Evaluation of different procedures for fertilization and larvae production in *Hediste diversicolor* (O.F. Müller, 1776) (Nereididae, Polychaeta)

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Abstract

As a further step toward the development of indoor farming systems, different experimental trials were performed to clarify some aspects of the biology of the polychaete *Hediste diversicolor* (O.F. Müller, 1776) valuable to set up appropriate breeding protocols. In particular, the trials were addressed to: evaluate the effectiveness of two fertilization conditions (*in vitro* and "natural-like"); induce gamete spawning by exposing mature individuals to thermal shock or to tissue homogenates; estimate the density effects on larval growth and survival; evaluate the most suitable parameters to be used as proxy for biomass assessment.

The highest percentages of fertilized eggs and larvae were obtained by the *in vitro* fertilization condition. Mature organisms were induced to spawn by exposure to thermal shock, although the spawned eggs revealed low rates of fertilization and hatching. The treatment with male tissue homogenates induced females to successful spawning, and the resulting eggs showed high fertilization and hatching rates. The density of larvae in the rearing phase had no effect on growth nor on survival rates of juveniles. Finally, an allometric study showed that fresh weight and L3 length are the most reliable parameters to be used as proxy for biomass assessment of this species.

Introduction

Several polychaete species are commercially exploited (most in the families Arenicolidae, Eunicidae, Glyceridae, Lumbrineridae, Nephtyidae and, mainly Nereididae) (Scaps, 2003), being economically attractive as marine baits in recreational fishing or as food supplement in aquaculture (Olive, 1999). Some species of polychaetes are used as dietary components due to their positive effects on Penaeid shrimp reproduction and their high levels of polyunsaturated fatty acid (Olive, 1999; Wouters, Lavens, Nieto & Sorgeloos, 2001). In particular, bloodworms (*Glycera* sp.), sandworm (*Perinereis* sp.) and mudworms (*Marphysa* sp.) are intensively used in marine shrimp broodstock maturation diets (Kawahigashi, 1998; Meunpol, Meejing & Piyatiratitivorakul, 2005). The commercialized worms are mostly harvested from field populations by anglers or professional diggers, which are particularly numerous in the South China Sea (Korea, China), the north-east coast of USA (Maine) and Europe (Netherlands, UK, Spain, Italy) (Olive, 1999; Scaps, 2003). The intense commercial harvesting of polychaete worms from natural environments have been indicated as the main reason for the depletion of this natural resource (Gambi et al. 1994; Olive, 1999; Pires, Gentil, Quintino & Rodriguez, 2012) and as one significant cause for the alterations of habitats and consequently of benthic communities (Anderson & Meyer, 1986; Beukema, 1995; Olive, 1999). The bait market demand for the European fishing is too high to be met by local supply, so most of the worms sold in Europe are imported from Eastern Asia, increasing the risk of introduction of non-native species (Scaps, 2003; Fidalgo e Costa et al., 2006). At the end of 1990's the European bait worm market had been estimated to value about 200 million euros (Olive, 1999) and more recent assessments indicates that the whole European recreational fishing industry is valued 8-10 billion euros (www.eaa-europe.org; Pawson, Glenn & Padda, 2008).

Consequently, economic interests and the need of technical solutions to be adopted for preventing ecological damages to natural habitats addressed scientific researches which have led to the establishment of companies engaged in indoor production of fishing baits in UK, The Netherlands, Australia, Asiatic Countries (www.ukmarinesac.org/activities/bait-collection; www.topsybaits.nl).

The ragworm *Hediste diversicolor* (O.F. Müller, 1776) is one of the most used and appreciated bait for recreational fishing in Italy, where it is actively harvested for commercial purposes from a number of natural littoral environments along the Adriatic and Tyrrhenian coastline (Ansaloni, Pellizzato, Prevedelli & Zunarelli Vandini, 1986; Gambi et al. 1994). In the wild, this species lives burrowed in the sediment and it is considered a deposit-feeder, i.e. it collects the food at the opening of gallery, by crawling and ingesting sediment, or by capturing the organic particulate by means of mucous secretions (Fauchald & Jumars, 1979; Reise, 1979). In addition, it may also take the typical alimentary filter feeder behavior, being able to select the particles trapped in the mucus which are subsequently passively conveyed to U or Y shaped burrows by the water circulation (Harley, 1950; Vedel, Andersen & Riisgård, 1994). Various studies had already suggested that *H. diversicolor* might be a suitable species for aquaculture purpose, proving to be easy to breed in indoor systems and to exhibit several favorable physiological traits, such as high tolerance to a wide range of environmental parameters (salinity, temperature, substrate grain size) and rapid growth rates, at least when fed with commercial fish foods (Scaps, Retiére, Desrosiers & Miron, 1993; Fidalgo e Costa, 1999; Fidalgo e Costa, Narciso & Cancela da Fonseca, 2000; Nesto, Simonini, Prevedelli & Da Ros, 2012). Moreover, *H. diversicolor* may be a potentially high quality fatty acids source for reared fish and shrimp as it has been

demonstrated that fat assimilation by ragworms reflects fat content of their diet (Santos et al., 2016). These same studies, although preliminary, have contributed to establish protocols for rearing *H. diversicolor* brood stock and for optimizing their growth through nutrition control. However, the indoor culture of *H. diversicolor*, as for any other polychaete breeding, to be commercially sustainable should also rely on procedures suitable to guarantee massive fertilization of eggs and sufficient production of larvae /juveniles, as well as on simple and inexpensive systems for rearing juveniles under optimal density (Olive, 1999). In some polychaete species, controlled reproduction can be achieved by manipulating fertilization process under controlled laboratory conditions (see review of Cross, 1984). The most effective results in this sector have been obtained with the ragworm *Alitta virens* (M. Sars, 1835), ultimately leading to patented procedures which have contributed to successful commercial businesses (Olive, 1999). To our knowledge, all these issues have not been comprehensively considered yet in relation to the possible indoor culture of *H. diversicolor.*

In many polychaetes species, body growth and gametogenesis are controlled by a hormone produced by the cerebral neuroendocrine system, which is also essential for segment regeneration processes (Golding, 1967; Andries, 2001; Lawrence & Soame, 2009). In nature, the transition from short to long photoperiod is an important factor for the start of the gametogenesis. In autumn the individuals able to breed show a decrease of juvenile hormone levels, probably also in response to increased levels of dopamine or melatonin. This allows oocytes to enter vitellogenic phase which is further increased by the production and secretion of vitellogenin by eleocytes, induced by the presence of oestradiol 17ß (Garcia-Alonso, Hoeger & Rebscher, 2006). The production of estradiol-

17ß and the ability to absorb vitellogenin are incremented by a second neurohormone in the cerebral ganglion of mature females, which has a gonadotrophic function (Lawrence & Olive, 1995). Moreover, a specific environmental signal or a hierarchical series of signals are often necessary to induce a synchronized spawning in wild populations (Caspers, 1984; Hardege et al., 1994; Watson, Williams & Bentley, 2000). Also chemical endogenous substances, such as pheromones, are known to induce gamete spawning at least in those polychaete species, e.g, the nereids *Alitta succinea* (Leuckart, 1847) and *Platynereis dumerilii* (Audouin & Milne Edwards, 1834), exhibiting behavioral gender interactions such as nuptial dances, (Hardege, Müller & Beckmann, 1997; Zeeck, Harder & Beckmann, 1998a, b; Ram, Müller, Beckmann & Hardege, 1999; Andries, 2001).

It is well known that reproduction of *H. diversicolor* is controlled both by environmental and endocrine factors (Scaps, 2002). Temperature and lunar periodicity are recognized to be the most effective parameters in determining spawning events, which typically occur at a temperature range of 5-11°C after a period of low temperature (Dales, 1950) and mainly during a new and full moon (Bartels-Hardége & Zeek, 1990). Fertilization takes place within maternal burrow; the male ejects its sperm in front of the female burrow; the sperms are soon after conveyed by the female inside the burrow, where the eggs had already been spawned. Larval development takes place entirely within the parental gallery, where larvae feed on the maternal body until they are able to exit and to replicate the burrowing behavior, typically when larvae reached the six setiger stage (Marty & Retièr, 1999). Natural populations of *H. diversicolor* have been observed to exhibit a variable breeding season according to the climatic conditions of their origin area (Abrantes, Pinto & Moreira, 1999). Life cycles characterized by a

single spawning event (preferably in spring), or extensive breeding seasons (with more spawning events over the year) have been also observed (Abrantes et al, 1999). In native populations of *H. diversicolor* from the Lagoon of Venice, Italy, one only annual reproductive cycle has been noticed (Casellato, Furlan & Bortolotto, 1999). In particular, according to these authors, gametogenesis begins in May and June. Since October and November it is possible to distinguish males from females (i.e mature males have a bright grass-green colours while mature females have a darker green colour) and the sex ratio is approximately 1:4 for females. Spawning is synchronous in the population and it takes place between the beginning of January and February, when water temperature ranged from 7 to 8°C. Being a semelparous species all individuals died after the spawning event (Dales, 1950). These studies have highlighted how *in situ* endogenous factors (i.e. temperature) and endogenous substances (i.e. hormones) may play an important role in determining the start of reproductive period and the spawning events in *H. diversicolor*. However, appropriate experiments carried out in controlled laboratory conditions aimed at implementing reproduction procedures easily transferable to aquaculture companies are still lacking for this species. With this in mind, the main purpose of the present study was to develop and test some procedures aiming at the production of larvae and juveniles of *H. diversicolor*, using mature organisms originally collected in the Venice lagoon. In particular, our goals were: 1) to test successful fertilization procedures in laboratory; 2) to evaluate the effectiveness of both exogenous and endogenous factors as spawning inducers, i.e. thermal shock and non-resolved mixture of natural substances (obtained by blending tissues of mature organisms); 3) to evaluate the effect of stocking density on biomass production; 4) to

monitor the growth of *H. diversicolor* juveniles in order to evaluate the most suitable parameters to be used as proxy for biomass assessment.

Materials and methods

Sampling and experimental conditions

At the begin of the experimental activities (February 2009), 150 adult specimens of *H. diversicolor* (weight 0.8 ± 0.2 g [mean ± SD]) were sampled along the Dese estuary, Lagoon of Venice, Italy (Lat. 45° 31' 6.5''N; Long. 12° 24' 47.02''E; water temperature = 10°C; salinity 10 gL^{-1}). Worms were maintained one week before the experiments in 15 L aquaria filled with filter and aerated seawater (FASW) at temperature 16 ±1°C for acclimatization, assuming that this is the optimum temperature value to achieve good fertilization rates, similarly to other Nereidids breeding in the early spring (Lewis, Olive Bentley & Watson, 2002). Salinity and photoperiod was always set at 16 gL^{-1} and 16:8 hr light/dark, respectively, being the values already known to be the most effective to achieve good growth and survival performances in juveniles of *H. diversicolor* (Nesto et al., 2012). Treated and control animals were not fed during the experiments. All the experiments with their related control tests were performed at these conditions, unless differently reported, using mature organisms only. The sexual maturity of the individuals was checked before each experiment by examination of a drop of gametes syringed *in vivo* from the parapodia under a binocular microscope (Wild M420). The eggs were measured by comparing their diameters with a micrometer slide. The

192 presence of free and motile spermatozoa in males and of large eggs in females (200-250
193 µm in diameter) were considered signals of sexual maturity (Dales, 1950).
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195 Effect of fertilization procedures on fertilization and hatching success
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197 The effects of two fertilization procedures, i.e. *in vitro* and "natural-like", on
198 fertilization and hatching success were evaluated in two different experiments which are
199 fully described in the following paragraphs. Fertilization and hatching percentages
200 obtained with the two procedures were statistically compared using the G-Test.
201
202 *In vitro* procedure
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204 The test was performed on eggs spontaneously spawned by mature females, whereas
205 the sperms suspension was obtained by dissecting mature males. To this end, two
206 mature females were singularly maintained in the aquarium within 50 ml tubes provided
207 with glass pipe as a shelter (0.5 cm diameter) until spawning, which occurred within one
208 week. The spawned eggs were then collected within 1 hour and immediately fertilized
209 by a fresh sperm suspension. The following fertilization tests were performed in beakers
210 filled with 500 ml of 0.2 µm FASW at same salinity and temperature as the
211 experimental tanks. In particular, after 10 min contact between eggs and sperms, the
212 sperm surplus was washed away by seawater double rinsing. Eggs were considered
213 fertilized at the onset of fertilization membrane, which normally occurred after 2 hours
214 from sperm contact (Dales, 1950). For each test, 300 fertilized eggs individually
215 counted were equally distributed into 3 replicated beakers (500 ml) provided with

FASW. All the hatching nectochaete were counted after 72 h. No water renewal was done during the test.

"Natural-like" procedure

The procedure relies on using naturally spawned gametes. To this end, 5 mature females and 5 mature males were positioned in a 2 L aerated seawater aquarium, provided with 10 glass pipes (0.5 cm diameter, 10 cm length) on the bottom as shelters to the organisms. The worms naturally spawned in the aquarium within 5 days. Half water was renewed every second day. The aquarium were daily checked for the presence of spawned organisms and free gametes by withdrawing triplicate 20 ml-water samples from the gravel using a pipette. When observed, fertilized eggs were isolated and counted to be distributed at a density of 0.2 cells/ml in six beakers filled with 500 ml of 0.2 µm FASW at the same salinity and temperature as the aquarium. Hatching nectochaete were numbered after 72 h.

Effects of spawning induction procedures on fertilization and hatching success

The effects of thermal shock and endogenous substances on spawning induction were evaluated in two different experiments. In both experiments, control and treated samples were daily checked up to 1 week, and the number of spawned organisms counted. An individual was considered spawned when completely drained and at same time free gametes were noticed in the medium.

In both experiments, eggs were collected soon after spawning to be cross-fertilized *in vitro*, i.e. following the procedure described in paragraph "*In vitro* procedure". For each test, 400 fertilized eggs were counted and distributed using a 100 µm mesh size sieve into four replicated 500 ml backers filled with FASW. Observations on larval development were carried out daily, up to day 4, under optical microscope (Leica DMLB).

Statistical comparisons between treated and controls animals and between the results of the test as percentage of both fertilized and hatching eggs were performed using the G-Test.

Exposure to thermal shock

Two replicated samples, each one consisting of five mature individuals, were set up considering separately males and females. Organisms were taken from the aquaria at 16°C and rapidly transferred to 15L aquaria at 5±1°C for 5 days. Each individual was subsequently relocated into a 50 ml tube provided with glass pipe as a shelter (diameter 0.5 cm). The 5 tubes were then positioned on the bottom of the aquaria at 16 ±1 °C for one week. A 10°C rise in seawater temperature on spawning capacity acts a proximate cue for spawning in *A. virens* (Lewis et al., , 2002). Moreover, the same procedure had proven to induce the spawning of gametes in cross fertilization experiments with nereidid *Perinereis rullieri* Pilato, 1974 from Venice Lagoon (Prevedelli & Cassai, 2001; D. Prevedelli, personal observation). Two control replicates of five individuals for each sex were similarly manipulated but always maintained in the aquaria at 16 ±1 °C.

263 Exposure to whole tissue homogenates

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265 Immediately after field sampling, 10 mature organisms (5 males and 5 females) were
266 minced individually using a Potter-Elvehjem homogenizing system. The homogenates
267 from each individual were subsequently stored at -80° C in 1 ml glass vials. Two
268 replicated samples of 7 males and 7 females per aquarium were separately placed within
269 50 ml tubes provided with glass pipes as shelter (diameter 0.5 cm), positioned on the
270 bottom of each 15 L aquarium. One ml of defrosted homogenate of contrasting sex
271 tissue was added to each replicate aquarium every second day by carefully diluting it in
272 water, up to one week. At the end of the experiment in each aquarium the homogenate
273 density was 0.2 ‰. This value is considered to be a threshold level above which the
274 water quality drastically worsens and the proliferation of protozoa increases (R.
275 Simonini, personal observation). For each sex two control replicates, each consisting of
276 7 individuals, were performed.

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278 Larval development and effect of stock density on survival and growth of juveniles

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280 Larval development of embryos rising from hatched eggs from *in vitro* procedure was
281 monitored daily under the microscope and time required to reach the following stages of
282 trocophora, metatrochophora, nechochaete and juvenile was recorded (Dales, 1950).
283 Juveniles at 6-10 setigers stage (1 month old) were subsequently selected and
284 distributed in five 10 L aquaria provided with FASW, biological filters and 2 cm of fine
285 sand sediment on the bottom (grain size 125-250 μm). The sediment, recovered from the
286 sandy shoreline of the Lido Island, was sieved, washed in tap and salted water and

maintained for 48 hours at 90° C to remove larger debris and native animals before being placed in acquaria. Initial densities were set at 5200 ind m^{-2} (in 3 replicates) and 10400 ind m^{-2} (in 2 replicates). Organisms were fed *ad libitum* with commercial fish feed (Classic C22 Hendrix®, 28% protein, 7% fat) three times a week; half of water was renewed every second day. Juvenile development was followed for 8 weeks. Temperature, salinity and photoperiod were fixed at 16 ± 1°C, 16 gL^{-1} and 16/8 hr light/dark, respectively (Nesto et al.,, 2012). Survival and wet weights were determined at the end of the experiment. Fresh weights were evaluated individually with a precision balance (0.0001 g) after wiping each organism for 1 min on absorbent paper.

Statistical comparisons between survival and wet weights evaluated in samples maintained at the two densities were performed using the non parametric Kruskal-Wallis test.

Allometric relationships

Juveniles obtained from the *in vitro* fertilization procedure were maintained in aerated aquaria provided with biological filter and 5 cm of natural sediment (previously treated as already described to obtain grain size 125-250 μm) at a density of 1000 individuals m^{-2}. They were fed *ad libitum* using commercial fish feed (Classic C22 Hendrix®) three time a week for 2 months. This feed had been already documented to promote the rapid growth of *H. diversicolor* under laboratory conditions (Nesto et al., 2012). Periodically, organisms were randomly collected and sacrificed for allometric measurements. As a whole, 80 worms of different sizes showing no regeneration signs were collected from the aquaria and anesthetized with 7% Ethyl 3-aminobenzoate methanesulfonate

(MS222-Sigma Aldrich). Total body length was determined by positioning on a millimetric graph paper each single organism after carefully wiping on adsorbent paper, segment numbers were counted individually under stereomicroscope and L3 length was evaluated through the imaging analysis (Image - Pro Plus software version 4.0.09) of the individual anterior portions (ACDSee free software). Fresh weights were determined as described above; dry weights were determined after 48 h in oven at 70°C and ash-free dry weight (AFDW) subsequently obtained after 4 h at 470°C (Durou, Mouneyrac & Amiard-Triquet, 2008).

Biometric measurements were used to determine allometric relationships based on regression models using Microsoft Office Software. In particular, the relationships between: i) individual fresh weight *vs* dry weight, ash-free dry weight (AFDW), total body length and number of setiger, respectively and ii) individual L3 length *vs* fresh weight, dry weight, total body length and number of setigers, respectively, were analyzed.

Results

Effect of *in vitro* vs "natural-like" fertilization procedure on fertilization and hatching success

The results of *in vitro* fertilization procedure demonstrated significantly higher fertilization and hatching percentages than those obtained by "natural-like" fertilization (G test, $p<0.001$; Tab. 1A). In particular, the highest percentages of fertilized and hatched eggs obtained through the *in vitro* fertilization ranged from 94 to 100 and from

94 to 99, respectively, whereas when the “natural-like” procedure was used values of the same parameters varied from 61 to 91 and from 46 to 88, respectively.

Effects of different spawning induction procedures on fertilization and hatching success

The procedure of thermal shock induced more frequent spawning events than in the controls (G-test, $p<0.01$); in particular, the recorded percentages of spawnings in the treated females and males were 90 and 100, respectively, whereas in the controls these values were 20 and 30, respectively (Fig. 1A).

The procedure of male homogenate exposure induced more frequent spawning events in treated females (57% in treated *vs* 14% in the controls; G Test, $p<0.05$). Conversely, no significant effects were observed in males after exposure to female homogenates (7% and 14% in treated and control groups, respectively; G Test $p>0.05$) (Fig. 1B).

Eggs spawned after thermal shock exhibited significantly lower percentages of fertilization (0-40%) (G test, $p<0.001$) in comparison with those released by females exposed to male homogenates (94-100%). Similarly, the hatching percentages of the fertilized eggs from thermal shock (0-32%) were lower than those from homogenate exposures (90-98%) (G test, $p<0.001$) (Tab. 1B).

Larval development and effect of two stock densities on survival and growth of juveniles

The results of the larval development were showed in Fig.2. The presence of the fertilization membrane was observed 1.5-2 hours after mixing the gametes and a spiral

cleavage process led to the achievement the first trocophora stage (190±10µm) 2 days after fertilization. The metatrocophora stage (220±10µm) was reached at day 3, first 3-setigers nechochaetes (425±30 µm) were observed after 4 days and 5-setigers juveniles (700±50µm) after 14 days.

After 8 weeks post-breeding, the fresh weights of juveniles were similar at the two tested densities, i.e. 30.4±0.8 mg at 5200 ind m^{-2} and 29.9±1.2 mg at 10400 ind m^{-2}. The survival percentages varied from 57 to 81 in the samples at lower density, and from 53 to 57% at higher density. No statistical differences between the two density conditions were evidenced for both growth and survival percentages (Kruskall Wallis Test, $p>0.05$) (Fig. 3).

Allometric relationship analysis

The relationships between fresh and dry weights and AFDW followed a linear pattern (Fig. 4A-B), whereas between fresh weight and length parameters (total body length and number of setigers) they were best fitted by power curves (Fig. 4C-D). Fresh weight parameter showed the highest coefficient of determination with dry weight ($R^2=0.91$) and ALDFG ($R^2=0.963$). The relationships between L3 length and fresh weight, and between L3 length and dry weight were described by exponential curves with different equations (Fig. 5A-B). Finally, relationships between L3 and other length variables (total body length and number of setigers) followed linear patterns (Fig. 5C-D). L3 length parameter showed the highest coefficient of determination with fresh weight ($R^2=0.818$) and total body length ($R^2=0.819$).

Discussion

The experimental work carried out in this study demonstrated the possibility for mature organism of *H. diversicolor* to be manipulated to carry out the artificial fertilization, a necessary procedure to have appropriate numbers of larvae and juveniles available throughout the year, thus overcoming temporal constrictions linked to natural reproduction cycles.

Our results suggest that the *in vitro* fertilization technique is most suitable for producing larvae, as shown by higher rates of fertilization. However, we cannot exclude that the poor results obtained through the "natural-like" fertilization have been mainly driven by unsuitable microbiological conditions, i.e presence of a number of free protozoans in the growing medium, which may have affected negatively larval development.

Temperature is a well-known key factor for the success of the fertilization process in polychaetes (Lewis et al., 2002). An increase in temperature within the tolerance limits of the gametes may raise the success of fertilization by stimulating egg-sperm collisions. However, fertilization rates may be reduced beyond certain temperature threshold, due to thermal negative effects on the cortical reaction (Allen & Hagstrom, 1955), and on microtubules distribution in the fertilized eggs (Harris, Clason & Prier, 1989). Various experiments have shown that best performances in *A. virens* and in the lugworms *Arenicola marina* (Linnaeus, 1758) and *Arenicola loveni* Kinberg, 1866 are reached when the fertilization process occurs at temperatures ranging between 15- 20° C, i.e. values comparable with the one used in our experiments (16° C). At these thermal conditions fertilization percentages ranged between 60 - 90 in *A. virens*, 50 - 70 in *A. marina* and 70 - 80 in *A. loveni* (Lewis et al., 2002; Lewis, 2005). The percentages of

fertilization achieved in our experiments are similar to those reported by Ozoh & Jones (1990) in an extensive study regarding the effects of temperature, salinity and copper contamination on various stages of *H. diversicolor* life cycle, whereas the hatching percentages were higher. Moreover, different fertilization tests performed in congeneric specie,(e.g. *Hediste japonica* (Izuka, 1908) and *Hediste. diadroma* Sato & Nakashima, 2003) exhibited similar results to ours (Tosuji & Sato, 2006). Also the first stages of larval development, recorded for *H. diversicolor* in the present study (the first free stage –trochophore, is reached at day 2 from fertilization), are similar to those reported by Tosuji & Sato (2006).

A further point to be considered for developing a successful indoor farming system is the setting up of procedures suitable to induce simultaneous gametes emissions, which are necessary to maximize larval production, and to obtain sustainable amount of cohorts of organisms to be used for sale or kept as a breeding stock (Olive, 1999). A number of studies have well documented that temperature may also widely influence spawning in marine invertebrates (see the review of Olive, 1995). Spawning events in *H. diversicolor*, which are generally synchronized, occur especially after exposure to low winter temperatures during full or new moon periods (Bartels-Hardège & Zeeck, 1990). In *A. virens* the maximum fertilization success was recorded at 15-18°C and a rise in the seawater temperature of 6-10°C was observed to act as a proximate cue for spawning (Lewis et al., 2002). Starting from these observations and considerations, our experimental approach was aimed at testing the effect of alternate exposure to lower (5±1°C) and higher (16±1°C) temperatures on spawning capacity. In our experiment, despite the prompt spawning of most treated organisms, the success of subsequent fertilization and hatching was scarce, thus indicating a poor quality of the induced

gametes, which were supposed to be likely negatively altered or damaged by the spawning procedures. Although exposure to rapid temperature variation up to 10°C are commonly used to induce spawning in bivalves and penaeid crustaceans (Paesanti & Pellizzato, 1994; Scovacricchi, 1994), a more gradual increase of temperature is probable needed for *H. diversicolor* to obtain good quality gametes, considering that in wild populations at our latitudes the pre-spawning period is characterized by a progressive rise in temperature.

We also tested the use of natural homogenates in which released endogenous organic substances would possibly act as chemical signals suitable to induce gamete spawning. Our approach was based on the findings of Andries (2001), who reported that environmental factors, such as temperature, photoperiod and lunar cycles are not sufficient to synchronize the reproductive events in marine polychaetes, particularly in epitoke Nereididae, and suggested that other endogenous factors may play a very significant role in inducing the spawning process. With this in minds, and considering at the same time the need of developing simplified reproduction techniques, our experimental mixture (homogenate of mature organism) was tested by simply diluting it into the water of the aquaria, not dissimilarly from methods already adopted in a previous paper (Watson, Bentley, Gaudron & Hardege, 2003). In particular, the results of these Authors, had shown that most of their tested organic substances on *A. succinea* (coelomic fluid and water containing spawned gametes) were suitable to induce spawning events, at least in males. In the present study, our test homogenate induced spawning in females only. This limited effect might be explained by hypothesizing that the sexual pheromone concentration in the homogenate was not sufficient to induce spawning in males; on the other hand, female organisms might also exhibit a lower

threshold response than males, as already known for different species (Hardege, Müller, Beckmann & Bartels-Hardege, 1998). However, the high percentages of fertilized and hatched eggs resulting from this procedure suggest the good quality of gametes.

The establishment of optimal breeding density values and the identification of the most effective diet formulation are other crucial steps to achieve sustainable biomass production and at the same time to contribute to the reproductive success in many polychaete species (Prevedelli, 1994; Olivier, Desrosiers, Caron, Retière & Caillou, 1996; Olive, 1999; Scaps, 2002; Safarik, Redden & Schreider, 2006). The results of the density effects on juveniles growth (starting from 6-10 setigers stage) showed similar growth and survival rates at the two experimental densities, suggesting that the levels of intra-specific competition in no-limiting food conditions are sufficiently low to provide good growth performance. In particular, the increasing number of setigers developed over the eight weeks of the experiment was estimated to be about two-fold higher than in *P. rullieri* nectochaetes maintained at a much lower density and fed with TetraMin, a fish feed enriched with proteins and vitamins (Prevedelli & Zunarelli Vandini, 1997).

The results of the allometric evaluations showed that L3 length is a parameter that can be used as a descriptor of the total length, at least when undamaged animals are unavailable. The calculated trends are linear in the relationships between weight or length parameters, and exponential in the ratios length/weight. The trend line type as well as the coefficents of determination (R^2) which are close to 1 for most of the equations, are in agreement with those reported in a study on a natural population of *H. diversicolor* of the Venice Lagoon (Cornello, Delaney, Cavallini & Volpi Ghirardini, 2001), and with the results obtained in a study aimed at evaluating biometric data of two populations of *H. diversicolor* (Durou et al., 2008).

In conclusion, our results highlight the great adaptability of *H. diversicolor* to laboratory conditions indicating that artificial fertilization and gamete spawning induction procedures may be successfully applied in this species. Unfortunately, thermal shock spawning procedures may produce low quality gametes, and further studies are needed to improve the method. On the other hand, the use of whole body homogenates to induce spawning in females is considered a promising procedure, sufficiently easy to be carried out without sophisticated laboratory equipment and for this reason it is suitable to be transferred to aquaculture companies. Juveniles may be successfully fed using commercial feed, commonly used also for adult forms (Nesto et al., 2012), and may be reared at a density of 10,000 ind. m^{-2}, at least up to reach individual fresh weights around 30 mg.

Overall, after considering various biological aspects influencing the breeding potential of this species, i.e the fast larval development associated with the lack of pelagic larval stages, the relatively simple facilities needed to perform a controlled reproduction, to maintain in aquaria both larval forms and juveniles and to obtain commercial sized individuals in few months (Nesto et al., 2012), we conclude that *H. diversicolor* should to be considered a promising species to be reared for commercial purpose within indoor farming systems.

Nevertheless, for the establishment of commercially applicable protocols further studies are necessary to improve the quality of gamete obtained through spawning induction and to investigate methods suitable to synchronize reproductive events through temperature and photoperiod conditioning experiments.

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References

Abrantes, A., Pinto, F., & Moreira, M.H. (1999). Ecology of the polychaete *Nereis diversicolor* in the Canal de Mira (Ria de Aveiro, Portugal): population dynamics, production and oogenic cycle. Acta Oecologica, 20, 267-283. doi:10.1016/S1146-609X(99)00139-3

Allen R.D. & Hagstrom B. (1955) Interruption of the cortical reaction by heat. *Experimental Cell Research* **9**, 157-162.

Anderson, F.E., & Meyer, L.M. (1986). The interaction of tidal currents on a disturbed intertidal bottom with a resulting change in particulate matter quantity, texture and food quality. Estuarine, Coastal and Shelf Science, 22, 19–29. doi:10.1016/0272-7714(86)90021-1

Andries, J.C. (2001). Endocrine and environmental control of reproduction in Polychaeta. Canadian Journal of Zoology, 79, 254-270. doi:10.1139/z00-197

Ansaloni, I., Pellizzato, M., Prevedelli, D., & Zunarelli Vandini, R. (1986). Policheti di interesse economico nella Laguna di Venezia. Nova Thalassia, 8, 641-642.

Bartels-Hardege, H.D., & Zeeck, E. (1990). Reproductive behaviour of *Nereis diversicolor* (Annelida: Polychaeta). Marine Biology, 106, 409-412. doi: 10.1007/BF01344320

527 Beukema, J.J. (1995). The long-term effects of mechanical harvesting of lugworms on
528 the zoobenthos community of a tidal flat in the Wadden Sea. Netherlands Journal of Sea
529 Research 33, 219–227. doi: 10.1016/0077-7579(95)90008-X
530 Casellato, S., Furlan, K., & Bortolotto, L. (1999). Ciclo riproduttivo di *Hediste*
531 *diversicolor* (O.F. Müller) nella laguna di Venezia. Biologia Marina Mediterranea, 6,
532 351-353.
533 Caspers, H. (1984). Spawning periodicity and habitat of the palolo worm *Eunice viridis*
534 (Polychaeta, Eunicidae) in the Samoan islands. Marine Biology, 79, 229– 236.
535 doi:10.1007/BF00393254
536 Cornello, M., Delaney, E., Cavallini, L., & Volpi Ghirardini, A. (2001). Indagini
537 biometriche su *Hediste diversicolor* O.F. Müller (Polychaeta: Nereididae) nella Laguna
538 di Venezia. *Biologia Marina Mediterrane,* 8, 535-538.
539 Cross, N. L. (1984). Fertilization in *Urechis caupo* and in polychaetes. Fortschritte der
540 Zoologie, 29, 149–166.
541 Dales, R.P. (1950). The reproduction and the larval development of *Nereis diversicolor*
542 O.F. Müller. Journal of Marine Biology Association of the United Kingdom, 29, 321-
543 360. doi:10.1017/S0025315400055405
544 Durou, C., Mouneyrac, C., & Amiard-Triquet, C. (2008). Environmental quality
545 assessment in estuarine ecosystems: use of biometric measurements and fecundity of the
546 ragworm *Nereis diversicolor* (Polychaeta, Nereididae). Water Research, 42, 2157–
547 2165. doi: 10.1016/j.watres.2007.11.028
548 Fidalgo e Costa, P. (1999). Reproduction and growth in captivity of the polychaete
549 *Nereis diversicolor* O.F. Müller, 1776, using two different kinds of sediments:
550 preliminary assays. Boletin Instituto Espaňol de Oceanografia, 15, 351-355.

Fidalgo e Costa, P., Gil, J., Passos, A.M., Pereira, P., Melo, P., Batista, F., & Cancela da Fonseca, L. (2006). The market features of imported non-indigenous polychaetes in Portugal and consequent ecological concerns. Scientia Marina**,** 70S3, 287-292. doi:10.3989/scimar.2006.70s3287

Fidalgo e Costa, P., Narciso, L., & Cancela da Fonseca, L. (2000). Growth, survival and fatty acid profile of *Nereis diversicolor* (O.F. Müller, 1776) fed on six different diets. Bulletin of Marine Science, 67, 337-343.

Fauchald, K., & Jumars, P. A. (1979). The diet of worms: a study of polychaetes feeding guids. Oceanography and Marine Biology: An Annual Review, 17, 193–284.

Gambi, M.C., Castelli, A., Giangrande, A., Lanera, P., Prevedelli, D., & Zunarelli Vandini, R. (1994). Polychaetes of commercial and applied interest in Italy: an overview. In: J.C. Dauvin, L. Laubier & D.J. Reish (Eds), Actes de la 4ème Conférence Internationale des polychètes, Memories du Muséum National d'Historie Naturelle (pp 593-603). Paris.

Garcia-Alonso, J., Hoeger, U., & Rebscher, N. (2006). Regulation of vitellogenesis in *Nereis virens* (Annelida: Polychata): effect of estradiol-17ß on eleocytes. Comparative Biochemistry and Physiology A, 143, 55-61. doi:10.1016/j.cbpa.2005.10.022

Golding, D.W. (1967). Endocrinology, regeneration and maturation in *Nereis*. Biological Bulletin of the Marine Laboratory of Woods Hole, 133, 567-577. doi:10.2307/1539918

Hardege, J.D., Bartels-Hardege, H.D., Yu, Y., Zhu, M.Y., Wu, B.L., & Zeeck, E. (1994). Environmental control of reproduction of *Perinereis nuntia* var. *brevicirrus*. Journal of Marine Biology Association of the United Kingdom, 74, 903-918.

574 Hardege, J.D., Müller, C., & Beckmann, M. (1997). A water-borne female sex
575 pheromone in the ragworm *Nereis succinea* (Anellida, Polychaeta). Polychaete
576 Research, 17, 18-21.
577 Hardege, J.D., Müller, C., Beckmann, M., & Bartels-Hardege, H.D. (1998). Timing of
578 reproduction in marine polychaetes: the role of sex pheromones. Ecoscience, 5, 395-
579 404. doi:10.1080/11956860.1998.11682477
580 Harley, M. B. (1950). Occurence of a filter-feeding mechanism in the polychaete *Nereis*
581 *diversicolor*. Nature, 165, 734–735. doi: 10.1038/165734b0
582 Harris, P., Clason, E.L., & Prier, K.P. (1989). Tubulin polymerisation in unfertilised sea-
583 urchin eggs induced by elevated temperature. Journal of Cell Science, 93, 9-18.
584 Kawahigashi, D.K. (1998). Overview of maturation technology in the Western
585 hemisphere. Anais de Aquicoltura, 98, 381-392.
586 Lawrence, A.J., & Olive, P.J.W. (1995). Gonadotrophic hormone in *Eulalia viridis*
587 (Polychaeta, Annelida): Stimulation of vitellogenesis. Invertebrate Reproduction and
588 Development, 28, 43-52. doi:10.1080/07924259.1995.9672462
589 Lawrence, A.J., & Soame, J.M. (2009). The endocrine control of reproduction in
590 Nereidae: a new multi-hormonal model with implications for their functional role in a
591 changing environment. Philosophical Transaction of the Royal Society B: Biological
592 Sciences, 364, 3363-3376. doi:10.1098/rstb.2009.0127
593 Lewis, C., Olive, P.J.W., Bentley, M.G., & Watson, G. (2002). Does seasonal
594 reproduction occur at optimal time for fertilisation in the polychaetes *Arenicola marina*
595 L. and *Nereis virens* Sars? Invertebrate Reproduction and Development, 41, 61-71.
596 doi:10.1080/07924259.2002.9652736

Lewis, C. (2005). Fertilization, post-fertilization development and larval biology of the South African polychaete *Arenicola loveni loveni* (Kinberg, 1866). Invertebrate Reproduction and Development, 48, 19-30. doi:10.1080/07924259.2005.9652167

Marty, R., & Retière, C. (1999). Larval-to-juvenile mobility activities of a holobenthic species, *Nereis diversicolor* (O.F.Müller) (polychaeta: Nereidae) — their involvement in recruitment. Bulletin of Marine Science, 65, 761–773.

Meunpol, O., Meejing, P., & Piyatiratitivorakul, S. (2005). Maturation diet based on fatty acid content for male *Penaeus monodon* (Fabricius) broodstock. Aquaculture Research**,** 36, 1216-1225. doi:10.1111/j.1365-2109.2005.01342.x

Nesto, N., Simonini, R., Prevedelli, D., & Da Ros, L. (2012). Effects of diet and density on growth, survival and gametogenesis of the polychaete *Hediste diversicolor* (O.F. Müller, 1776). Aquaculture, 362-363, 1-9. doi:10.1016/j.aquaculture.2012.07.025

Olive, P.J.W. (1995). Annual breeding cycles in marine invertebrates and environmental temperatures: probing the proximate and ultimate causes of reproductive synchrony. Journal of Thermal Biology, 20, 79-90. doi:10.1016/0306-4565(94)00030-M

Olive, P.J.W. (1999). Polychaete aquaculture and polychaete science: a mutual synergism. Hydrobiologia, 402, 175–183. doi:10.1023/A:1003744610012

Olivier, M., Desrosiers, G., Caron, A., Retière, C., & Caillou, A. (1996). Juveniles growth of *Nereis diversicolor* (O.F. Müller) feeding on a range of marine vascular and macroalgal plant sources under experimental conditions. Journal of Environmental Marine Biology and Ecology, 208, 1-12. doi:10.1016/S0022-0981(96)02654-8

Ozoh, P.T.E., & Jones, N.V. (1990). Capacity adaptation of *Hediste (Nereis) diversicolor* embryogenesis to salinity, temperature and copper. Marine Environmental Research, 29, 227-243. doi:10.1016/0141-1136(90)90035-M

621 Paesanti, F., & Pellizzato, M. (1994). Tapes philippinarum. Ente Sviluppo Agricolo del
622 Veneto Ed.
623 Pawson, M.G., Glenn, H., & Padda, G. (2008). The definition of marine recreational
624 fishing in Europe. Marine Policy, 32, 339–350. doi:10.1016/j.marpol.2007.07.001
625 Pires, A., Gentil, F., Quintino, V., & Rodriguez, A.M., (2012). Reproductive biology of
626 *Diopatra neapolitana* (Annelida, Onuphidae), an exploited natural resource in Ria de
627 Aveiro (Northwestern Portugal). Marine Ecology, 33, 56–65. doi:10.1111/j.1439-
628 0485.2011.00463.x
629 Prevedelli, D. (1994). Influence of temperature and diet on the larval development and
630 growth of juveniles *Marphysa sanguinea* (Montagu) (Polychaeta, Eunicidae). In: J.C.
631 Dauvin, L. Laubier & D.J. Reish (Eds), Actes de la 4ème Conférence Internationale des
632 polychètes. Memoires Memoirs du Museum d'Histoire Naturelle (pp 521-526). Paris.
633 Prevedelli, D., & Cassai, C. (2001). Reproduction and larval development of *Perinereis*
634 *rullieri* Pilato in the Mediterranean Sea (Polychaeta: Neredidae). Ophelia, 54, 133-142.
635 doi:10.1080/00785236.2001.10409461
636 Prevedelli, D., & Zunarelli Vandini, R. (1997). Survival and growth rate of *Perinereis*
637 *rullieri* (polychaeta, Nereididae) under different salinities and diets. Italian Journal of
638 Zoology, 64, 135-139. doi:10.1080/11250009709356186
639 Ram, J.M., Müller, C.T., Beckmann, M., & Hardege, J.D. (1999). The spawning
640 pheromone cysteine-glutathione disulfide (nereithione) arouses a multicomponent
641 nuptial behaviour and electrophysiological activity in *Nereis succinea* males. The
642 FASEB Journal, 13, 945-952.
643 Reise, K. (1979). Spatial configurations generated by motile benthic polychaetes.
644 Helgoländer wiss. Meeresunters.**,** 32, 55–72.

645 Santos, A., Granada, L., Baptista, T., Anjos, C., Simões, T., Tecelão, C., Fidalgo e Costa,
646 P., Lino Costa, J., & Pombo, A. (2016). Effect of three diets on the growth and fatty
647 acid profile of the common ragworm *Hediste diversicolor* (O.F. Müller, 1776).
648 Aquaculture, 465, 37–42. doi:10.1016/j.aquaculture.2016.08.022
649 Safarik, M., Redden, A.M., & Schreider, M.J. (2006). Density-dependent growth of the
650 polychaete *Diopatra aciculata*. Scientia Marina**,** 70S3, 337-341.
651 Scaps, P. (2002). A review of the biology, ecology and potential use of the common
652 ragworm *Hediste diversicolor* (O.F. Müller) (Annelida: Polychaeta). Hydrobiologia,
653 470, 203-218. doi:10.1023/A:1015681605656
654 Scaps, P. (2003). Exploitation et élevage des vers marins. Bulletin de la Société
655 zoologique de France, 128, 21-33.
656 Scaps, P., Retiére, G., Desrosiers, G., & Miron, G. (1993). Effets de la ration
657 alimentaire, de la densitè intraspécifique et des relations entre individus sur la
658 croissance des juvèniles de l'espèce *Nereis diversicolor* (Annelida: Polychaeta).
659 Canadian Journal of Zoology, 71, 424-430. Scovacricchi, T. (1994). Crostacei Penaeidi.
660 Riproduzione Controllata Allevamento Larvale. Ente Sviluppo Agricolo del Veneto Ed.
661 Tosuji, H., & Sato, M. (2006). Salinity favourable for early development and gamete
662 compatibility in two sympatric estuarine species of the genus *Hediste* (Polychaeta:
663 Nereididae) in the Ariake Sea, Japan. Marine Biology**,** 148, 529 –539.
664 doi:10.1007/s00227-005-0079-1
665 Vedel, A., Andersen, B. B., & Riisgård, H. U. (1994). Field investigation of pumping
666 activity of the facultatively filter-feeding polychaete *Nereis diversicolor* using an
667 improved infrared phototransducer system. Marine Ecology Progress Series**,** 103, 91–
668 101.

669 Watson, G.J., Williams, M.E., & Bentley, M.G. (2000). Can synchronous spawning be
670 predicted from environmental parameters? A case study of the lugworm *Arenicola*
671 *marina*. Marine Biology, 136, 1003-1017. doi:10.1007/s002270000283
672 Watson, G.J., Bentley, M.G., Gaudron, S.M., & Hardege, J.D. (2003).The role of
673 chemical signals in the spawning induction of polychaete worms and other marine
674 invertebrates. Journal of Experimental Marine Biology and Ecology, 294, 169– 187.
675 doi:10.1016/S0022-0981(03)00264-8
676 Wouters, R., Lavens, P., Nieto, J., & Sorgeloos, P. (2001). Penaeid shrimp broodstock
677 nutrition: an updated review on research and development. Aquaculture, 202, 1–21.
678 doi:10.1016/S0044-8486(01)00570-1
679 Zeeck, E., Harder, T., & Beckmann, M. (1998a). Inosine, L-glutamic acid and L-
680 glutamine as components of a sex pheromone complex of the marine polychaete *Nereis*
681 *succinea* (Annelida: Polychaeta). Chemoecology, 8, 77-84. doi:10.1007/PL00001807
682 Zeeck, E., Harder, T., & Beckmann, M. (1998b). The sperm release pheromone of the
683 marine polychaete *Platinereis dumerilii*. Journal of Chemistry and Ecology, 24, 13-22.
684

685 **Figure legends**

686

687 Fig. 1 - Spawning percentages (Mean ± SD) in males and females exposed to thermal
688 shock (A) and mature organism homogenate (B). Statistical comparison between treated
689 and controls: G-test, * = $p<0.05$; ** = $p<0.01$.

690

691 Fig. 2 - Larval development of *H. diversicolor*: (A) day 0 – fertilised eggs; (B) day 1 –
692 cleavage eggs; (C) day 2 – trochophore; (D) day 3 – metatrochophore; (E) day 4 –
693 nectochaete with three chaetigers; (F) day 14 – juvenile with five chaetigers.

694

695 Fig. 3 – Fresh weight (mg) and survival (%) of *H. diversicolor* (Mean ± SD) maintained
696 for eight weeks at two rearing densities. Statistical comparison between two densities:
697 Kruskal-Wallis test, not significant.

698

699 Fig. 4 – Relationship between fresh weight and dry weight (A), ash-free dry weight
700 (AFDW) (B), total body length (C) and number of setigers (D).

701

702 Fig. 5 - Relationship between L3 length and fresh weight (A), dry weight (B), total body
703 length (C) and number of setigers (D).

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