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

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19  
20 Running title: Reproductive procedures for *Hediste diversicolor*

21  
22 Key-words: Polychaetes, *Hediste diversicolor*, breeding technique, spawning induction,  
23 fertilization, larval development.

25     **Abstract**

26

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

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

43

44

45

46     **Introduction**

47

48 Several polychaete species are commercially exploited (most in the families  
49 Arenicolidae, Eunicidae, Glyceridae, Lumbrineridae, Nephtyidae and, mainly  
50 Nereididae) (Scaps, 2003), being economically attractive as marine baits in recreational  
51 fishing or as food supplement in aquaculture (Olive, 1999). Some species of  
52 polychaetes are used as dietary components due to their positive effects on Penaeid  
53 shrimp reproduction and their high levels of polyunsaturated fatty acid (Olive, 1999;  
54 Wouters, Lavens, Nieto & Sorgeloos, 2001). In particular, bloodworms (*Glycera* sp.),  
55 sandworm (*Perinereis* sp.) and mudworms (*Marphysa* sp.) are intensively used in  
56 marine shrimp broodstock maturation diets (Kawahigashi, 1998; Meunpol, Meejing &  
57 Piyatiratitivorakul, 2005). The commercialized worms are mostly harvested from field  
58 populations by anglers or professional diggers, which are particularly numerous in the  
59 South China Sea (Korea, China), the north-east coast of USA (Maine) and Europe  
60 (Netherlands, UK, Spain, Italy) (Olive, 1999; Scaps, 2003). The intense commercial  
61 harvesting of polychaete worms from natural environments have been indicated as the  
62 main reason for the depletion of this natural resource (Gambi et al. 1994; Olive, 1999;  
63 Pires, Gentil, Quintino & Rodriguez, 2012) and as one significant cause for the  
64 alterations of habitats and consequently of benthic communities (Anderson & Meyer,  
65 1986; Beukema, 1995; Olive, 1999). The bait market demand for the European fishing  
66 is too high to be met by local supply, so most of the worms sold in Europe are imported  
67 from Eastern Asia, increasing the risk of introduction of non-native species (Scaps,  
68 2003; Fidalgo e Costa et al., 2006). At the end of 1990's the European bait worm market  
69 had been estimated to value about 200 million euros (Olive, 1999) and more recent  
70 assessments indicates that the whole European recreational fishing industry is valued 8-  
71 10 billion euros ([www.eaa-europe.org](http://www.eaa-europe.org); Pawson, Glenn & Padda, 2008).

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

77 The ragworm *Hediste diversicolor* (O.F. Müller, 1776) is one of the most used and  
78 appreciated bait for recreational fishing in Italy, where it is actively harvested for  
79 commercial purposes from a number of natural littoral environments along the Adriatic  
80 and Tyrrhenian coastline (Ansaloni, Pellizzato, Prevedelli & Zunarelli Vandini, 1986;  
81 Gambi et al. 1994). In the wild, this species lives burrowed in the sediment and it is  
82 considered a deposit-feeder, i.e. it collects the food at the opening of gallery, by  
83 crawling and ingesting sediment, or by capturing the organic particulate by means of  
84 mucous secretions (Fauchald & Jumars, 1979; Reise, 1979). In addition, it may also  
85 take the typical alimentary filter feeder behavior, being able to select the particles  
86 trapped in the mucus which are subsequently passively conveyed to U or Y shaped  
87 burrows by the water circulation (Harley, 1950; Vedel, Andersen & Riisgård, 1994).  
88 Various studies had already suggested that *H. diversicolor* might be a suitable species  
89 for aquaculture purpose, proving to be easy to breed in indoor systems and to exhibit  
90 several favorable physiological traits, such as high tolerance to a wide range of  
91 environmental parameters (salinity, temperature, substrate grain size) and rapid growth  
92 rates, at least when fed with commercial fish foods (Scaps, Retière, Desrosiers & Miron,  
93 1993; Fidalgo e Costa, 1999; Fidalgo e Costa, Narciso & Cancela da Fonseca, 2000;  
94 Nesto, Simonini, Prevedelli & Da Ros, 2012). Moreover, *H. diversicolor* may be a  
95 potentially high quality fatty acids source for reared fish and shrimp as it has been

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

113 In many polychaetes species, body growth and gametogenesis are controlled by a  
114 hormone produced by the cerebral neuroendocrine system, which is also essential for  
115 segment regeneration processes (Golding, 1967; Andries, 2001; Lawrence & Soame,  
116 2009). In nature, the transition from short to long photoperiod is an important factor for  
117 the start of the gametogenesis. In autumn the individuals able to breed show a decrease  
118 of juvenile hormone levels, probably also in response to increased levels of dopamine or  
119 melatonin. This allows oocytes to enter vitellogenic phase which is further increased by  
120 the production and secretion of vitellogenin by eleocytes, induced by the presence of  
121 oestradiol 17 $\beta$  (Garcia-Alonso, Hoeger & Rebscher, 2006). The production of estradiol-

122 17β and the ability to absorb vitellogenin are incremented by a second neurohormone in  
123 the cerebral ganglion of mature females, which has a gonadotrophic function (Lawrence  
124 & Olive, 1995). Moreover, a specific environmental signal or a hierarchical series of  
125 signals are often necessary to induce a synchronized spawning in wild populations  
126 (Caspers, 1984; Hardege et al., 1994; Watson, Williams & Bentley, 2000). Also  
127 chemical endogenous substances, such as pheromones, are known to induce gamete  
128 spawning at least in those polychaete species, e.g, the nereids *Alitta succinea* (Leuckart,  
129 1847) and *Platynereis dumerilii* (Audouin & Milne Edwards, 1834), exhibiting  
130 behavioral gender interactions such as nuptial dances, (Hardege, Müller & Beckmann,  
131 1997; Zeeck, Harder & Beckmann, 1998a, b; Ram, Müller, Beckmann & Hardege,  
132 1999; Andries, 2001).

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

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

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

171

## 172 **Materials and methods**

173

### 174 Sampling and experimental conditions

175

176 At the begin of the experimental activities (February 2009), 150 adult specimens of *H.*  
177 *diversicolor* (weight  $0.8 \pm 0.2$  g [mean  $\pm$  SD]) were sampled along the Dese estuary,  
178 Lagoon of Venice, Italy (Lat.  $45^{\circ} 31' 6.5''$ N; Long.  $12^{\circ} 24' 47.02''$ E; water temperature  
179 =  $10^{\circ}$ C; salinity  $10$  gL<sup>-1</sup>). Worms were maintained one week before the experiments in  
180 15 L aquaria filled with filter and aerated seawater (FASW) at temperature  $16 \pm 1^{\circ}$ C for  
181 acclimatization, assuming that this is the optimum temperature value to achieve good  
182 fertilization rates, similarly to other Nereidids breeding in the early spring (Lewis, Olive  
183 Bentley & Watson, 2002). Salinity and photoperiod was always set at  $16$  gL<sup>-1</sup> and 16:8  
184 hr light/dark, respectively, being the values already known to be the most effective to  
185 achieve good growth and survival performances in juveniles of *H. diversicolor* (Nesto et  
186 al., 2012). Treated and control animals were not fed during the experiments. All the  
187 experiments with their related control tests were performed at these conditions, unless  
188 differently reported, using mature organisms only. The sexual maturity of the  
189 individuals was checked before each experiment by examination of a drop of gametes  
190 syringed *in vivo* from the parapodia under a binocular microscope (Wild M420). The  
191 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  $\mu\text{m}$  in diameter) were considered signals of sexual maturity (Dales, 1950).

194

195 Effect of fertilization procedures on fertilization and hatching success

196

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

203

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  $\mu\text{m}$  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

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

218

219 “Natural-like” procedure

220

221 The procedure relies on using naturally spawned gametes. To this end, 5 mature females  
222 and 5 mature males were positioned in a 2 L aerated seawater aquarium, provided with  
223 10 glass pipes (0.5 cm diameter, 10 cm length) on the bottom as shelters to the  
224 organisms. The worms naturally spawned in the aquarium within 5 days. Half water was  
225 renewed every second day. The aquarium were daily checked for the presence of  
226 spawned organisms and free gametes by withdrawing triplicate 20 ml-water samples  
227 from the gravel using a pipette. When observed, fertilized eggs were isolated and  
228 counted to be distributed at a density of 0.2 cells/ml in six beakers filled with 500 ml of  
229 0.2  $\mu\text{m}$  FASW at the same salinity and temperature as the aquarium. Hatching  
230 nectochaete were numbered after 72 h.

231

232 Effects of spawning induction procedures on fertilization and hatching success

233

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

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

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

248

249 Exposure to thermal shock

250

251 Two replicated samples, each one consisting of five mature individuals, were set up  
252 considering separately males and females. Organisms were taken from the aquaria at  
253 16°C and rapidly transferred to 15L aquaria at 5±1°C for 5 days. Each individual was  
254 subsequently relocated into a 50 ml tube provided with glass pipe as a shelter (diameter  
255 0.5 cm). The 5 tubes were then positioned on the bottom of the aquaria at 16 ±1 °C for  
256 one week. A 10°C rise in seawater temperature on spawning capacity acts a proximate  
257 cue for spawning in *A. virens* (Lewis et al., , 2002). Moreover, the same procedure had  
258 proven to induce the spawning of gametes in cross fertilization experiments with  
259 nereidid *Perinereis rullieri* Pilato, 1974 from Venice Lagoon (Prevedelli & Cassai,  
260 2001; D. Prevedelli, personal observation). Two control replicates of five individuals for  
261 each sex were similarly manipulated but always maintained in the aquaria at 16 ±1 °C.

262

263 Exposure to whole tissue homogenates

264

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.

277

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

279

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

287 maintained for 48 hours at 90° C to remove larger debris and native animals before  
288 being placed in aquaria. Initial densities were set at 5200 ind m<sup>-2</sup> (in 3 replicates) and  
289 10400 ind m<sup>-2</sup> (in 2 replicates). Organisms were fed *ad libitum* with commercial fish  
290 feed (Classic C22 Hendrix®, 28% protein, 7% fat) three times a week; half of water was  
291 renewed every second day. Juvenile development was followed for 8 weeks.  
292 Temperature, salinity and photoperiod were fixed at 16 ± 1°C, 16 gL<sup>-1</sup> and 16/8 hr  
293 light/dark, respectively (Nesto et al., 2012). Survival and wet weights were determined  
294 at the end of the experiment. Fresh weights were evaluated individually with a precision  
295 balance (0.0001 g) after wiping each organism for 1 min on absorbent paper.  
296 Statistical comparisons between survival and wet weights evaluated in samples  
297 maintained at the two densities were performed using the non parametric Kruskal-  
298 Wallis test.

### 300 Allometric relationships

301

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

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

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

325

## 326 **Results**

327

### 328 Effect of *in vitro* vs “natural-like” fertilization procedure on fertilization and hatching 329 success

330

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

335 94 to 99, respectively, whereas when the “natural-like” procedure was used values of  
336 the same parameters varied from 61 to 91 and from 46 to 88, respectively.

337

338 Effects of different spawning induction procedures on fertilization and hatching success

339

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

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

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

353

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

356

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

359 cleavage process led to the achievement the first trocophora stage ( $190\pm 10\mu\text{m}$ ) 2 days  
360 after fertilization. The metatrocophora stage ( $220\pm 10\mu\text{m}$ ) was reached at day 3, first 3-  
361 setigers nechochaetes ( $425\pm 30\mu\text{m}$ ) were observed after 4 days and 5-setigers juveniles  
362 ( $700\pm 50\mu\text{m}$ ) after 14 days.

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

369

#### 370 Allometric relationship analysis

371

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

382

383 **Discussion**

384

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

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

395 Temperature is a well-known key factor for the success of the fertilization process in  
396 polychaetes (Lewis et al., 2002). An increase in temperature within the tolerance limits  
397 of the gametes may raise the success of fertilization by stimulating egg-sperm  
398 collisions. However, fertilization rates may be reduced beyond certain temperature  
399 threshold, due to thermal negative effects on the cortical reaction (Allen & Hagstrom,  
400 1955), and on microtubules distribution in the fertilized eggs (Harris, Clason & Prier,  
401 1989). Various experiments have shown that best performances in *A. virens* and in the  
402 lugworms *Arenicola marina* (Linnaeus, 1758) and *Arenicola loveni* Kinberg, 1866 are  
403 reached when the fertilization process occurs at temperatures ranging between 15- 20°  
404 C, i.e. values comparable with the one used in our experiments (16° C). At these thermal  
405 conditions fertilization percentages ranged between 60 - 90 in *A. virens*, 50 - 70 in *A.*  
406 *marina* and 70 - 80 in *A. loveni* (Lewis et al., 2002; Lewis, 2005). The percentages of

407 fertilization achieved in our experiments are similar to those reported by Ozoh & Jones  
408 (1990) in an extensive study regarding the effects of temperature, salinity and copper  
409 contamination on various stages of *H. diversicolor* life cycle, whereas the hatching  
410 percentages were higher. Moreover, different fertilization tests performed in congeneric  
411 specie,(e.g. *Hediste japonica* (Izuka, 1908) and *Hediste. diadroma* Sato & Nakashima,  
412 2003) exhibited similar results to ours (Tosuji & Sato, 2006). Also the first stages of  
413 larval development, recorded for *H. diversicolor* in the present study (the first free stage  
414 –trochophore, is reached at day 2 from fertilization), are similar to those reported by  
415 Tosuji & Sato (2006).

416 A further point to be considered for developing a successful indoor farming system is  
417 the setting up of procedures suitable to induce simultaneous gametes emissions, which  
418 are necessary to maximize larval production, and to obtain sustainable amount of  
419 cohorts of organisms to be used for sale or kept as a breeding stock (Olive, 1999). A  
420 number of studies have well documented that temperature may also widely influence  
421 spawning in marine invertebrates (see the review of Olive, 1995). Spawning events in  
422 *H. diversicolor*, which are generally synchronized, occur especially after exposure to  
423 low winter temperatures during full or new moon periods (Bartels-Hardège & Zeeck,  
424 1990). In *A. virens* the maximum fertilization success was recorded at 15-18°C and a  
425 rise in the seawater temperature of 6-10°C was observed to act as a proximate cue for  
426 spawning (Lewis et al., 2002). Starting from these observations and considerations, our  
427 experimental approach was aimed at testing the effect of alternate exposure to lower  
428 (5±1°C) and higher (16±1°C) temperatures on spawning capacity. In our experiment,  
429 despite the prompt spawning of most treated organisms, the success of subsequent  
430 fertilization and hatching was scarce, thus indicating a poor quality of the induced

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

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

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

458 The establishment of optimal breeding density values and the identification of the most  
459 effective diet formulation are other crucial steps to achieve sustainable biomass  
460 production and at the same time to contribute to the reproductive success in many  
461 polychaete species (Prevedelli, 1994; Olivier, Desrosiers, Caron, Retière & Caillou,  
462 1996; Olive, 1999; Scaps, 2002; Safarik, Redden & Schreider, 2006). The results of the  
463 density effects on juveniles growth (starting from 6-10 setigers stage) showed similar  
464 growth and survival rates at the two experimental densities, suggesting that the levels of  
465 intra-specific competition in no-limiting food conditions are sufficiently low to provide  
466 good growth performance. In particular, the increasing number of setigers developed  
467 over the eight weeks of the experiment was estimated to be about two-fold higher than  
468 in *P. rullieri* nectochaetes maintained at a much lower density and fed with TetraMin, a  
469 fish feed enriched with proteins and vitamins (Prevedelli & Zunarelli Vandini, 1997).

470 The results of the allometric evaluations showed that L3 length is a parameter that can  
471 be used as a descriptor of the total length, at least when undamaged animals are  
472 unavailable. The calculated trends are linear in the relationships between weight or  
473 length parameters, and exponential in the ratios length/weight. The trend line type as  
474 well as the coefficients of determination ( $R^2$ ) which are close to 1 for most of the  
475 equations, are in agreement with those reported in a study on a natural population of *H.*  
476 *diversicolor* of the Venice Lagoon (Cornello, Delaney, Cavallini & Volpi Ghirardini,  
477 2001), and with the results obtained in a study aimed at evaluating biometric data of two  
478 populations of *H. diversicolor* (Durou et al., 2008).

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

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

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

501

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503

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507

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684

## 685 **Figure legends**

686

687 Fig. 1 - Spawning percentages (Mean  $\pm$  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  $\pm$  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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