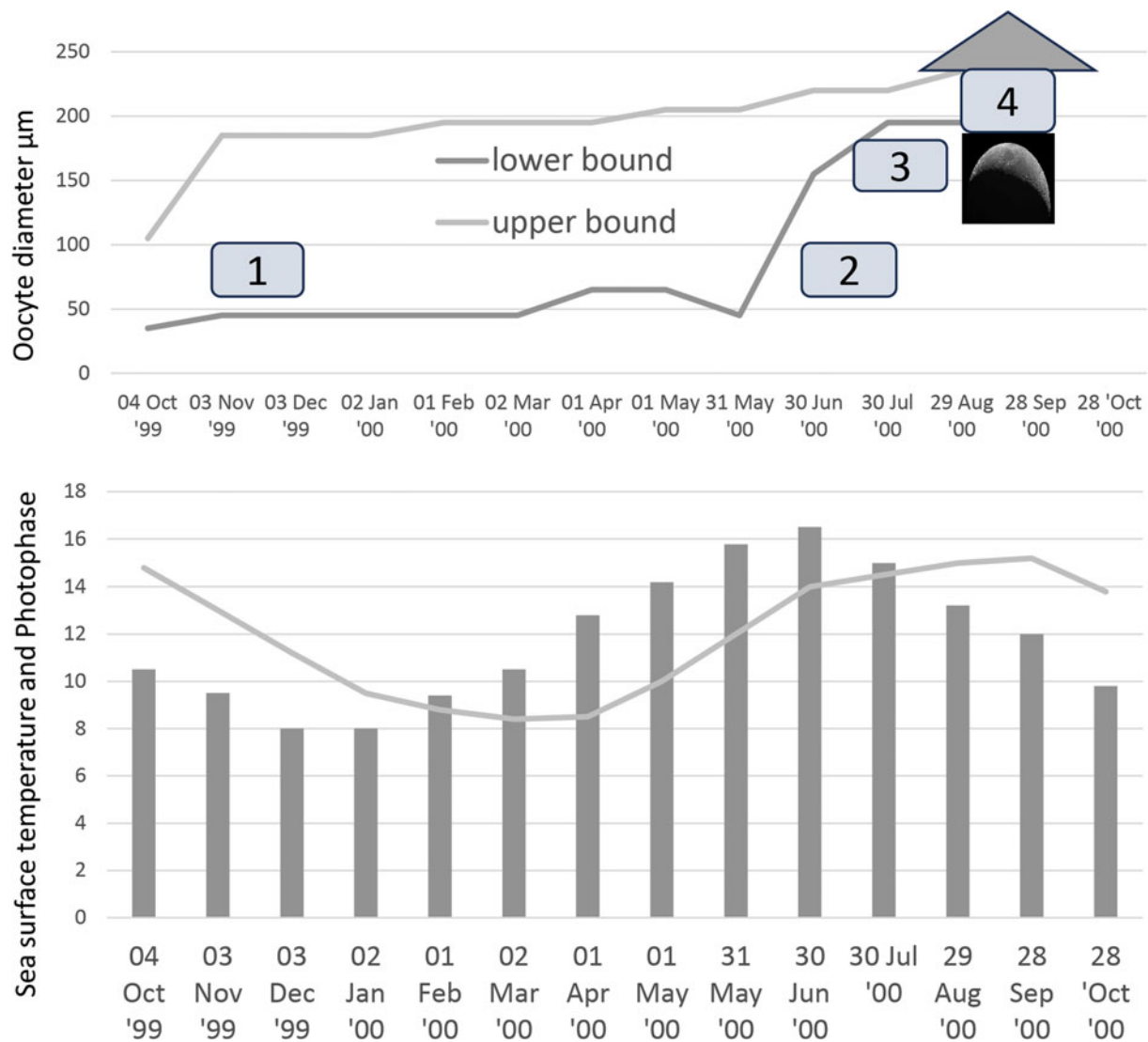
Figure 3. Schematic representation of the reproductive cycle of female *Marphysa sanguinea* during the observed year 1999–2000 at Edgecombe Bay, Plymouth, UK (upper panel), in relation to environmental parameters (lower panel – surface sea temperature °C, continuous line; photophase duration hr, vertical columns). The following principle phases of the reproductive cycle are indicated in the upper panel: (1) Extended period of ovarian proliferation, during which successive batches of oocytes in individual females are released from the ovary and grow to a sub-mature threshold size of c.180 μm. (2) Period of 'catching up' during which further ovulation is suppressed, and remaining oocytes proceed to the sub-mature size threshold. (3) Period of oocyte maturation, during which oocytes proceed through the later stages of prophase meiosis I, to diplotene/diakinesis, and increase in size to the mature size, mean diameter 215 μm. (4) Spawning period, when individual females release the entire complement of coelomic oocytes and enter a brief resting period (≤1 month) prior to initiation of the next cycle of oocyte proliferation. In addition to the two annual cycles shown here, the individuals would also be subject to diel (24 h) cycles, tidal cycles (12.4 h) and to the complex cycle of spring and neap tides (semilunar) cycle throughout the entire reproductive cycle.

near 17°C which then subsequently declines. In a study of a Mediterranean species of *Marphysa*, Prevedelli (1989) demonstrated an important link between the timing of reproduction and larval development, as spawning occurred when ambient seawater temperature was around 24°C and laboratory experiments showed that the rate of larval growth was reduced at 18°C and suppressed at 13°C.

Post settlement larvae and young adults of *M. sanguinea* in Plymouth Sound would then encounter autumn and winter with low temperature, short photophase and low light intensity. This is similar to the temporal organisation of the reproductive cycle observed in the lugworm *A. marina*, which also spawns in a highly synchronised manner in September around the British Isles (Howie, 1959; Watson *et al.*, 2000b). Such a pattern contrasts with that of the many spring-breeding polychaetes, the young adults of which would first encounter spring and summer conditions. One such species is *A. virens* (Sars, 1835), which, like the two former species, has a lecithotrophic larva with a short pelagic

phase of only a few days, but in which spawning occurs in the spring. Spawning may not deliver larvae into optimum conditions for either their fertilisation or larval growth however, but may enhance pre-emptive competition (Olive *et al.*, 2000, 2001).

In conclusion, our observations show the breeding and annual reproductive cycle of *M. sanguinea* is highly synchronised to a short period in the late autumn, and that the processes of gametogenesis are constrained to generate a uniformly mature population of coelomic germ cells all ready for spawning at that time. Our observations also reinforce the concept that only a short period of pelagic larval development, amounting only to a few days is likely. These observations are not compatible with the species having a widespread geographical distribution as has been formerly suggested. We strongly recommend that investigators finding specimens superficially resembling *M. sanguinea* should adopt the most rigorous taxonomic procedures that have recently been developed for this genus, requiring detailed examination of chaetae and parapodial structures, preferably at scanning electron

microscopical scale along the length of the body and to examine in detail the complex jaw apparatus. If possible investigators should also devise non-invasive mechanisms for determining the timing of reproduction in the populations from which their specimens are derived. It may, for instance, be possible to create larval traps that would capture larvae immediately after spawning or at settlement. Such procedures would enable a re-evaluation of the genus and lead to a more comprehensive and meaningful interpretation of changes in the distribution of *Marphysa* spp. consequent on changing climatic conditions.

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