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Embryonic and larval development in the semelparous Nereid polychaete *Hediste diversicolor* (OF Müller, 1776) in Norway: Challenges and perspectives

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Abstract

Knowledge of broodstock manipulation, gametogenesis, artificial fertilization and larval nutrition is a prerequisite to reach a large-scale production of the polychaete *Hediste diversicolor*. In this study, the characteristics of oogenesis and spermatogenesis and embryonic and larval development were observed. Moreover, the effect of temperature on embryonic development and effect of diets and sediment on earlier juvenile development were tested. Mature oocytes were characterized by a spherical shape with a diameter of about 200 μm. They showed radial symmetry with a large centric nucleus surrounded by four distinctive layers. Embryo cleavage started between 4 and 10 hr after fertilization, and developed to the swimming trochophore stage after 5–6 days after fertilization at 11°C. The rate of embryonic development increased with a temperature increase from 6.1 to 21.2°C. The trochophore larvae increased in length from day 9 and commenced differentiation into 3-setiger stage larvae at day 12. The length growth of larvae until 7-setiger stage was 0.43 mm/day when fed with fish feed, while those fed shellfish diet and smolt sludge both grew around 0.21–0.23 mm/day. The results suggest that fish feed is a superior diet compared to shellfish diets and smolt sludge for the early larval stage of *H. diversicolor*.

KEYWORDS

embryonic development, *Hediste diversicolor*, juvenile growth, larval development, oocyte

1 | INTRODUCTION

Hediste diversicolor (OF Muller, 1776) inhabits mucous burrows in brackish coastal areas and is widely distributed from the Norwegian coast in the north to the Mediterranean in the south (Budd, 2008). In recent years, the polychaete *H. diversicolor* has been intensively studied as a novel candidate for recycling aquaculture waste and producing substitutable aquafeed ingredients. Integration of polychaete biomass production with use of waste from land-based fish aquaculture is suggested to create environmentally sustainable businesses (Bischoff, Fink, & Waller, 2009; Brown, Eddy, & Plaud, 2011; Fang et al., 2017; Wang et al., 2019).

Hediste diversicolor is rich in fatty acids and has a balanced amino acid profile which makes this species an interesting

candidate for producing feed ingredients (Bischoff et al., 2009; Wang et al., 2019). To enter and sustain the increasing aquafeed market with polychaete raw materials, comprehensive, reliable year-round production of polychaetes is required. Intensive production calls for knowledge-based detailed production protocols covering all aspects of the reproductive cycle. Year-round supply of seeding material (i.e. juveniles) is considered as one of the major bottlenecks. Larvae production outside the reproductive season will therefore be critical for developing year-round polychaete cultivation.

For semelparous polychaetes such as *H. diversicolor*, sexual maturation means entering irreversibly the terminal phase of their life cycle (Bentley & Pacey, 1992). Unlike most members of the Nereidae family, for example, *Platynereis dumerilii* (Mercer & Dunne, 1973), *Nereis virens* (Creaser & Clifford, 1982), *H. japonica* and *H. diadroma* (Hanafiah, Sato, Nakashima, & Tosuji, 2006), and *H. diversicolor* do not undergo morphological and behavioural transformation during the reproduction season (Bartels-Hardege & Zeeck, 1990; Peixoto & Santos, 2016). This represents a possible difficulty when it comes to the collection of mature males and females for artificial fertilization and year-round production of juveniles.

While Wilson (1991) classified *H. diversicolor* as free spawning with lecithotrophic larvae, Bartels-Hardege and Zeeck (1990) described that the males of *H. diversicolor* crawl and release sperm outside the tubes of mature females, which actively introduce the sperm into their burrows by ventilation behaviour and then release their eggs into their tubes. Larvae and postlarvae were guarded by the females, and the benthic larvae fed on the mucus and microbial film covering the surface of the maternal tube. Because of the reproductive traits of *H. diversicolor* with brooding inside the tube without pelagic larvae, Bartels-Hardege and Zeeck (1990) found it difficult to collect natural spawned eggs or larvae. Dales (1950) and Smith (1964) did not observe natural spawning under laboratory conditions, but Dales (1950) distinguished ripe females as reddish brown and the males as bright green which enabled the selection of broodstock for artificial fertilization. Nesto, Simonini, Prevedelli, and Da Ros (2018) used naturally spawned eggs in the laboratory to breed, but the natural spawned eggs had lower fertilization rates compared with in vitro fertilization.

Ozoh and Jones (1990) found that fertilization of *H. diversicolor* was not affected in the ranges of temperature from 12 to 22°C, while the capacity adjustment of embryonic cleavage to increasing temperature deceased with increasing salinity. The developmental pace and success of polychaete *H. diversicolor* under different temperature were still lesser-known, which would need further exploration.

Smith (1984) reported that *H. diversicolor* larvae started feeding at the 4- to 5-setiger stage. Marty and Retière (1999) indicated that the larvae acquired burrowing ability at the 6-setiger stage, and Bartels-Hardege and Zeeck (1990) found that larvae developed to 7 to 8 segmented larvae 10–14 days post fertilization, where the female died and the larvae left the burrow.

The aim of this study was to supplement the information on reproduction and early larval development of *H. diversicolor*. The results from several trials on selected culture conditions of broodstock, oogenesis, in vitro fertilization, embryonic development and larval rearing are presented. This will provide information to develop year-round production of this species.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

The use of worm in this study was under the guidelines of the National Committee for Research Ethics in Science and Technology (NENT), Norway, and Directive 2010/63/EU.

2.2 | Broodstock sampling and handling

The broodstock ($n \approx 60$) were collected from the natural field in Leangenbukta Bay, Trondheim, Norway, in mid-February 2019. At the laboratory, the worms were kept in tanks with sediment (ceramic clay with grain size 0.5–2 mm, Alt, Keramikk AS, Norway), with gradually increasing daylength from 8 to 13 hr and temperature from 2 to 6°C as shown in Figure 1. The change in daylength and temperature in the laboratory mimicked the local conditions. Upon maturation in the middle of March 2019, worms were distinguishable and were separated into different holding cups (~200 ml) to prevent the broodstock from spontaneous, non-synchronized spawning. Maturing specimens were thereafter removed from the gender distinct holding containers upon demand and used for artificial, in vitro propagation. Great care was taken during handling to avoid body wall ruptures. The males were more fragile than the females, and they easily released premature sperm if moved during the water changes. During the broodstock rearing period, the natural population at the sampling location were also observed twice.

2.3 | Histological observations of the gametes

Worms were sampled from natural population at the same place as above in October 2017 to February 2018 for histological observations. The worms were anaesthetized using 4% $MgCl_2$ solution and then fixed in 4% formaldehyde for later examination. The tissue was dehydrated by a tissue processor (Leica TP1020) in ascending concentrations of alcohol from 80% to 100%. The samples were further embedded in paraffin wax (Leica EG1120, Leica Industries) for histological sectioning (RM2255), and then the sections (4 μm thick) were air-dried for more than 30 min and put in an oven at 37°C overnight.

The sections were stained with haematoxylin (Mayer's hemalum solution) and eosin Y-solution 0.5% aqueous (Fischer, Jacobson, Rose, & Zeller, 2008; Ross & Pawlina, 2017). After staining, the sections were covered using Neo-Mount as a binding medium. They were scanned in one layer at 40x magnification using a digital slide

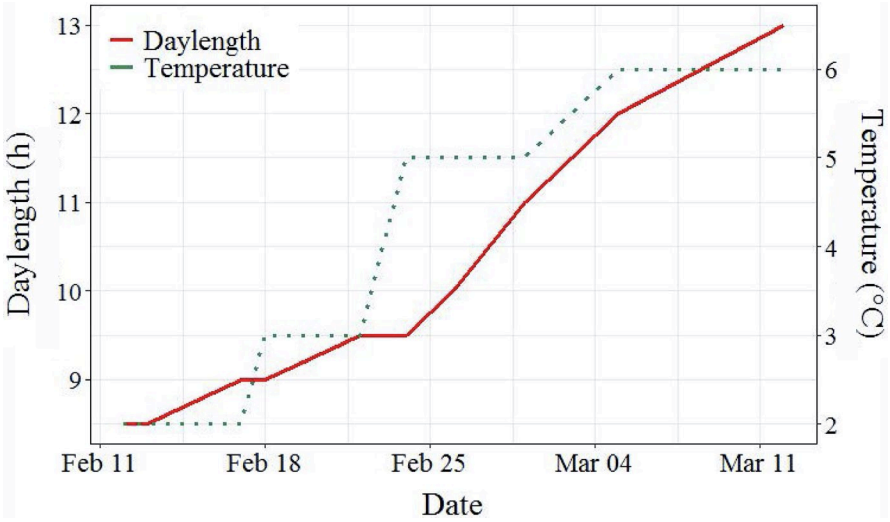


FIGURE 1 Light and temperature regimes used for maintaining *H. diversicolor* broodstock animals in the lab during the acclimation period until maturation [Colour figure can be viewed at wileyonlinelibrary.com]

scanner (Hamamatsu NanoZoomer) and then examined with an image viewing software (Hamamatsu NDP.view2).

2.4 | Induction of spawning

Natural spawning was not observed for the broodstock worms. Several different treatments were tested without success in spawning in males and females:

1. Temperature instantly increased from 5 to 10°C with 1-week post-exposure observation.
2. Temperature instantly decreased from 6 to 2°C for 3 days and thereafter an instant increase to 6°C with 3-day post-exposure observation.
3. Co-culture of mature females and males with 7-day post-exposure observation.
4. Air exposure for 0.5 to 2 hr (Appendix Figure A1) was the only treatment that induced spawning, though only for two of seven females for 1–2 hr.

2.5 | Characteristics of *H. diversicolor* development

Six female worms with mature oocytes were sacrificed by chopping them with a scalpel to release mature eggs (uniform spherical oocytes in a diameter of 200 μm) in pasteurized natural seawater (PSW, 34‰ salinity). The sperm was easily released by slightly touching the worm with a tweezer, and their vitality was examined under a microscope (200–400× magnification). Several drops of sperm solutions with high vitality obtained from two males were mixed with the oocytes. The fertilized embryos were washed through a sieve (100 μm opening with PSW) two to three times after 30–60 min post fertilization to remove protozoan and sperm. Afterwards, the embryos were transferred to an autoclaved conical flask (200 ml) with aeration till 3-setiger larvae. The water in the flasks was changed every second day.

The characteristics of polychaete *H. diversicolor* at various developmental stages were described according to those of polychaete *Platynereis dumerilii* by Fischer, Henrich, and Arendt (2010) and *Galeolaria hystrix* by Nelson, Liddy, and Lamare (2017). The 12-, 16- and 20-segmented juveniles were captured under a microscope (Nikon SMZ1000), while the other images were captured by microscope (Eclipse TS100) with the software (NIS-Elements BR version 4.30). Drawings of embryo, larvae and juveniles from Dales (1950) were used as a reference.

2.6 | Embryonic development under different temperature

Embryonic development was evaluated for five temperature regimes (6.1, 10.1, 15.3, 21.2 and 24.5°C) using a modified temperature gradient device (Thomas, Scotten, & Bradshaw, 1963). Fertilized embryos (n = 400), initially fertilized at 10°C, were transferred to 1 L beakers filled with PSW (250 ml) under static culture conditions. Half of the water volume was changed every day, and the new PSW was kept to the same temperature at each treatment. Each treatment was replicated four times. The temperature table was initially set to 10°C, where after the temperature was gradually adjusted over a course of 4 hr. The samplings were conducted at 6, 10, 16 and 30 hr post fertilization, and then, the sampling interval was set to every 24 hr until 16 days post fertilization. About 10 embryos were removed from each replicate for microscope examination to determine the developmental status.

2.7 | The effect of two kinds of sediments and three different diets on the growth of juvenile *H. diversicolor*

The parental worms were collected (as described in Section 2.2) at the end of March 2017 and reared in a laboratory to investigate the optimal sediment and feeds for early juvenile development. Two types of sediments (natural sediment and chamotte) and three different diets to the juveniles (~7 segments) were evaluated. The

natural sediment was the upper layer sediment (0–5 cm) from the collection site of the polychaete. The grain size distribution of the natural sediment was measured using a set of stacked sieves with a mesh size between 75 μm and 1.4 mm. The chamotte sediment was obtained by use of the same filter to get the grain size as for the natural sediment (Appendix Table A1). Three different diets were compared, fish feed (ground to powder with mortar and pestle), smolt sludge (ground with a blender, obtained from a land-based recirculating aquaculture system (RAS) facility) and shellfish diets 1800 ® (Reed Mariculture), were used in this experiment.

Worms reproduced spontaneously in the holding tanks. Juveniles from this spontaneous reproduction were collected in June 2017. To collect the worms, the flow-through system was stopped, and half of the surface water was drained. A hole was dug into the sediment, and the water in the hole was filtered immediately through a 120 μm mesh. The juveniles (around seven segments) were rinsed in seawater and placed in beakers. The process was repeated until we collected a sufficient amount of juvenile worms. The juveniles were reared in six-well plates (Sigma® cell culture plate) with one juvenile per well. The juveniles were fed ad libitum for 38 days on a bi-daily basis. Each treatment had 10 replicates. The length and segments of the juveniles were recorded weekly using a stereo microscope (Nikon SMZ1000), and growth rates were calculated according to Equation (1) until they reached around 29 segments and became too large for microscopy. From that time forward, the juveniles were photographed with a ruler (as reference) and measured using Image J software (Appendix Figure A2). The water was changed, and the leftover feed and faeces were cleaned and new feed supplied every 2 days.

Length-specific growth rate (LSGR) was calculated by using the equation:

$$\mathrm{LSGR} = \frac{\ln(L_t) - \ln(L_{t0})}{t} \quad (1)$$

where L_t is the length of juveniles at the experimental time (t), L_{t0} is the length of juveniles at start, and t is the duration between two measurements (days).

The relationship between length and segment was expressed by the equation:

$$L = aS^b \quad (2)$$

where L is length of the worm (μm), S was the number of segments, and 'a' and 'b' are constants. The equation was transformed into a linear form: $\ln L = \ln a + b \ln(S)$, to estimate the values 'a' and 'b'.

2.8 | Statistical analysis

T tests were used to compare the LSGR means between different feed and sediment treatments (only data with more than three replicates was included), as missing information during LSGR calculation resulted from not all the juveniles were successfully found at each sampling time. Linear regression with maximum likelihood was used to evaluate the general length and segment growth rate of the juveniles fed with three kinds of feeds under two kinds of substrates. The length and number of segments of juveniles under the same substates were pooled for correlation analysis. The statistical analysis and graph were carried out in R 3.6.2.

3 | RESULTS

3.1 | Broodstock sex differentiation

Because of the lack of distinct morphological changes when the worms reach sexual maturity, sexual differentiation of *H. diversicolor* sampled between February and April 2019 could only be determined based on colour differences. During maturation, the females showed a yellowish, dark green colour (Figure 2a,b). The males were grass green when they were close to maturity and contained splitting sperm plate (Figure 2c), and turned milky green as once they fully matured (Figure 2d). The epidermis of matured females was more transparent compared to that of immature specimens, and the chaetae easily fell off the parapodia when they were handled (Figure 2b). However, observation on worms from breeding seasons showed that all immature worms were reddish brown (Figure 2e), and females could not be distinguished from males.

3.2 | Gametogenesis in *H. diversicolor*

The oocyte cluster was found at the early reproductive period (Figure 3a,b). In histological samples, clustered germ cells (Figure 3a) were detected at the distal end of the parapodia for immature worms, which were later released into the coelom to mature. The size and shape of the oocytes were initially heterogeneously distributed and became more uniform towards maturity (Figure 4a,b). Four distinct circular structures were detected for the mature oocytes by histology and microscopy observations (Figures 3c and 4b): (a) the first peripheral, cortical layer (oocyte envelope) with cortical alveoli where the spermatozoa penetrate the egg to reach the cytoplasm during fertilization; (b) a bright layer filled with lipid droplets and yolk granules; (c) the cytoplasm; and (4) surrounding the nucleolus and the nucleus (Figure 3c).

The spermatozoa developed from sperm plates (Figures 5a and 6a) through spermatids. The spermatids contained clustered motionless spermatozoa (Figures 5b and 6b). Finally, they developed into spermatozoa floating freely in the coelomic fluid (Figure 6c).

3.3 | Development of *H. diversicolor*

Successful in vitro fertilization occurred during February and March, and embryonic, larval and juvenile development was documented for fertilized eggs to the 3-segmented larval stage, which happened over 8–13 days at 11°C. Photograph documentation of the different

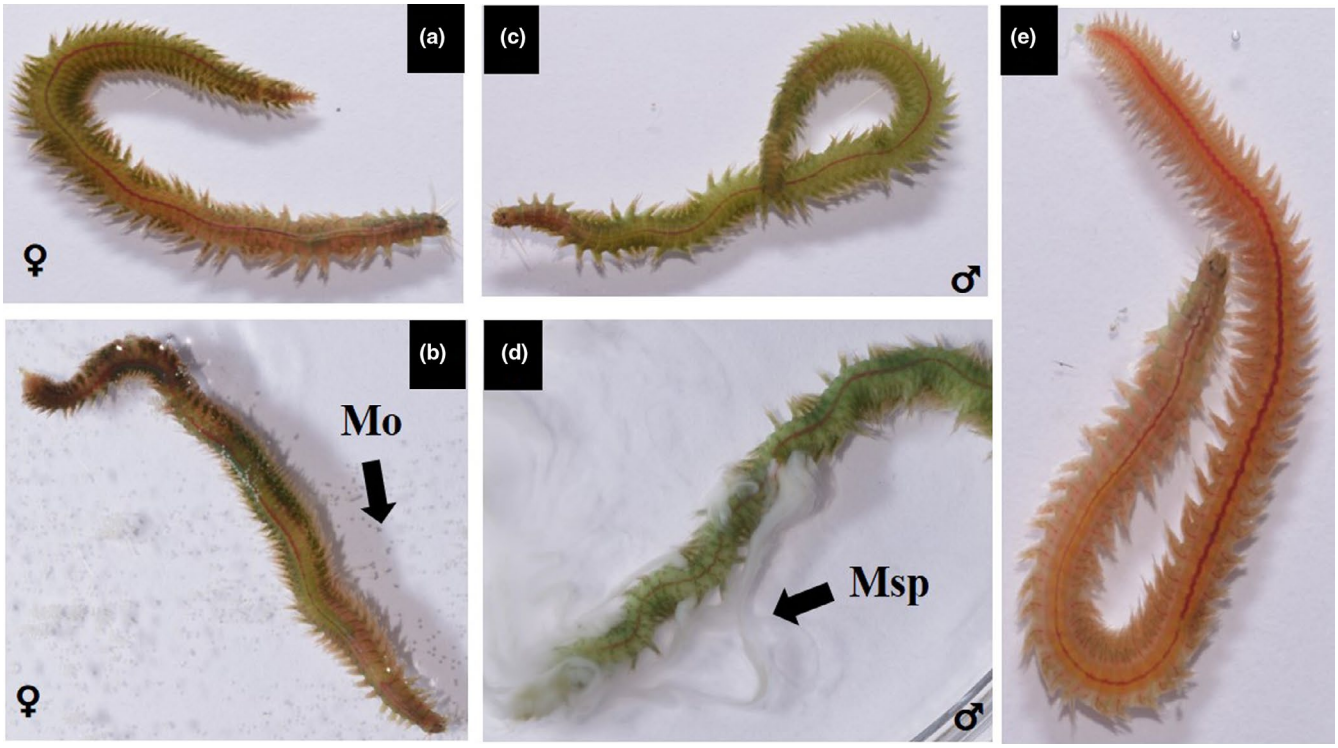


FIGURE 2 The characteristics of worms at different stages, (a) maturing female, (b) spawning female, (c) maturing male, (d) spawning male and (e) juvenile worms (immature worms). Mo: released, mature sperm; Msp: released, mature oocyte. Pictures taken by Bjarne Kvæstad, SINTEF [Colour figure can be viewed at wileyonlinelibrary.com]

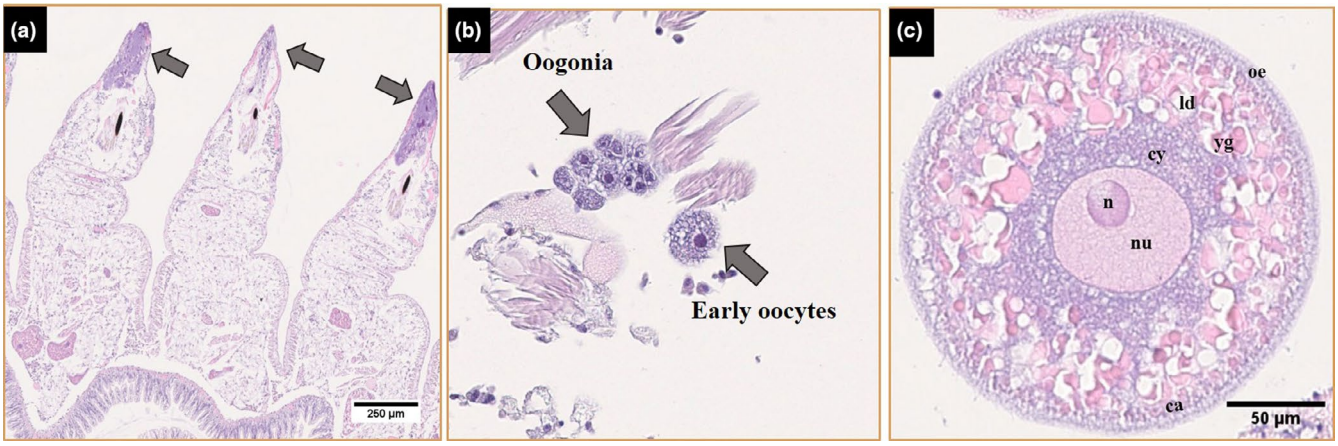


FIGURE 3 Histological sections of female *H. diversicolor*. (a) Early oocytes, arrow indicated *Pn-vasa* cell cluster, (b) histological sections across an ovary in nonproliferative phase showing oogonia and early oocytes, and (c) mature oocytes. Note: (c) oe: oocyte envelope; ca: cortical alveoli; yg: yolk granules; ld: lipid droplets; cy: cytoplasm; nu: nucleus; n: nucleolus [Colour figure can be viewed at wileyonlinelibrary.com]

stages of embryonic, larval and juvenile development observed in our study, and descriptive characteristics were shown in Table 1.

3.4 | Embryonic development under different temperature

Hediste diversicolor embryos showed faster developmental rates at higher water temperature (Figure 7), but the cultures were contaminated with ciliates. The embryos kept at 24.5°C showed abnormal development from the beginning, and the yolk surface did not appear smooth as in normally developing embryos (Figure 8). The embryos at 21.2°C were contaminated by lots of ciliates already at day 2. Even though the larvae were washed with PSW to remove ciliates, the ciliates resulted in 100% embryo mortality at the trochophore stage. The embryos at 15.3°C developed to 3-setiger juveniles after 9 days. The ciliates emerged later at lower temperatures than at higher temperatures, but ciliate

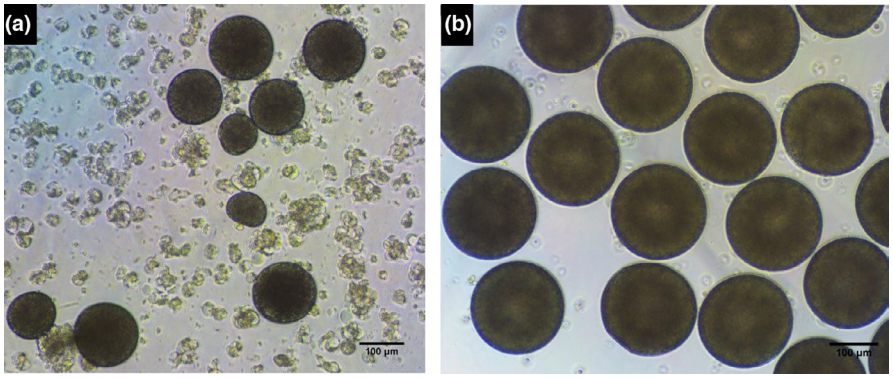


FIGURE 4 Microscopic images of live oocytes of polychaete *H. diversicolor*, (a) immature oocytes, (b) mature oocytes [Colour figure can be viewed at wileyonlinelibrary.com]

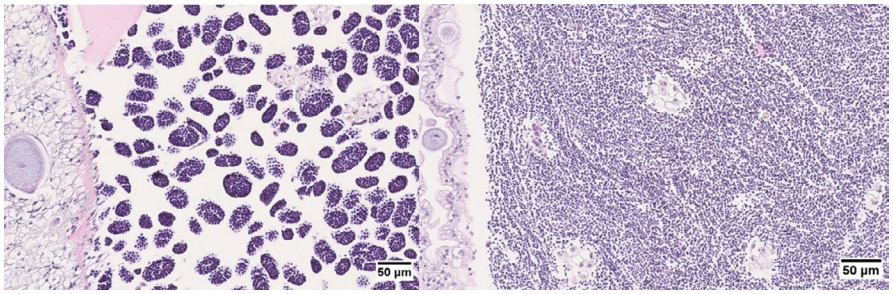


FIGURE 5 Histological section of *H. diversicolor* showing spermatogenesis. (a) spermatocytes (sperm plate), (b) mature spermatozoa (sperm) [Colour figure can be viewed at wileyonlinelibrary.com]

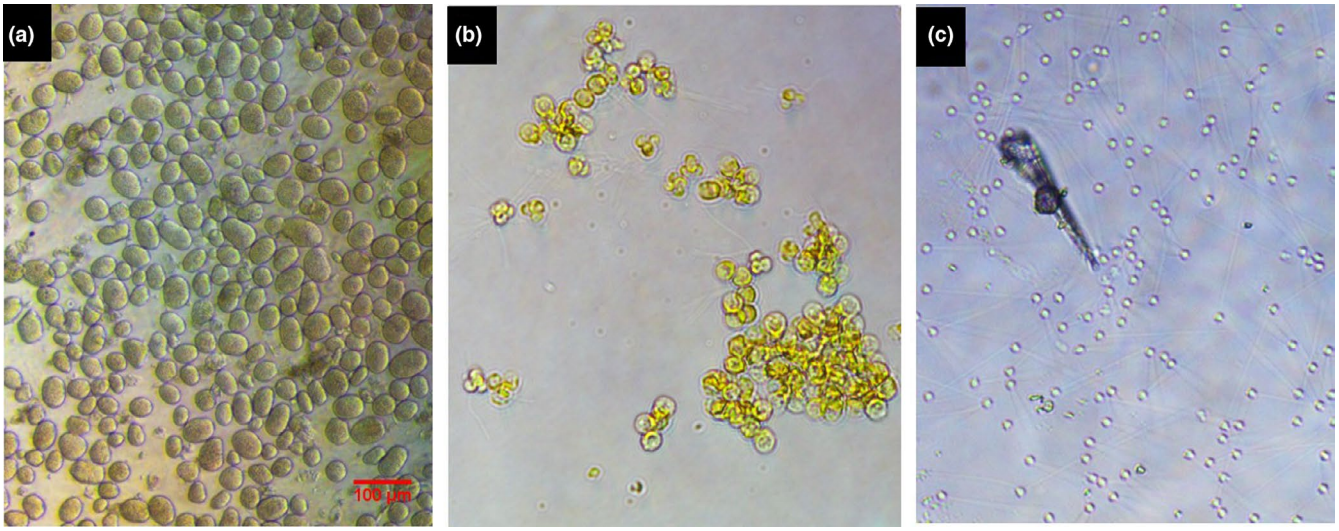


FIGURE 6 Spermatogenesis of *H. diversicolor*, from (a) spermatocytes (sperm plate), to (b) spermatids, to (c) mature free-swimming spermatozoa (sperm) [Colour figure can be viewed at wileyonlinelibrary.com]

contamination resulted in mortality of larvae in all treatments. The embryos at 10.1°C developed to Nectochaete at day 12, while the embryos remained trochophore till 16 days at 6.1°C. All treatments had high mortality and were finished when all embryo had died.

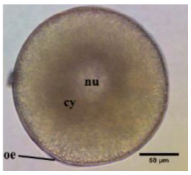
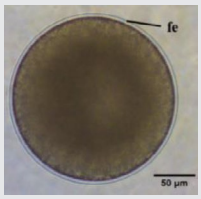
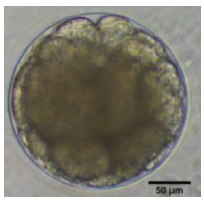
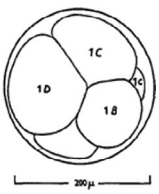
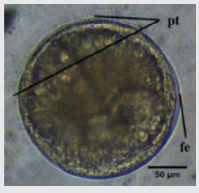
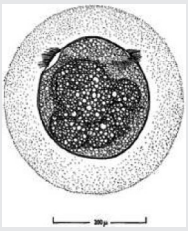
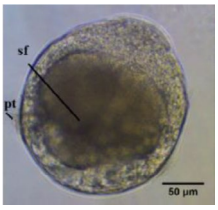
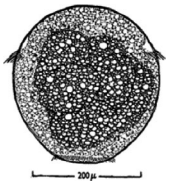
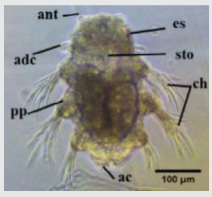
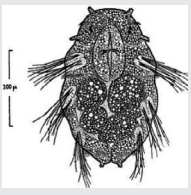
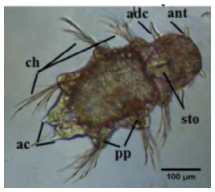
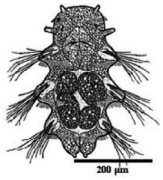
3.5 | Growth of juvenile *H. diversicolor* with two kinds of sediments and three different diets

Before the worms got a size of 20 segments, they had a relatively straight gut from mouth to anus. Larger worms with around 20 segments had obvious septum and pigments (Figure 9), which defined the worms as adult mode.

The juveniles reared in natural sediments fed fish feed showed higher LSGR than those fed with shellfish diet (0.06 ± 0.01 day^{-1} vs. 0.02 ± 0.02 day^{-1}, $p < .05$, Figure 10) at day 10. The juveniles reared in natural sediments fed fish feed and shellfish diet showed the highest LSGR at day 17 at 0.09 ± 0.03 day^{-1} ($n = 2$) and 0.09 day^{-1} ($n = 1$) respectively.

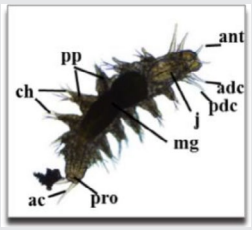
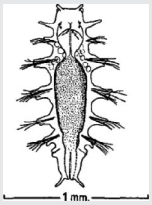
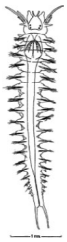
There was a clear gradual increase in the total length of the juveniles for all treatments (Figure 11). The general length increment rate of juveniles was highest for those fed fish feed reared in natural and chamotte substrate were 0.42 and 0.37 mm/day respectively.

TABLE 1 The characteristics of the developmental stages of polychaete *H. diversicolor* [Colour figure can be viewed at wileyonlinelibrary.com]

Developmental stages photographs	Drawings Dales (1950)	Characteristics
		**Maturing egg** Visible gelatinous oocyte surface and vitelline envelopes Apparent nucleus surrounded by dense cytoplasm.
		**Fertilized egg (0.5–3 hpf)** Fertilized egg before cell cleavage. Jelly envelope formed within in around 0.5 hr at 10°C.
		**8- to 64-cell embryo (16–24 hpf)** Spiral cleavage before blastula stage Visible cell cleavage.
		**Protrochophore (72 hpf–5 dpf)** Girdled by a ring of cilia The prototroch enables the larvae to rotate in the jelly.
		**Trochophore (5–6 dpf)** Embryos rotate around their anterior axis, and rotations are faster than in protrochophore. Early trochophore: anterior part was out of jelly membrane. Late trochophore: distinct stomodeum opening surrounded by stomodeum rosette, first chaetae appear visible inside the trunk as sign of first segmentation (Fischer et al., 2010).
		**Late 3-chaetae Nectochaete (9–12 dpf)** Adult eyes clearly visible. Elongation of the trunk. Stomodeal opening becomes slit-like. Antenna, anterior dorsal cirrus and anal cirrus appear. Jaws start forming. The trunk is still filled with lipid droplets.
		**3-segmented juvenile (12–13 dpf)** The shape of the head is more distinguishable from the trunk as a constriction. Antenna, anterior dorsal cirrus and anal cirrus increase in length. The gut directs till the end proctodeum. Lipid droplet in the gut was hardly found. Settlement metamorphosis completed. Food can be found in the barrel-shaped gut (Fischer et al., 2010).

(Continues)

TABLE 1 (Continued)

Developmental stages photographs	Drawings Dales (1950)	Characteristics
	 6-segmented	**7-segmented juvenile (–)** Cephalic metamorphosis becomes more complex. One pair of prostomial cirri is visible The jaws harden.
 12–16- and 20-segmented	 19-segmented	**12-, 16- and 20-segmented juvenile (–)** Four pairs of peristomial cirri are clearly visible at 12-segmented juvenile. Dales (1950) demonstrated 4 pairs of peristomial cirri developing till about 16-segemented juvenile. Blood pigmentation was visible near the tail at 12-segmented juvenile. Septum is clear in 20-segmented juveniles with gut-shaped beaded chains.

Abbreviations: ac: anal cirrus; adc: anterior dorsal cirrus; ant: antenna; apt: apical tuft; bl: blastocoel; ch: chaetae; cy: cytoplasm; dpf: days post fertilization; es: eye spot; fe: fertilization envelope; hpf: hours post fertilization; j: jaw; mg: midgut; mm: macromere; nu: nucleus; oe: oocyte surface and vitelline envelope; pdc: posterior dorsal cirrus; pp: parapodia; pro: proctodeum; pt: prototroch; sf: stomodeal field; sto: stomodeum. Juveniles after the 4-segmented stage were observed in the broodstock tanks, so the post fertilization duration was not documented.

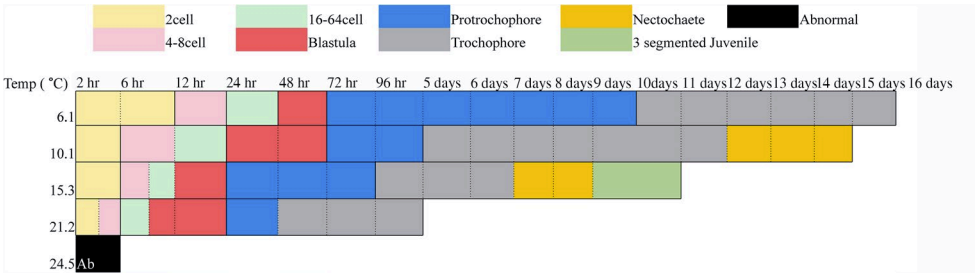


FIGURE 7 Embryonic and larval development of *H. diversicolor* reared at different temperatures [Colour figure can be viewed at wileyonlinelibrary.com]

The juveniles fed shellfish diets reared in natural sediments and chamotte grew slightly slower, showing an LSGR of 0.23 and 0.15 mm/day respectively. The LSGR for juveniles fed smolt sludge reared in natural sediments and chamotte were 0.23 and 0.21 mm/day respectively (Table 2).

The number of juvenile segments increased throughout the experiment was highest in fish feed, followed by that for juveniles fed smolt sludge. The lowest growth was found for juveniles fed shellfish diets (Table 2; Figure 12). For juveniles reared in natural sediment and chamotte fed fish feed, the number of segments increased by 0.75 and 0.78 chaeta per day^{-1} respectively. Juveniles reared in natural sediment fed smolt sludge showed the highest growth (0.78 chaeta per day), whereas those kept in chamotte grew the poorest, showing a growth of 0.57 chaeta per day. The larvae fed shellfish diet increased around 0.42–0.51 chaeta per day for both sediments (Figure 12). Juveniles fed with shellfish diet and smolt sludge grew faster in natural sediments compared to those at chamotte.

A strong correlation was found between number of segments and length (μm) for the juvenile *H. diversicolor* (Figure 13) using the equation $L = 44.1\ S^{1.6}$ ($R^2 = 0.96$, $p < .05$), where L is the length of the juveniles (μm) and S is the number of segments.

4 | DISCUSSION

4.1 | Worm stock management

The spawning season of the polychaete *H. diversicolor* in the Trondheim area in Central Norway was found to be in March–April, which is relatively similar to those in several other geographical

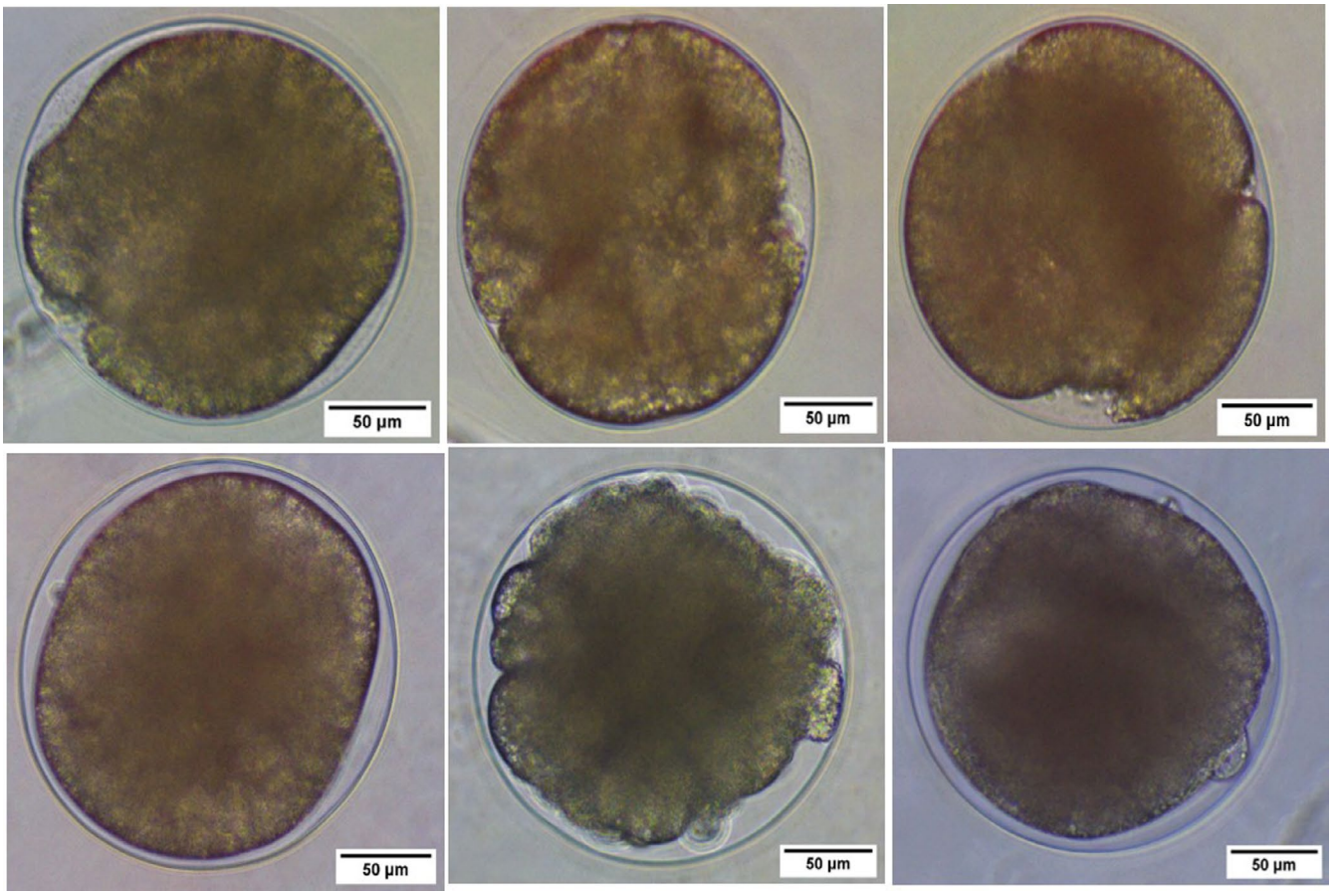


FIGURE 8 Abnormal development of embryos of *H. diversicolor* at 24.5°C [Colour figure can be viewed at wileyonlinelibrary.com]

locations (Table 3), whereas *H. diversicolor* in Portugal was found to have two or more spawning seasons during the year.

The coloration of the adult worm can be picked out 1 or 2 months before the spawning seasons, which was considered the suitable time to collect the parental worms. Nesto et al. (2018) kept the maturing worms in glass pipes (5 mm) placed in 50 ml tubes to prevent asynchronous spawning. We tested several glass pipettes with a diameter of around 5 mm placed in a plastic rectangle box to shelter the parental worms (Appendix Figure A3). However, several worms were found to share one tube, while the other tubes remained empty. This may be due to the size difference between the worms used in the current study and the ones described in Nesto et al. (2018; 0.3 g vs. 0.8 g). The worms were less aggressive when sharing the tube, but an appropriate-sized tube only fit one worm would be better. Alternatively, cultivation of individual worms with a layer of sediment could also be a solution (Appendix Figure A3).

The photoperiod and temperature in laboratory broodstock holding tanks were gradually increased, so the worms in the laboratory could mature earlier than those in the field. It was demonstrated that regulation of maturation could be conducted successfully in controlled conditions. Batista et al. (2003) found that in optimal laboratory conditions with enough food supply, *H. diversicolor* could reach sexual maturation during 3 months, which was shorter compared to the natural reproduction process (1–3 years; Chambers & Milne, 1975; Kristensen, 1984; Olive & Garwood, 1981). Chu and Levin (1989) found that in early fall, the transition from long to short daylength was a better cue for inducing sexual maturation than the temperature for the polychaete *Streblospio benedicti*, whereas in spring increasing temperature or increasing daylength promoted brood size and fecundity. It was reported that food availability can override photoperiod regulation of the reproductive cycle on field populations (cited in Chu & Levin[, 1989]). Accordingly, nutrient supply, photoperiod and temperature seemed to contribute greatly to early maturation in artificial rearing.

None of the induction methods for spawning tested in our study was ideal. The temperature during the spawning season in Trondheim was around 10°C, and we tried not to expose the worms to excessive temperatures to ensure good egg quality. The temperature shift to induce spawning did not show any effect, probably because the temperature difference was not large enough to stimulate spawning.

Lin, Tung, Yu, and Su (2016) found that spawning in the acorn worm *Ptychodera flava* could be induced by a temperature increase in 8°C, from 22 to 30°C, and that addition of sperm water (supernatant from sperm centrifuged at 6,400 *g* for 15 min at 4°C) that contained heat-stable but trypsin-digestible molecule increased the spawning ration from 10% to 50%. Hardege and Bentley

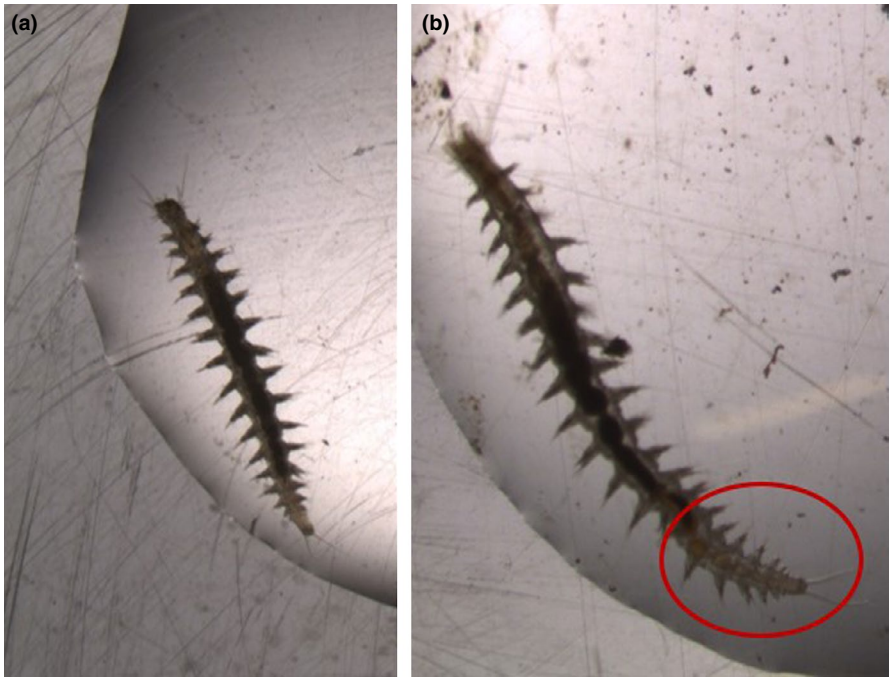


FIGURE 9 Juvenile *H. diversicolor*. (a) 16-segmented, and (b) 20-segmented pigment and septum can be clearly seen (red circle) [Colour figure can be viewed at wileyonlinelibrary.com]

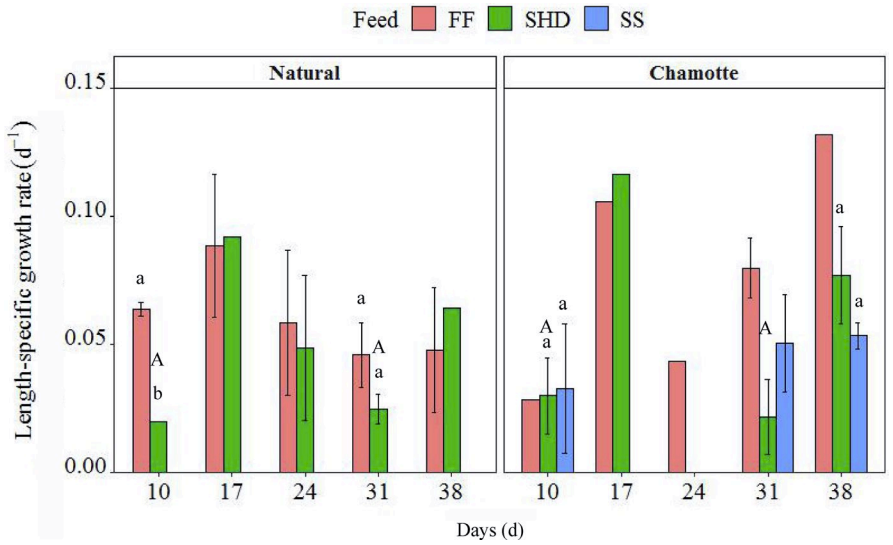


FIGURE 10 LSGR (day^{-1}) of juveniles of *H. diversicolor* fed with three different diets and cultivated in two different substrates changed over the time. FF, SHD, and SS are-fish feed, shellfish diet and smolt sludge respectively. Small letters denote significant differences within sediments and between diets, capital letters denote significant differences within diets between sediments [Colour figure can be viewed at wileyonlinelibrary.com]

(1997) mentioned that sperm water and male coelom fluid had positive effects on female spawning in the polychaete *Arenicola marina*. Nesto et al. (2018) tried two ways to induce spawning in *H. diversicolor*: (a) thermal shock with an 11°C decrease (from of 16 to 5°C) for 5 days followed by an 11°C increase again for 1 week, and (b) tissue homogenate addition for a week. They found that eggs spawned by thermal shock induction showed low fertilization rates (0%–40%), while tissue homogenates worked well on females and resulted in high fertilization and hatching rates compared to thermal shock induction. Based on our trials and previous studies, temperature could be increased earlier in spring to shorten the duration of maturation, coupled with chemical cues (such as sperm water) to induce spawning when worms have really matured. This could possibly lead to higher fertilization rates and better egg quality.

4.2 | Gametogenesis of *H. diversicolor*

We found that the size and shape of *H. diversicolor* oocytes changed during maturity from heterogeneous at the beginning to a more uniform shape when approaching breeding season, which was consistent with the results by Olive and Garwood (1981). Smith (1950) also reported that early oocytes of polychaete *Neanthes lighti* developed asynchronously in cluster, and their development became synchronized later during maturation and finally fertilizable synchronously. In our study, we also found cell clusters at parapodia that we classified as stem cells of oocytes. Maceren-Pates, Kurita, Pates, and Yoshikuni (2015) used a gene marker to trace *Pn-vasa*-positive cell clusters at the distal end of the parapodia and indicated that germ cells settle in the parapodia and then move into the coelom to develop into oocytes. The mature oocytes had distinct characteristics, and their

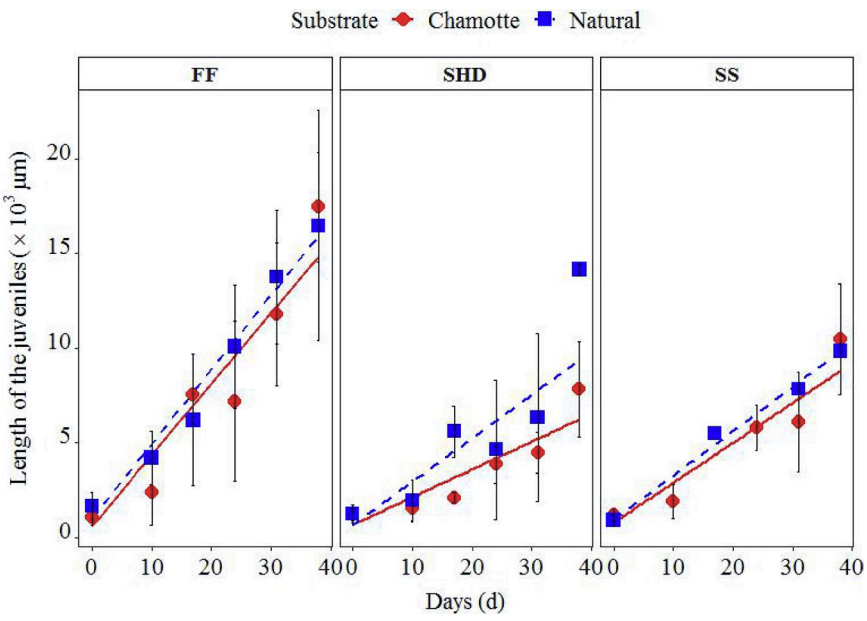


FIGURE 11 Length of juvenile *H. diversicolor* fed with three different feeds (FF: fish feed; SHD: shellfish diet; SS: smolt sludge) in two different substrates (natural sediment and chamotte) [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Length or segment change of juvenile *H. diversicolor* change over time: *n* is the numbers of samples in the linear regression analysis

Dependent variable	Substrate	Feed	Regression equation	r^2	*N*	*p*
Length (mm)	Natural	FF	$y = 0.42x + 0.76$	0.75	38	<.001
		SHD	$y = 0.23x + 0.66$	0.55	27	<.001
		SS	$y = 0.23x + 0.93$	0.99	13	<.001
	Chamotte	FF	$y = 0.37x + 0.60$	0.84	21	<.001
		SHD	$y = 0.15x + 0.66$	0.72	31	<.001
		SS	$y = 0.21x + 0.78$	0.77	23	<.001
Segment	Natural	FF	$y = 0.75x + 9.16$	0.81	38	<.001
		SHD	$y = 0.51x + 7.06$	0.63	27	<.001
		SS	$y = 0.78x + 6.08$	0.99	13	<.001
	Chamotte	FF	$y = 0.78x + 6.17$	0.88	21	<.001
		SHD	$y = 0.42x + 6.69$	0.75	31	<.001
		SS	$y = 0.57x + 6.61$	0.87	23	<.001

histologic characteristics were found similar to that of *Nereis virens* (Kostyuchenko & Dondua, 2000).

The spermatids in our observations were either paired or consisted of several pairs. Finally, the spermatids split, and the spermatozoa gained activity. Free spermatozoa were formed when breeding season was reached, and they could be found in the coelom about 2 weeks before spawning. Males' germ cells originate from the seminiferous epithelium and are released into the coelom at an early stage (Dales, 1950). Lu, Aitken, and Lin (2017) found that the spermatids consisted of paired spermatocytes connected by a cytoplasmic bridge. Spermatids in early development were without tails, which developed over time. The spermatogenesis of *H. diversicolor* took 6 months to complete, which is considerably shorter than the 1–3 years for oogenesis (Olive & Garwood, 1981).

The oogenesis of *H. diversicolor* was found to be stimulated by photoperiod or temperature transition in fall, while increasing temperatures advanced oocyte size synchronously in spring (own unpublished data). Males were observed to mature earlier than females when kept at the same condition. In a previous study, it was indicated that gametogenesis can be influenced by hormones, whose production and release are often stimulated by exogenous factors in marine invertebrate (Gianasi, 2017). Among many factors, photoperiod and temperature are the two main factors that control the timing of the reproductive cycle (Olive, 1995). Bartels-Hardege and Zeeck (1990) demonstrated that the worms' spawning activity could not be synchronized in a specific period by directly increasing temperature from 7 to 16°C without experiencing winter condition (lower than 5°C). Besides temperature and photoperiod, feed was also a factor triggering gametogenesis onset. Nesto, Simonini, Prevedelli, and Da Ros (2012) tested diets differing in their nutritional profiles on 2-month-old juveniles. The authors found that a protein-rich diet (66% protein and 14% fat) could trigger onset of gametogenesis. Therefore, further research on how to improve synchronized gametogenesis and spawning will help realize larvae production in artificial systems outside the normal reproductive season.

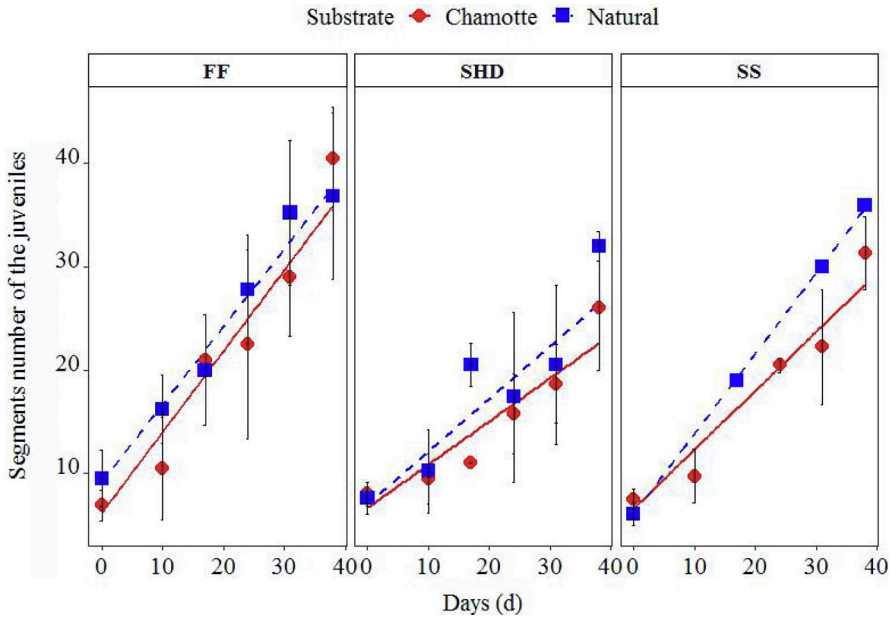


FIGURE 12 The number of segments of juvenile *H. diversicolor* fed with three different feeds (FF: fish feed; SHD: shellfish diet; SS: smolt sludge) in two different substrates (natural sediment and chamotte) [Colour figure can be viewed at wileyonlinelibrary.com]

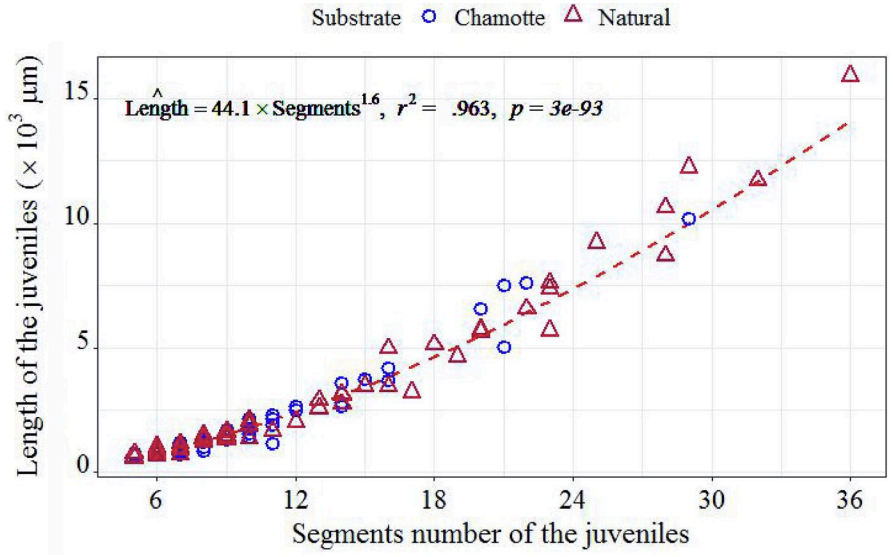


FIGURE 13 Relationship between length and segment number of *H. diversicolor* juveniles (n = 131) [Colour figure can be viewed at wileyonlinelibrary.com]

4.3 | Fertilization, embryonic and larval development

We found that the oocytes could not be squeezed out of the mature females, so the release of the oocytes from the worms was done by dissecting several segments, which was also the method reported by Ozoh and Jones (1990). Smith (1964) tried to suck the eggs through the muscular body to bring out more eggs (several hundreds), and with this method, one female can stay alive to supply oocytes in 3–5 days.

Smith (1964) reported that *N. diversicolor* developed to 3-setiger larvae in 5.2 days at 18°C at 18‰, and Dales (1950) observed larvae from natural habitats developing within 3 weeks to 3-setiger larvae at temperatures between 1 and 10°C. In our study, ciliate infection probably caused complete mortality at all temperatures. Therefore, it is important to modify the fertilization procedure by washing the fertilized eggs for two or three times to avoid ciliate contamination. The modified method worked well in later trials. Ozoh and Jones (1990) added one adult worm in an embryo incubation dish to ingest protozoan and bacterial infections.

4.4 | The effect of temperature on the embryonic development of *H. diversicolor*

Temperature can significantly affect the pace of embryonic and larval development of polychaetes. In our study, temperatures higher than 21°C were considered not suitable for embryonic development of *H. diversicolor*. The rate of embryonic development increased with a temperature increase from 6.1 to 21.2°C.

However, the tolerance of the embryos to temperature may be related to the parental rearing conditions or regional

TABLE 3 The spawning time of *H. diversicolor* population and the temperature at various geographical locations

Locations	Spawning season	Temperature (°C)	References
Trondheim, Norway	March–April	5–10[a]	Observation at sampling site
Turku, Finland	April–May	5–11.5	Smith (1964)
Kristineberg, Sweden	April	12–17	Smith (1964)
Göteborg, Sweden	May	>10	Möller (1985)
Aarhus, Denmark	New recruitment: March–May/July–August	>10	Kristensen (1984)
Scotland, UK	January–March/June–August	7–8/11–14[b]	Chambers and Milne (1975)
St. Andrews, UK	March	7[b]	Cited by Dales (1950)
Cowpen, UK	Late March–April	6–7[b]	Olive and Garwood (1981)
Grimsby, UK	April–May	8–10[b]	Ozoh and Jones (1990)
Stiffkey, UK	May–June	10–13[b]	Nithart (1998)
Chalkwell, UK	February	5–8.8	Dales (1950)
Jadebusen, Germany	February	12	Bartels-Hardege and Zeeck (1990)
Belgium	May–December (peak in August)	8–20[c]	Heip and Herman (1979)
Venice, Italy	January–February	7–8	Nesto et al. (2018)
Ria de Aveiro, Portugal	March–April/September–October	15	Abrantes, Pinto, and Moreira (1999)
Odeceixe/Aljezur, Portugal	Larvae recruitment all the year	12–21/12.7–24.7	Fidalgo e Costa, Sarda, and da Fonseca (1998)

[a]The temperature was estimated from https://www.seatemperature.org/europe/norway/trondheim-april.htm.
[b]The temperature was estimated from https://www.seatemperature.org/europe/united-kingdom/scotland/.
[c]The temperature was estimated from https://seatemperature.info/december/belgium-water-temperature.html.

differences within the same species. Santos et al. (2016) mentioned that the broodstock of *H. diversicolor* was held at 25°C, and the larvae were also reared at 25°C. Nesto et al. (2018) cultivated larvae of *H. diversicolor* successfully at 16°C. In our study, exposure to abruptly increased or decreased temperature may have resulted in abnormal or slow development. Oocytes and sperm from parental worms reared at different temperatures may be fertilized to test the effect of temperature on embryonic development in the future.

Furthermore, temperature may affect the adaptation of the embryos to other factors like, for example salinity. Ozoh and Jones (1990) found that increasing temperature in the range from 12 to 22°C will decrease the adaptation of the cleaving embryos of *H. diversicolor* to increasing salinity, and the hatching rate of the embryos was found to decrease with increasing temperature.

4.5 | The effect of different sediments and diets on the growth of juvenile *H. diversicolor*

In general, the larvae fed with shellfish diet grew slower, and the feeding behaviour of the juveniles indicated that deposit feeding was more attractive. We observed that larvae began to feed at the 4-setiger stage at a body length of about 500 μm. Our results suggest an earlier onset of feeding than previously reported at about 2 mm length (about 10–11 setigers) by Dales (1950). The length of larvae that fed fish feed increased faster than those fed shellfish diet and salmon sludge. In general, larvae fed all three diets showed positive growth in length. The length-specific growth rate of larvae that fed with shellfish diet peaked at day 17 followed by a decrease suggesting that the shellfish diet did not provide enough nutrition for growth at the later stage around 11–20 segments.

The length of juveniles in our study increased at 0.42 and 0.37 mm/day in natural and chamotte substrate, respectively, which was lower than 0.97 and 0.84 mm/day found by Fidalgo e Costa (1999) for *H. diversicolor* grown in sand (~80% of 0.5–1 mm granules) and mud (~80% of 62.5–125 μm granules). This difference might be caused by different treatments before measurement that the worms in Fidalgo e Costa (1999) were anaesthetized with MS-222, while in our study, photographs of the live worms were taken for measurement. Sediments with different constituents or with grain size ranging 62.5 to 1 mm did not seem to affect the growth of juveniles.

5 | CONCLUSIONS

The broodstock collection of *H. diversicolor* from natural populations was found to be best 1 or 2 months before maturation when the coloration of parental worms can be distinguished. Ripe oocytes and sperm have distinctive characteristics, but the activity of the sperm

needs to be checked before in vitro fertilization. Embryonic development increased with increasing temperature within the range of 6–21°C. Bacterial and protozoan may be prevented by washing the embryos several times before rearing in pasteurized seawater. The larvae growth was found to be affected by food source and not by sediment type.

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CONFLICT OF INTEREST

All authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

AM, AHN and HW conceived and designed the experiment. HW and MU contributed to the experiment implementation. HW drafted the manuscript. AM, AHN, KIR and AHA aided with the writing. All authors agreed with the final version submitted for publication.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researchers.

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APPENDIX

TABLE A1 Grain size composition of surficial natural sediments and chamotte used for the juvenile rearing experiment

Grain size (mm)	Percentage (%)
1.18–1.4	1.8
0.5–1.18	14.3
0.25–0.5	20.0
0.15–0.25	25.2
0.125–0.15	10.9
0.075–0.125	14.9
<0.075	12.8

TABLE A2 The length-specific growth rate of larvae fed with three different diets at two different substrates

Substrate	Feed	Date	n	LSGR	SSGR
Natural	FF	10	4	0.06 ± 0.00	0.03 ± 0.02
		17	2	0.09 ± 0.03	0.05 ± 0.00
		24	4	0.06 ± 0.03	0.04 ± 0.01
		31	3	0.05 ± 0.01	0.03 ± 0.01
		38	4	0.05 ± 0.02	0.02 ± 000
Natural	SHD	10	4	0.02 ± 0.02	0.01 ± 0.02
		17	1	0.09	0.05
		24	2	0.05 ± 0.03	0.03 ± 0.01
		31	4	0.02 ± 0.01	0.01 ± 0.01
		38	2	0.06 ± 0.07	0.03 ± 0.02
Chamotte	FF	10	2	0.03 ± 0.03	0.02 ± 0.02
		17	1	0.11	0.06
		24	1	0.04	0.04
		31	2	0.08 ± 0.01	0.04 ± 0.01
		38	1	0.13	0.05
Chamotte	SHD	10	6	0.03 ± 0.01	0.03 ± 0.01
		17	1	0.12	0.01
		31	3	0.02 ± 0.01	0.04 ± 0.01
		38	3	0.08 ± 0.02	0.02 ± 0.00
Chamotte	SS	10	3	0.03 ± 0.03	0.02 ± 0.01
		31	2	0.05 ± 0.02	0.03 ± 0.01
		38	3	0.05 ± 0.01	0.03 ± 0.01

FIGURE A1 Several females were exposed to air in order to induce spawning. (a) At beginning of exposure; (b) 1–2 hr [Colour figure can be viewed at wileyonlinelibrary.com]

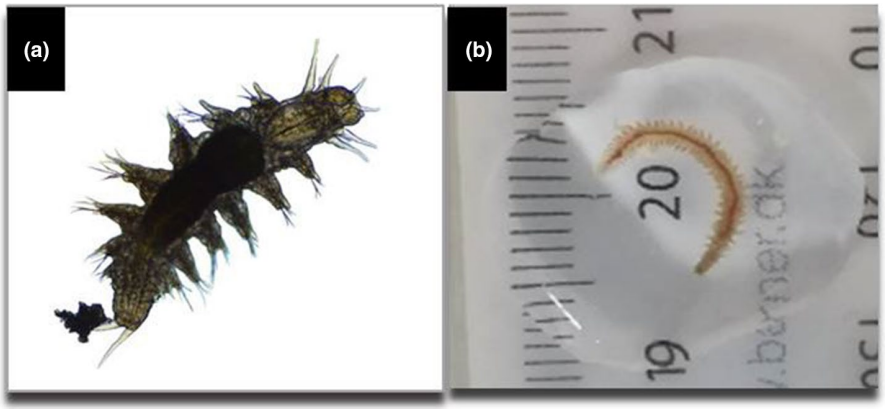

FIGURE A2 (a) The 7-segmented juvenile worm *H. diversicolor* at initiation of the experiment and (b) one 43-segmented juvenile [Colour figure can be viewed at wileyonlinelibrary.com]

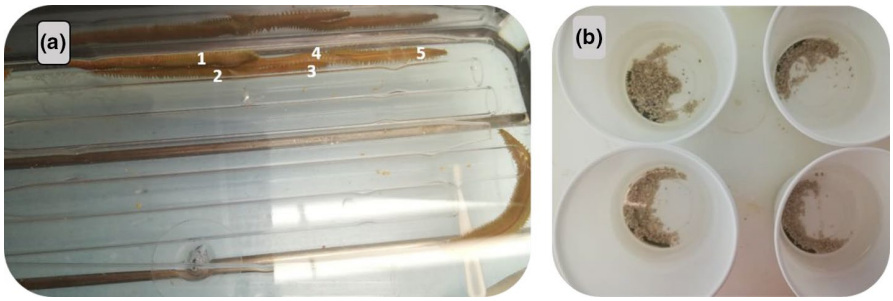

FIGURE A3 Maturing worms (a) in glass tubes; (b) with a layer of sediment [Colour figure can be viewed at wileyonlinelibrary.com]