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Oocyte development in *Hediste diversicolor* (O.F. Müller, 1776) and influence of manipulated light cycles

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Marine Coastal Development

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Abstract

In developing a sustainable aquaculture industry, it will be important to recycle wastes and discards to minimize negative environmental impacts and increase the total value creation. The polychaete *Hediste diversicolor* is a promising species to utilize salmon waste and recycle such wastes into valuable new raw biomass. To establish an intensive production of the species, it is crucial to obtain protocols for reproduction. To ensure controlled production and availability of worms for cultivation, maturation and spawning needs to be manipulated and controlled.

In the present study a histological approach was used to investigate the oocyte development in *H. diversicolor*. The possibility of accelerating the maturation process by use of a manipulated light cycle was investigated. Oocyte development was investigated in samples taken directly from the field (Leangbukta, Trondheim). Effect of a simulated natural light cycle treatment and a compressed light cycle treatment, both kept at constant temperature (8 °C), was investigated on two different experimental groups. The compressed light cycle was reduced to 40 % of the simulated natural light cycle, which means that the daylength fluctuations in the simulated natural treatment over 118 days was reduced to 46 days.

The oocyte development in wild sampled *H. diversicolor* showed that the oocytes got rounder in shape as the oocyte size increased. In the present study, the size of the oocytes was measured by drawing a closed polyline along the cell membrane of the oocytes. By performing this measurement, both the area (μm^2) and the perimeter (µm) was measured for each oocyte. This made it possible to calculate the roundness of the oocyte, and a shape factor was derived.

The present study showed that the oocytes in the compressed light treatment were significantly less mature than the oocytes in the simulated natural light treatment during the experimental duration (%). In conclusion, the compressed light cycle did not accelerate the oocyte maturation in *H. diversicolor*.

Sammendrag

I utviklingen av en bærekraftig oppdrettsindustri vil det være viktig å gjenvinne avfall og utkast for å minimere de negative miljøpåvirkningene og øke den totale verdiskapingen. Børstemarken *Hediste diversicolor* er en lovende art for å utnytte avfall fra lakseoppdrett og resirkulere slikt avfall for å skape verdifull, ny biomasse. For å etablere en intensiv produksjon av arten vil det være avgjørende å oppnå protokoller for reproduksjon. For å sikre en kontrollert produksjon og tilgjengelighet av børstemark for kultivering, må modnings- og gytingsprosessen kunne manipuleres og kontrolleres.

I den nåværende studien ble en histologisk tilnærming brukt for å undersøke oocyttutviklingen i *H. diversicolor*. Muligheten for å akselerere modningsprosessen ved bruk av en manipulert lyssyklus ble utforsket. Oocyttutviklingen ble undersøkt i prøver som var tatt direkte fra felt (Leangbukta, Trondheim). Effekten av en simulert naturlig lyssyklus behandling og en komprimert lyssyklus behandling, begge holdt ved en konstant temperatur (8 °C), ble undersøkt for de to eksperimentelle behandlingene. Den komprimerte lyssyklusen var redusert til 40 % av den simulerte naturlige lyssyklusen, som betyr at variasjonen i daglengde i den simulerte naturlige lyssyklusen over 118 dager ble redusert til 46 dager.

Oocytt utviklingen i *H. diversicolor* fra felt viste at oocyttene ble rundere på form når størrelsen på oocyttene økte. I den nåværende studien ble størrelsen på oocyttene målt ved å tegne en lukket polylinje langs cellemembranen til oocyttene. Ved å utføre denne målingen, ble både arealet (μm^2) og perimeteren (µm) målt for hver oocytt. Dette gjorte det mulig å kalkulere rundheten av oocytten, og en form faktor ble utledet.

Den nåværende studien viste at oocyttene i den komprimerte lyssyklus behandlingen var signifikant mindre modne enn oocyttene i den simulerte naturlige lyssyklus behandlingen gjennom den eksperimentelle perioden (%). Det ble konkludert at den komprimerte lyssyklusen ikke akselererte oocyttmodningen i *H. diversicolor*.

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Trondheim, January 2019 – Marianne Uhre

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Abbreviations

ANCOVA	Analysis of covariance
Compressed light treatment (C)	Experimental treatment group with a compressed light cycle
DHA	Docosahexaenoic acid (22:6n-3)
EPA	Eicosapentaenoic acid (20:5n-3)
FAO	Food and agriculture organization
Field (F)	Polychaetes sampled directly from Leangbukta
n	Number of individuals
SE	Standard error
Simulated natural light treatment (SN)	Experimental treatment group with a simulated natural light cycle
RGR	Relative growth rate (day^{-1})
WW	Wet weight (mg)

1 Introduction

The amount of fish produced in aquaculture surpassed the amount of wild-caught fish for human consumption in 2014 (Figure 1.1) (FAO, 2016). The aquaculture industry is the fastest growing food producing sector in the world, increasing with an annual growth rate of 3.2 % in the period 1961-2013 (FAO, 2016). In order to develop a more sustainable aquaculture industry, the industry needs a development for closing the production cycle (Duarte et al., 2009). This entails that the feed requirements for aquaculture species should be provided by the industry itself and not from wild populations, or agriculture (Duarte et al., 2009). Out of the feed given to reared salmon, approximately 70 % of carbon, 62 % of nitrogen and 70 % of phosphorous are not incorporated in the fish, but released into the environment (Wang et al., 2012). As the industry is developing, the amount of waste produced by the fish increases (Martins et al., 2010). In other words, there is great potential for increased utilisation of discards and wastes.

The wastes can be used as a secondary flow to new biomass production, which is a form of integrated multitrophic aquaculture (IMTA) (Barrington et al., 2009, Martins et al., 2010). IMTA is a practice where by-products from one cultured species are used as food or fertilizer for other species, at different trophic levels (Barrington et al., 2009). Production of the target species is combined with secondary bioproduction of species, for instance seaweed, to take up inorganic materials. Additionally, species such as polychaetes are used to feed on particulate organic matter. Collected wastes can be utilized as a resource for cultivation of polychaetes (Barrington et al., 2009).

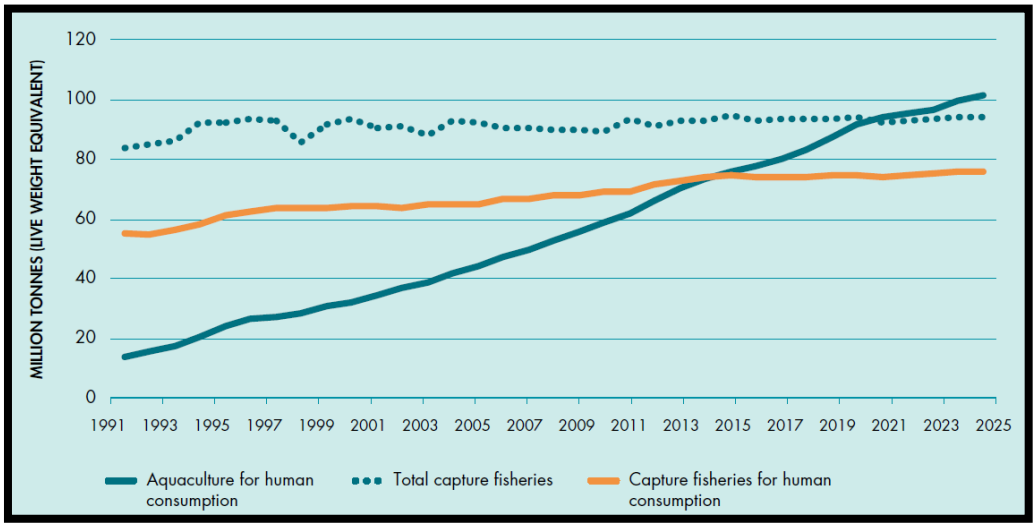


Figure 1.1 Global marine resources for human consumption (FAO, 2016, p. 175). Aquaculture has increased since 1991, whereof fisheries has stagnated.

1.1 Cultivation of polychaetes as a feed resource in aquaculture

Polychaetes have a high lipid content, ranging from 11 to 20 % depending on the feed (Pajand et al., 2017) and a mean omega-3:omega-6 ratio of 2:1 (in *Hediste diversicolor*) (Luis & Passos, 1995)). Moreover, they have a relatively high protein content, ranging from 41 to 55 % depending on the feed (Pajand et al., 2017). Because of their nutritional composition they can be used as food supplement in feed for aquaculture species (Olive, 1999). They are commercially harvested and also commercially produced in Europe, in the United Kingdom (Shoreline Polychaetes Farms LLP, 2018, Sustainable Feeds Ltd., 2018) and in the Netherlands (Delta Farms, 2018) as bait for recreational fishing (Olive, 1999). Polychaetes such as Bloodworms (*Glycera dibranchiata* and *Americonuphis reseii*) and Sandworms (*Perinereis* sp. and *A. virens*), are used as feed supplements in diets for marine shrimp broodstocks (Meunpol et al., 2005, Sustainable Feeds Ltd., 2018, Wouters et al., 2001). Polychaetes for commercial purposes are often harvested from natural populations (Olive, 1999). It is estimated that there was 120 000 tonnes of polychaetes harvested globally in 2015, and that the biomass value was 5.9 million British pounds (£) (Watson et al., 2017). Vigorous harvesting of polychaetes can result in shifts in the biodiversity and population structure, and impair ecosystems (Gambi et al., 1994).

In order to utilize polychaetes for bioremediation, and ease pressure of wild populations, an intensive production must be established (Olive, 1999). The production needs to be initiated comprising of optimization of growth, broodstock rearing, and eventually procedures for optimisation of fertilization. To establish a year-round production of larvae, it is crucial that maturation and spawning events can be manipulated and controlled. In order to manipulate the time of maturation, it is important to know how environmental and physiological variables may affect maturation and the time of spawning in the particular polychaete species. Temperature and daylength are variables that are known to affect the time of spawning in polychaetes (Olive, 1999). It has been shown that the polychaete *Alitta virens* (previously *Nereis virens* (Sars, 1835), Nereididae family) respond to a relative duration of the diurnal light/dark cycle below 13 hours of light to induce oocyte maturation (Olive et al., 1998).

Nesto et al. (2012) suggested that *Hediste diversicolor* (Müller, 1776) is a suitable species for commercial exploitation in indoor farming systems. Moreover, *H. diversicolor* is a promising source of high quality fatty acids for reared fish and shrimp (Santos et al., 2016). Further studies

on *H. diversicolor* has been conducted by Seekamp (2017) and Berntsen (2018) to assess its ability to grow on waste effluents from land-based salmon farms. The studies imply that *H. diversicolor* is a promising species to treat salmon waste, and utilize such wastes to produce valuable raw materials (Berntsen, 2018, Seekamp, 2017).

1.2 Species description of *Hediste diversicolor*

Figure 1.2 *Hediste diversicolor* (Photo: Marianne Uhre, 2018).

Hediste diversicolor (Figure 1.2), previously referred to as *Nereis diversicolor*, is a polychaete species that lives in sediments in shallow marine or brackish waters in the North temperate zone of the Atlantic ocean (Scaps, 2002). The species is widespread along the coasts of Europe, from the Mediterranean in the south to the coast of Helgeland in the north (Moen & Svensen, 2014, Scaps, 2002). The species is found in the intertidal zone in estuaries and lagoons in sheltered areas (Budd, 2008). This infaunal species creates U, J or Y shaped burrows in soft sediment (Esselink & Zwarts, 1989, Scaps, 2002). Burrowing depth varies with the size of the worms, seasonal temperature and sediment composition, and it can be up to 20 cm deep (Budd, 2008, Esselink & Zwarts, 1989). Size-range of the polychaete differs due to the wide geographical distribution, and the size increases with declining latitude (Scaps, 2002). The species is commercially harvested in at least Italy (Gambi et al., 1994), Portugal (Carvalho & Santos, 2013) and Algeria (Younsi et al., 2010). The commercial size of the polychaete is around 10 cm long, which corresponds to a mean fresh weight of ca 0,5 gram (Gambi et al., 1994). The maximum size of the species can be up to 20 cm long (Gambi et al., 1994, Nesto et al., 2012). The species has a normal life span of one to two years, although Olive and Garwood (1981) reported that *H. diversicolor* in the North-East England could have a three-year life span.

H. diversicolor is an omnivorous species (Budd, 2008, Scaps, 2002). The polychaete can change between two feeding tactics, depending on seasonality and the food availability. For the first

feeding tactic the polychaete crawl outside its burrow and catch food with its jaws, which is characterized as predatory feeding or deposit feeding (Budd, 2008, Scaps, 2002). For the second feeding mode, the ragworm uses mucus secretions to capture food, which is later ingested inside the burrow (Costa et al., 2006, Scaps, 2002). The secreted mucus can be used to trap particles and phytoplankton, and the polychaete can then be characterized as a secondary filter feeder (Scaps, 2002). In the period between May and August, filter feeding is preferred and can be as high as 50-100 %, but during early spring and autumn the polychaete only uses filter feeding 5-20 % of the time (Vedel et al., 1994). Food availability is one of the most important factors influencing growth (Scaps, 2002). When rearing polychaetes for broodstock purposes, high protein contents in the food can induce earlier gametogenesis and maturity (Nesto et al., 2012).

H. diversicolor can reach a population density of up to 3000 individuals m^{-2} in the wild (Vedel et al., 1994), but the optimal density for aquaculture purposes would be between 300 to 1000 ind. m^{-2} (due to higher growth rates) (Batista et al., 2003, Nesto et al., 2012). Moreover, the species has a high tolerance to variations in salinity (range from freshwater to 70 ppt, however they cannot reproduce at a salinity < 5 ppt (Oglesby, 1970)). Optimum temperature is 5 to 16 °C (Kristensen, 1983). They can also survive conditions of hypoxia (Kristensen, 1981). The high stress tolerance, high population density and high growth rate makes the species suitable for aquaculture purposes (Nesto et al., 2012).

1.2.1 Sexual maturation

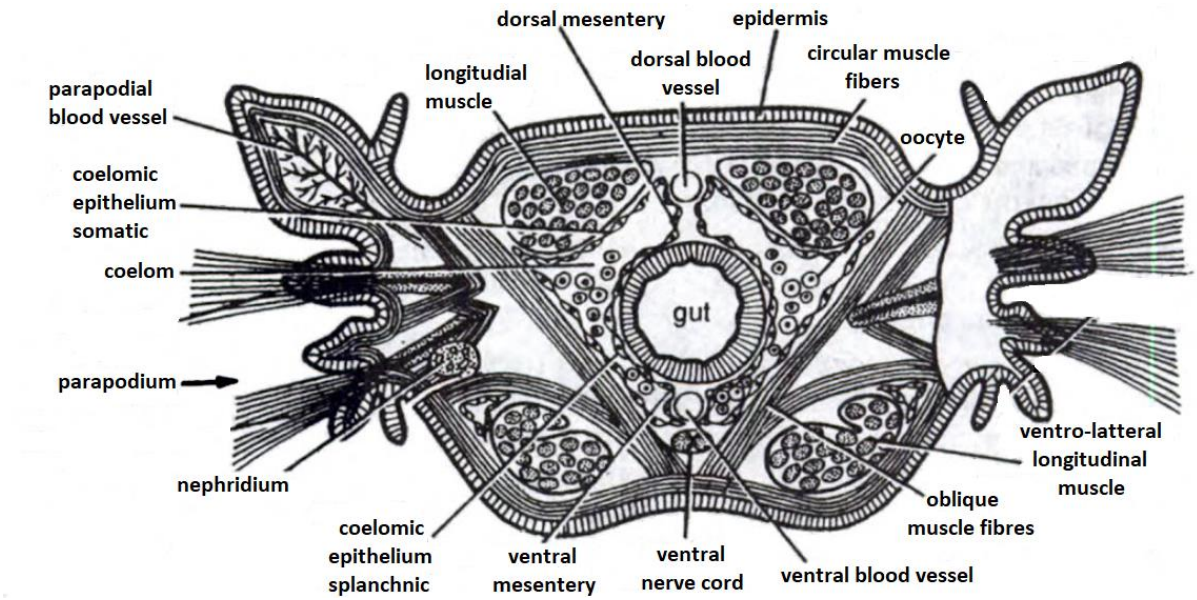


Figure 1.3 Cross section of a polychaete (*Neanthes*, figure modified from Jordan & Verma (2001)). The coelom is filled with oocytes and lined with coelomic epithelium.

Polychaetes has a greater variety in ovarian morphology than most metazoan classes (Eckelbarger, 2005). Most polychaetes have ovaries that are retroperitoneal (the ovary is behind the layer of peritoneum, lining the coelomic cavity rear wall (Kåss, 2018)), and the ovaries are often repeated in each setiger (body segment) of the female (Eckelbarger, 2005). The ovaries position varies between families, but they are most commonly found in the parapodia or in the ventral region of the body (Eckelbarger, 2005, Purschke, 2006). The ovaries in the Nereididae family has not been located, and the family is presumed to lack discrete ovaries (Eckelbarger, 2005). This family has an extraovarian oogenesis (Figure 1.4A), which means that previtellogenic oocytes in clusters are released into the coelom (body cavity lined with tissue derived from mesoderm (Reece et al., 2011)) at an early stage (Eckelbarger, 2005). The previtellogenic oocytes are densely packed in a cluster that are surrounded by a thin layer of sheath cells originating from peritoneum (Eckelbarger, 2005, Fischer, 1975). The clusters are free floating in the coelom of nereids. Prior to vitellogenesis (the process of yolk deposition), the oocytes rupture through the epithelium of the cluster. The oocytes are then floating freely in the coelom (Figure 1.3 and Figure 1.4). Further development of the oocytes precedes in a "solitary" fashion, which entails that the oocytes develop without permanent contact to other cells (Eckelbarger, 2005, Fischer, 1975). The female's coelom starts filling with previtellogenic oocytes during late autumn (Dales, 1950).

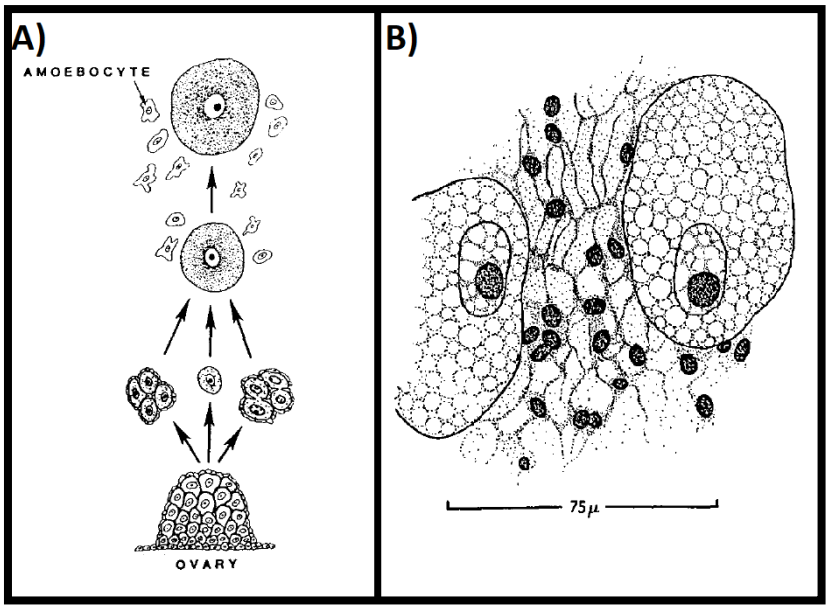


Figure 1.4 A) The extraovarian development of the Nereididae family (the figure is modified from Eckelbarger (1988)). The ovary is shown in this figure, but Nereididae family is presumed to lack discrete ovaries. Amoebocytes are cells that possesses amoeboid abilities in form and behaviour (Beesley et al., 2000). **B)** Oocytes in an early vitellogenic stage, imbedded in the parenchyma (figure taken from Dales (1950)).

Vitellogenesis is the accumulation of yolk material in to the cytoplasm of the oocyte (Eckelbarger, 2005). Eleocytes (type of coelomocyte, which is an amoebocyte present in the coelom; mobile cell (Beesley et al., 2000)) in the coelom synthesize the major yolk protein (vitellogenin) which is further secreted into the coelomic fluid where the oocytes take up and incorporates the protein in their cytoplasm (Baert, 1986, Baert & Slomianny, 1987, Fischer et al., 2003). The yolk composition varies between species of polychaete, but may contain lipid droplets and yolk granules, and sometimes glycogen granules (Eckelbarger, 2005). The yolk granules often consist of membrane-bound lipoglycoproteins (vitellin) (Eckelbarger, 2005). There are two types of vitellogenesis in polychaetes: an autosynthetic process and a heterosynthetic mechanism (Porchet et al., 1989). Autosynthesis is when the oocyte produces vitellin itself by directly taking up precursors of low molecular weight (amino acids, fatty acids, monosaccharides), passing them through RER and the Glogi apparatus and eventually incorporating them into yolk globules (Eckelbarger, 2005). Heterosynthesis is when vitellogenin is transported into the oocyte via endocytosis and further assembled into vitellin in the oocyte (Eckelbarger, 1988, Eckelbarger, 2005, Porchet et al., 1989).

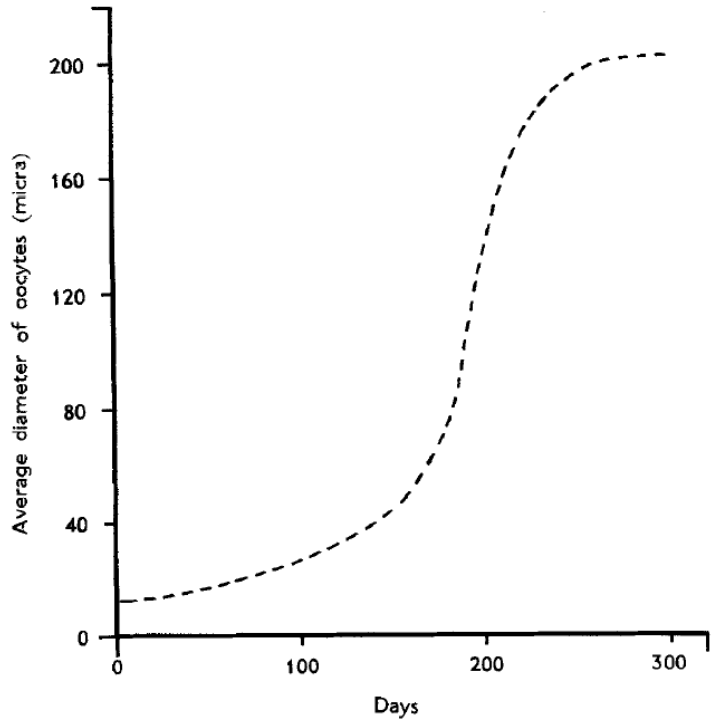


Figure 1.5 The average diameter (μm) of oocytes in *H. diversicolor* from the time they are shed into the coelom until maturation. The figure is taken from Clark & Ruston (1963). The oocytes from the study were not fixated and they were examined directly after sampling.

Early in the oocyte development, the coelom is filled with coelomic corpuscles (free floating cells, including eleocytes (Boolootian & Giese, 1958)) which later forms as loose parenchyma (mesodermal tissue, filling spaces between organs, muscles and other tissues (Beesley et al., 2000)) (Dales, 1950). Formation of parenchyma seems to be correlated to the development of the female's oocytes, and its cells appears to somehow be responsible for the deposition of yolk into the oocytes (Dales, 1950). There are two phases of activity of the coelomic corpuscle. The first phase is the formation of loose parenchyma. The oocytes are then quite small, and their nuclei are irregular in shape. The final phase is when the oocytes are nearly mature. In this phase the coelomic parenchyma disappears, and histolysis (or dissolution) of the muscle layer in the female starts. At this point in the maturation process, the coelom is filled with mature oocytes, but also some fragments of muscle fibres and coelomocytes (Dales, 1950).

When the oocytes are mature, the diameter is approximately ≥ 200 μm (Dales, 1950). The nucleus of the oocytes are now spherical (Dales, 1950), and the shape of the oocytes becomes spherical shortly before spawning (Möller, 1985, Olive & Garwood, 1981). The oocytes are arrested in the prophase I when they are released from the ovary, and precedes into metaphase I after spawning (Olive, 1971, Stricker et al., 2015). Oocyte differentiation in nereidids is relatively slow (Figure 1.5) (Clark & Ruston, 1963, Eckelbarger, 2005). The process from the time when previtellogenic oocytes are present in the coelom until mature oocytes are present in the coelom takes generally a year (minimum 9 months to maximum 18 months) (Clark & Ruston, 1963, Eckelbarger, 2005, Olive & Garwood, 1981).

In males, sperm plates originate from the coelomic epithelium, and break away into the coelom at an early stage (Figure 1.6) (Dales, 1950). The sperm plates, which are flattened groups of cells, are then floating freely in the coelomic fluid. Free spermatozoa (Figure 1.6) are formed when the normal breeding season is reached, and can be found in the coelom just two weeks before spawning (Dales, 1950). The duration time of spermatogenesis in *H. diversicolor* takes up to 6 months for completion (Olive & Garwood, 1981).

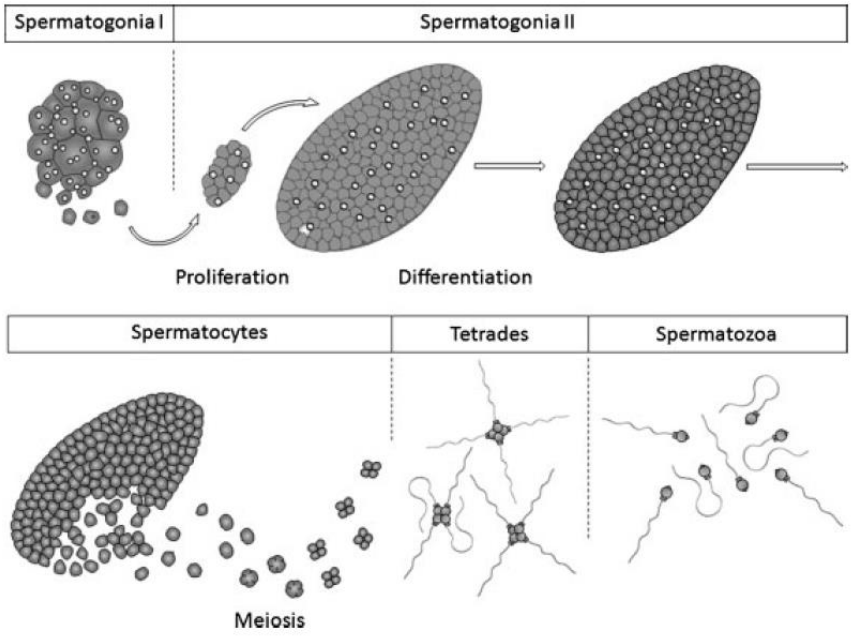


Figure 1.6 Spermatogenesis in Alitta virens (previously Nereis virens). *A. virens* is from the same family (Nereididae) as *H. diversicolor* (figure taken from Schenk & Hoeger (2010)).

In all natural population of *H. diversicolor* there is a majority of females, but the gender distribution varies between populations (Scaps, 2002). The gender distribution in normally around 16 % males and 84 % females (Abrantes et al. (1999) 16 % males and 84 % females, Dales (1950) 10 % males and 90 % females, Olive & Garwood (1981) 18 % males and 82 % females, Smith (1976) 15 % males and 85 % females). The genders are externally indistinguishable during summer and autumn, and the worms has a reddish brown colour (Dales, 1950, Dales & Kennedy, 1954). At the time of maturation, the colour changes to a bright green colour in males and a darker green colour in females (Dales, 1950, Dales & Kennedy, 1954).

1.2.2 Spawning

H. diversicolor is semelparous, which means that the worms dies after reproduction (Dales, 1950). There is no contact between the sexes during reproduction. The worms release its gametes by rupture of the body wall (female and male) or through the nephridia in males (paired organs used for excretion (Beesley et al., 2000)) (Dales, 1950). The female spawns her eggs inside her burrow and starts performing an intense ventilation activity (Bartels-Hardege & Zeeck, 1990). The male senses the female, and ejects his sperm outside the entrance of the

burrow (Bartels-Hardege & Zeeck, 1990). The female brings the sperm into the burrow with her proboscis (extensible pharynx), and the eggs are then fertilized (Bartels-Hardege & Zeeck, 1990, Purschke, 2006). The female remains inside the burrow, guarding the eggs. The hatched larvae remain in the burrow for 10 to 14 days (Bartels-Hardege & Zeeck, 1990).

Temperature and lunar periodicity are factors that are known to affect spawning period in nereid species (Bartels-Hardege & Zeeck, 1990). Spawning is induced in *H. diversicolor* when the temperature rises above 6 °C, after a period of low temperature, in early spring (Bartels-Hardege & Zeeck, 1990). The temperature to induce spawning is presumably between 5 to 8.8 °C (Dales 1950: 5-8.8 °C, Bartels-Hedge & Zeeck 1990: above 6 °C). Additionally, spawning seem to occur mainly around new and full moon, which coincides with the spring tides (Bartels-Hardege & Zeeck, 1990, Dales, 1950).

Spawning season varies due to the wide geographical distribution of the species, where temperature plays a key role (Scaps, 2002). The species also show great variation in the duration of the spawning period, from a few weeks of spawning to spawning throughout the year (Möller, 1985, Scaps, 2002). Durou et al. (2007) suggested that spawning is induced in only one short spawning period in Northern Europe and over two periods in Southern Europe, but there have been recorded some exceptions to this assumption, such as Chambers & Milne (1975) who reported two spawning periods in Ythan (Scotland) and Kristensen (1984) who recorded a prolonged spawning period during early spring and summer in Norsminde Fjord (Denmark).

1.3 Aims and hypothesis of the study

The overall aim of the study was to investigate the oocyte development in *H. diversicolor* and investigating the effect of light on the maturation process. The secondary aims were to:

1. Provide a description of oocyte development in *H. diversicolor* for the stages observed in the study, based on histological methods.
2. Evaluate the possibility of accelerating the oocyte maturation of *H. diversicolor* with use of a compressed light cycle.
3. Compare the development in the experimental treatments to samples taken directly from field.

The polychaetes were kept in two different laboratory conditions, one group of polychaetes were exposed to a simulated natural light cycle and the other group were exposed to a compressed light cycle (the simulated natural light cycle reduced to 40 %). Both light cycle treatments were applied in a climate chamber at a constant temperature (8 °C). The treatments were compared to polychaetes sampled from a natural population in Leangbukta. The length and number of setigers of the polychaetes were measured for each individual. A histological assessment of the structure, size and shape of the oocytes was performed. Developmental stages of the oocytes were measured by size assessments (area and perimeter).

The hypotheses for the study were:

1. The oocyte development of the simulated natural light cycle will coincide with samples taken from field.
2. The compressed light cycle will accelerate the oocyte development in *H. diversicolor*.

The study was part of the project “Cultivation of Polychaeta as raw material for feed” (POLYCHAETE, 280836/E40).

2 Materials and methods

The sampling and maturation experiment were conducted in collaboration with Haiqing Wang, PhD candidate at NTNU department of Biology and SINTEF Ocean AS.

2.1 Field sampling

Wild-caught polychaetes of the species *H. diversicolor* were used in the experiment. The polychaetes were collected from Leangbukta (63.4390549 N, 10.4705787 E), a beach east of Trondheim, Norway (Figure 2.1). The worms were dug up from loamy sand at a depth from 0 to 25 cm, at low tide. The biggest individuals were selected for sampling. This was conducted since it was likely that only the largest worms had initiated oogenesis. The polychaetes sampled for the experiment were collected from Leangbukta the 23rd of October 2017. They were kept at normal daylengths in the experimental set-up for four days to acclimatize, before the experimental treatment started at the 28th of October 2017.

Figure 2.1 The red dot marks the sampling site (Leangbukta) for *H. diversicolor* (Google Maps, 2018).

2.2 Maturation experiments

A climate chamber (Aralab Curing Test Chambers, Fitoclima S600 PHCI) was used to keep the temperature stable at 8 °C for the two experimental treatment groups. The experimental treatments temperature (8 °C) was set based on the measured temperature from the first sampling, and literature stating that spawning can be induced between 6 to 8.8 °C (Bartels-Hardege & Zeeck, 1990, Dales, 1950). The temperature in the cabinet was not measured manually. The different light cycle treatments were kept in two different compartments within

the cabinet, with no light contamination between them (Figure 2.2). The light source for both treatments was LED -modules at 400-735 nm, with equal intensity in each spectrum, and a total intensity of around 5-10 $\mu molm^{-2}s^{-1}$(Heliospectra RX 30, Sweden). In order to get an even light distribution in the cabinet, the six containers in the middle right underneath the light source, were covered with a thin sheath of paper packed in plastic (Figure 2.2).

The polychaetes were rinsed in seawater and weighed in groups of eight, before they were put into 1 L containers (length x height x width = 120 x 105 x 80mm, total volume = 1 L). Each treatment had 20 containers with eight individuals in each container. The density of polychaetes used in the experiment was 833 individuals/m^2. The containers had 575 ml of sediment (chamotte sand, 6 cm depth), and around 600 ml of seawater. The water was constantly aerated by supplying atmospheric air through air stones. The salinity was measured regularly with a refractometer, and it was kept around 30 ppt. The entire water volume was exchanged every second day. The polychaetes were fed salmon feed (10-15 pellets in each container, 1 mm pellet size) every second day. The containers were inspected for dead polychaetes every second day. If dead polychaetes were found, they were removed to avoid deterioration of the water quality (mortality in the experiment is shown in Appendix 2). The daylength was adjusted daily for both treatments on a computer connected to the LED modules.

Figure 2.2 Experimental set-up in the climate chamber.

2.2.1 Field sampling – natural condition

Samples from field were used to compare the natural conditions to the experimental conditions. For each sampling from the field, the temperature in the sediment was measured at different depths (from 0 to 20 cm, with a 5 cm interval) at two different locations within the sampling area (temperature measurements in Appendix 1). The polychaetes were transported to the lab in a box with seawater and natural substrate (sediments or macroalgae) to minimize stress. The sampling times from field are shown in Table 2.1. A total number of 471 individuals were collected from Leangbukta during the whole experiment.

Table 2.1 Overview of field sampling of wild *H. diversicolor*.

Sampling	Date	Number of individuals
0	23.10.2017	331*
1	10.11.2017	16
2	25.11.2017	19
3	09.12.2017	16
4	26.12.2017	16
5	06.01.2018	17
6	22.01.2018	16
7	06.02.2018	17
8	22.01.2018	23

*11 worms for field, and 320 worms for the experimental treatment groups

2.2.2 Simulated natural light cycle treatment

All environmental parameters except the daylength were kept constant throughout the experiment. Every day the light was gradually turned on in three steps for both treatments, shown in Table 2.2. Dim lights were turned on at sun rise, and the light increased in two steps. In the evening the lights were dimmed in two steps before sunset. At sunset the lights were completely shut off. The simulated natural light cycle treatment (hereafter referred to as simulated natural light treatment, Figure 2.3 and Table 2.3) were simulated from the natural daylength in Trondheim (timeanddate.no, 2018).

2.2.3 Compressed light cycle treatment

The compressed light cycle treatment (hereafter referred to as compressed light treatment, Figure 2.3 and Table 2.3) reflected the natural daylength in Trondheim comprised to 40 %, hence comprising 118 days into 46 days. The compressed light treatment received a total of 295 hours and 18 minutes of light, and the simulated natural light treatment received a total of 746 hours and 33 minutes of light during the experiment.

Table 2.2 Light regime in the two light cycle treatments. For both treatments, the light was gradually turned on in three levels. Dim lights were turned on at sun rise, and the light increased in two steps; 5 minutes (step 2) and 60 minutes (step 3) after sunrise. In the evening the lights were dimmed in two steps;10 minutes (step 3) and 5 minutes before sunset (step 2). At sunset the lights were completely shut off.

Treatment	Light regime
Sunrise	on
Step 2	5 min
Step 3	60 min
Step 3	10 min
Step 2	5 min
Sunset	off

Table 2.3 Overview of the sampling for the two experimental treatments. Sampling number (No), the date, number of days (# Days), percent experimental duration (%) and daylength (hours) from the start of the experiment are shown for each sampling.

	Simulated natural				Compressed			
No	**Date**	**#Days**	**%**	**Daylength**	**Date**	**#Days**	**%**	**Daylength**
1	10.11.2017	14	11.8	7.2	01.11.2017	5	10.8	7.5
2	25.11.2017	29	24.6	5.78	07.11.2017	11	23.9	6
3	09.12.2017	43	36.4	4.81	13.11.2017	17	37	4.83
4	26.12.2017	60	50.8	4.55	19.11.2017	23	50	4.66
5	06.01.2018	71	60.1	5.03	24.11.2017	28	60.8	5
6	22.01.2018	87	73.7	6.31	30.11.2017	34	73.9	6.5
7	06.02.2018	102	86.4	7.81	06.12.2017	40	86.9	8
8	22.02.2018	118	100	9.5	12.12.2017	46	100	9.5

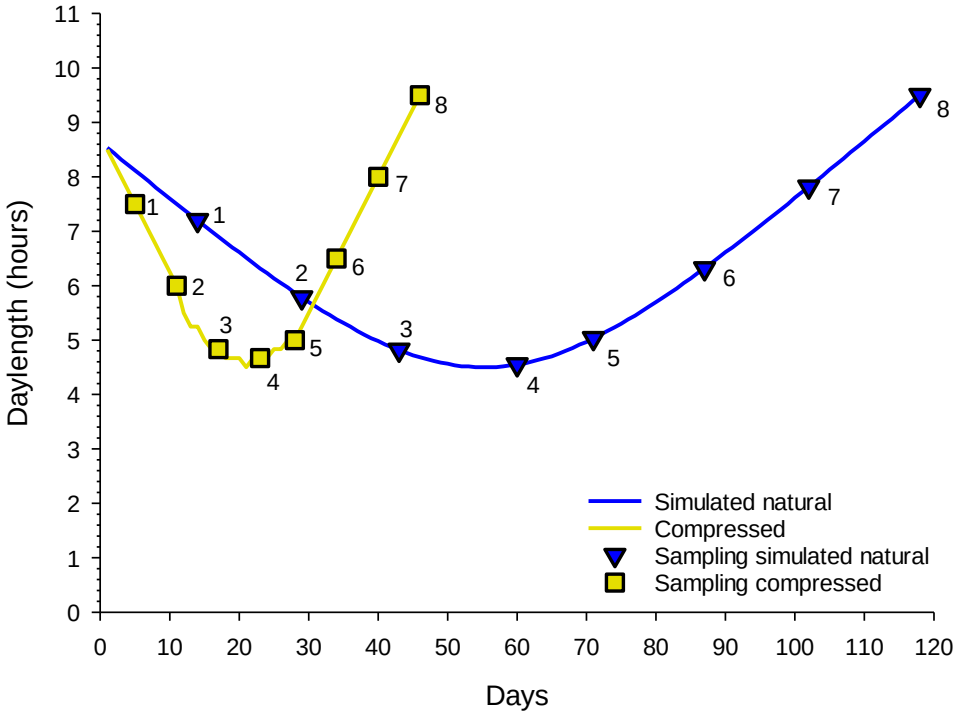


Figure 2.3 Daylength plotted against the experimental days for the two light cycle treatments. The comparable sampling times are marked 1 to 8. Day 1 represents the 28th of October 2017 and day 118 represents the 22rd of February 2018.

2.3 Sampling regime

Sampling frequency from the field study and the simulated natural light treatment was approximately every 15th day, at the same day. The compressed light treatment was sampled with an interval of 5 to 6 days. The different treatments were sampled at approximately the same daylength, so that it would be possible to compare the development of the oocytes (sampling dates are shown in Table 2.1 and 2.3). The sampling from field were conducted in the morning at low tide. Samples from the experimental treatments were dug up in the evening, before the lights went out in the climate chamber. When sampling from the experimental treatment, two containers with approximately eight individuals in each, were dug up at the day of sampling. To exclude sand and gravel the sampled polychaetes were rinsed overnight in a fresh container with artificial sediment (small mesh nets) and a constant aeration through an air stone. This was conducted to avoid problems during histological sectioning of the polychaetes.

The morning after sampling, individuals from each container were weighed in groups of eight, and thereafter sedated with 4 % $MgCl_2$ (M2670, BioXtra, ≥99.0 %, Merck) in seawater (Fauchald, 1977, Grimmel et al., 2016, Zajac, 1985) for around 30 minutes, until their movement slowed down. Polychaetes from the same container were sedated together. After sedation, the polychaetes were photographed in groups of four in a transparent plate with four oblong pits, and with a ruler in the background for scaling. Each well was marked with an individual number and the number of the container, photographed, and put into tissue cassettes for fixation. The polychaetes were fixated in 4 % formaldehyde in phosphate buffer (pH 6.9, Merck, Germany) and stored cold (4 °C) in plastic boxes for minimum 2 days.

A total number of 400 individuals were sampled throughout the experiment (initially 471, 47 died during the experiment and 24 where left after the completed experiment), where 206 worms were sectioned for histological analysis. 194 worms where used for oocyte extraction (oocyte diameter and quantity measurements of fixated oocytes, performed by PhD candidate Haiqing Wang).

2.4 Analyses

2.4.1 Length and setiger measurements

The pictures taken of the polychaetes while they were sedated (4 %$MgCl_2$ in seawater) were used to measure the length and width of the individuals, using an image processing program (ImageJ, illustrated in Figure 2.4). Length measurements were conducted by using the option "segmented line" in ImageJ and then retracing the dorsal blood vessel in the centre of the worm. The total number of setigers in each individual was counted by using the "Multi-Point Tool" in ImageJ. Due to bad quality in some of the pictures, the number of setigers in a few of the sampled polychaetes was not counted.

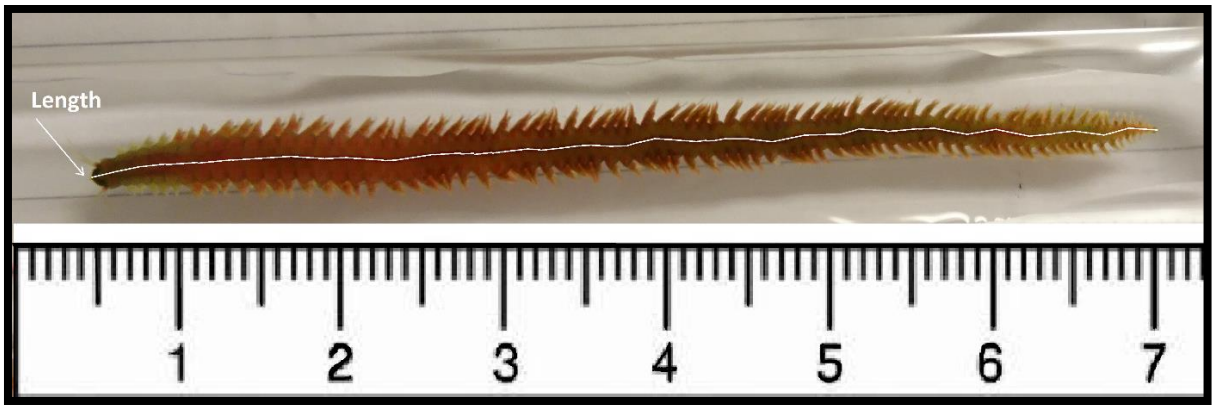


Figure 2.4 Illustration of the length measurement preformed in ImageJ.

2.4.2 Extraction of fixated oocytes

Approximately half of the individuals sampled in the experiment were used for oocyte size and quantity measurements. Polychaetes that had been fixated in 4 % formaldehyde in phosphate buffer, were further rinsed in seawater and cut open into small pieces to wash out the oocytes inside the polychaetes. The tissue was rinsed in saltwater several times to ensure that there were no oocytes left inside. The coelomic fluid with the oocytes were then sieved (100 µm mesh opening) to avoid contamination from other materials in the coelom (coelomic corpuscles and parenchyma), and further transferred into a sample glass (30 mL) and diluted to 20 mL. Ten droplets of 50 µL were taken by using a pipette (Eppendorf, Germany). The number of oocytes in each droplet was counted in a microscope (Eclipse TS100), and the number of oocytes in each individual was calculated. The diameter of the oocytes was measured by use of a microscope camera (Nikon digital sight (DS-U3)) attached to a microscope and analysed with an imaging software (NIS-Elements BR version 4.30). The diameter of fifty oocytes was measured per individual. Oocyte diameter measurements and quantity calculations was performed by Haiqing Wang, PhD candidate at NTNU department of Biology and SINTEF Ocean AS.

2.4.3 Histological sectioning and analyses

Half of the samples from the experiment were used for histological sectioning. A tissue processor (semi-enclosed tissue processor, Leica TP1020) dehydrated the tissue in ascending concentrations of alcohol (starting from 80 % to 100 %), cleared it with Tissue-clear (Tissue-Tek ® Tissue-Clear ® Xylene Substitute, Sakura, UK) to remove alcohol, and further infiltrated it with paraffin (Tissue-Tek® III Embedding wax, Sakura, UK). The samples were further embedded in a casting frame with hot paraffin wax from a dispenser (Leica EG1120, Leica

industries, Germany). The polychaetes were oriented correctly within the paraffin blocks using forceps (Leica EG F). Afterwards, the paraffin was cooled on a cooling element for a few seconds to fix the polychaete in its position. The bottom of the cassette was placed over the casting frame, and more paraffin was added to fix the wax to the cassette. Finally, the cassette was placed on a cooling element to harden the paraffin wax.

Both cross- and longitudinal sections of the worms were investigated. Longitudinal sections were chosen because it gave the best overview of the tissue. In order to get the whole polychaete in the same plane, it was divided into two to four pieces as shown in Figure 2.5. The polychaetes were embedded with their dorsal side towards the cut side of the block (furthest out from the cassette). The digestive tract was used as a reference point for the sections. A minimum of two different sections were taken for each polychaete. One section when the digestive tract was present, and one when most of the digestive tract was present in most parts of the polychaete. The sections that were used were sectioned with a minimum of 200 µm distance from each other. A total number of 201 individuals (116 females) were histologically sectioned. The paraffin blocks were sectioned (4 µm thick) using a Leica fully automated rotary microtome (RM2255, Leica, Germany) with a CoolClamp (RM CoolClamp, Leica, Germany). The sections were mounted on glass microscope slides and then air dried for ≥ 30 minutes, and thereafter dried in an oven (Termaks, Norway) at 37 °C overnight.

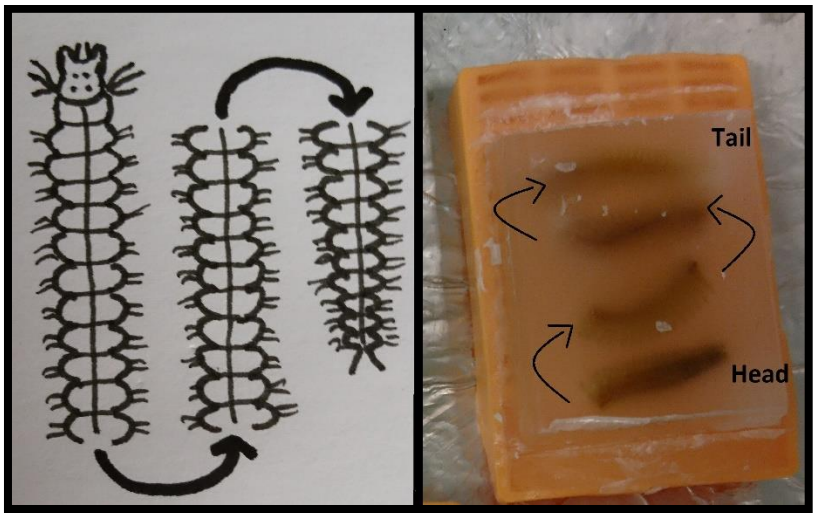


Figure 2.5 Illustration of orientation of polychaetes in paraffin blocks used for histological sectioning.

The sections were stained with haematoxylin (Mayer`s hemalum solution (Merck, Germany)) and Eosin Y-solution 0,5 % aqueous (Merck, Germany) (staining protocol in Appendix 3). This is the most common staining procedure used in histology, and it is primarily used to display structural features (Ross & Pawlina, 2016). Hematoxylin is a basic dye, that stains nucleic acids with a deep blue-purple colour. The counterstain, Eosin, is an acidic dye that stains proteins, cytoplasm and the extracellular matrix in different shades of pink (Fischer et al., 2008, Ross & Pawlina, 2016). In the oocytes of this study the nucleus had a dark purple colour (Haematoxylin) and the rest of the cell tissue was stained in varying shades of pink (Eosin). Lipid vacuoles were not stained by this procedure, and they were present as empty spaces within the oocyte.

After completing the staining procedure, the sections were covered using Neo-Mount® (Merck KGaA, Darmstadt, Germany) as a binding medium. The sections were scanned in one layer at 40x magnification using a digital slide scanner (Hamamatsu NanoZoomer, Hamamatsu, Japan). The sections were examined using an image viewing software (Hamamatsu NDP.view2).

To assess the level of oocyte development of the individuals, the perimeter (µm) and area (μm^2) of the oocytes was measured in the sections where the nucleolus was visible. The measurements were performed in regions with a high oocyte density, and setiger by setiger, so that all the oocytes present in each setiger was measured. The measurements were conducted in the NanoZoomer program by using the "Freehand region tool" and retracing the cell membrane (Figure 2.6) by using a drawing tablet (Wacom Bamboo Create Pen and Touch Tablet, CTH-670). As described by Olive et al (1998), optimally 50 oocytes (minimum of 25 oocytes) were measured from each polychaete ($n_{females}$ =116). Oocytes with broken cell walls were not measured. Females with oocytes ≤ 40 % (mean oocyte size < 8000 μm^2) of the average oocyte area from the particular sampling day in the different treatment groups, were omitted from the results and grouped as immature (non-maturing) (Appendix 4).

The area measurement was used to calculate the diameter of the oocytes. This was only an estimate of the diameter and not a perfect measure. The calculation was conducted to compare the results to the fixated oocytes and to literature since the only size measurement found of mature oocytes was in diameter (≥200 µm).

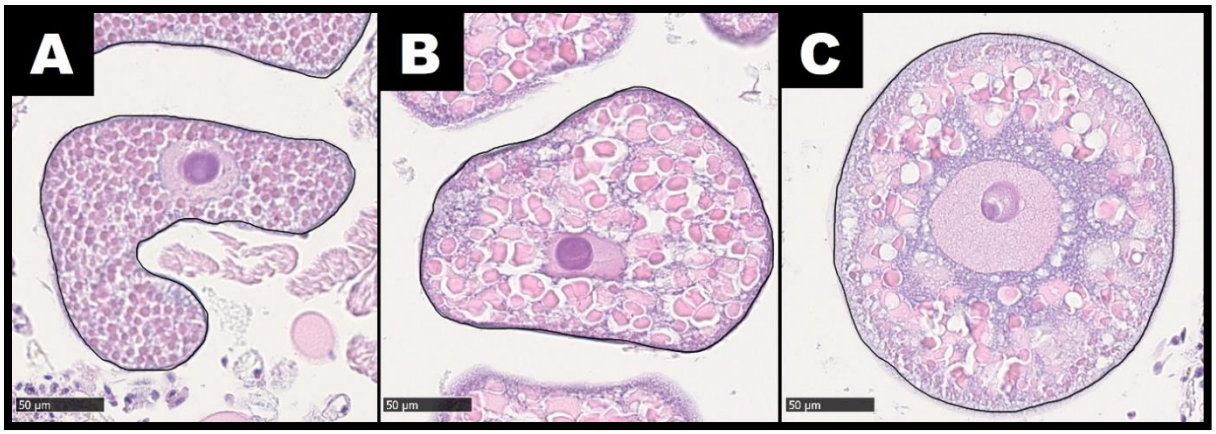


Figure 2.6 Illustration of the perimeter and area measurements of the oocytes. The calculated shape factor for the oocytes are: A) 0.49, B) 0.75, and C) 0.98. Scale bars 50 µm.

2.4.4 Shape factor

Because the oocytes get rounder in shape as they developed (Olive & Garwood, 1981), a shape factor was developed. Since a circle is the geometric shape that has the largest area for a given perimeter, a shape factor can be derived. The formula applies the area and the perimeter as a measurement for the shape of a circle. The shape factor ranges from 0 to 1, where 1 equals a circle. The shape factor was calculated from the following formula:

$$\frac{A}{P^2} = \frac{\pi * r^2}{(\pi * 2r)^2} = \frac{\pi * r^2}{4\pi * \pi * r^2} = \frac{1}{4\pi} \quad (1)$$

$$\frac{A}{P^2} * 4\pi = \frac{1}{4\pi} * 4\pi \quad (2)$$

$$\frac{A}{P^2} * 4\pi = 1 \quad (3)$$

Where A is the area, P is the perimeter, and r is the radius.

2.4.5 Relative growth rate of the polychaetes

The relative growth rate was calculated from the mean initial and final bulk weights (≤ 16 worms) of the polychaetes from the experimental treatment groups. The initial weight of the polychaetes was performed at the 23rd of October. The growth rate specifies how large the relative change in weight was over time (days). The relative growth rate (RGR) was calculated from the following formula:

$$\boldsymbol{RGR} = \frac{\left(\frac{W_F - W_I}{W_I}\right)}{\Delta t} \quad (5)$$

Where W_I is the initial weight, W_F is the final weight and Δt is the number of days between the initial weight and the final weight.

2.5 Statistics

Microsoft Excel 2013 was used to calculate the mean, standard deviation and standard error of the data. Sigmaplot 14.0 was used for making graphs and formatting regression lines with r^2-values. The data for the area and the shape factor of the oocytes were tested for normality by using a Shapiro-Wilk test, and Levene's test of equality of error variances. The data was further tested for significant differences at the same daylengths by using ANCOVA, where the experimental duration time (%) was set as the covariate. To compare the different treatments with the actual days of the experiment an ANCOVA was used, with days as the covariate. To check for correlation between number of setigers and polychaete length, mean oocyte area and mean oocyte shape factor, and polychaete length and number of oocytes, the Pearson correlation test was used. All the statistical analyses were performed in IBM SPSS Statistics v25.0 (SPSS Inc., Chicago, USA) for Windows. A significance level of 0.05 was used for all tests, except Pearson's correlation where the level of significance was 0.01.

3 Results

3.1 Temperature

Figure 3.1 shows the measured temperature in the field (Leangbukta) from 23rd of October 2017 to 22nd of February 2018. The experimental temperature is illustrated by the yellow line in the figure. The temperature in field changed drastically from 23rd of October to 10th of November. Temperature measurements for the 25th of November was not conducted. The next measured temperature on the 9th of December was identical to the 10th of November. After the 9th of December the temperature was steadily decreasing, until the 22nd of January. The temperature on the 22nd of January was the lowest measured temperature. The temperature then started to increase until the 6th of February and seemed to level off to the 22nd of February.

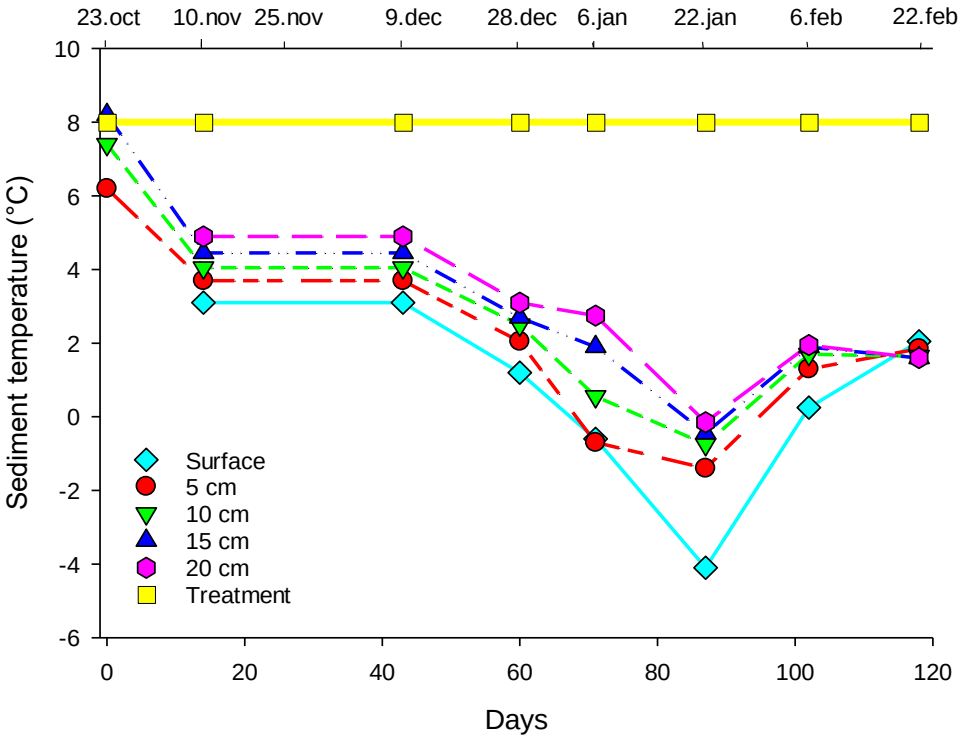


Figure 3.1 Average sediment temperature, at different depths, from field sampling at Leangbukta. The temperature in the two experimental treatment groups is illustrated by the yellow line. Temperature measurements were not conducted on the 25th of November. Sampling times are shown on the secondary x-axis.

3.2 Size and growth measurements of the polychaetes

Figure 3.2 shows the measured length against the measured number of setigers for all polychaetes in the experiment. The measured length ranged from 29.2 mm to 97.6 mm, and the number of setigers ranged from 35 to 88. Pearsons correlation test showed that there was a positive correlation between length and number of setigers in all treatment groups. Out of all the sampled polychaetes in the experiment, 48.5 % of polychaetes had lost their posterior setigers. This made it difficult to estimate the correct size of the individual polychaetes.

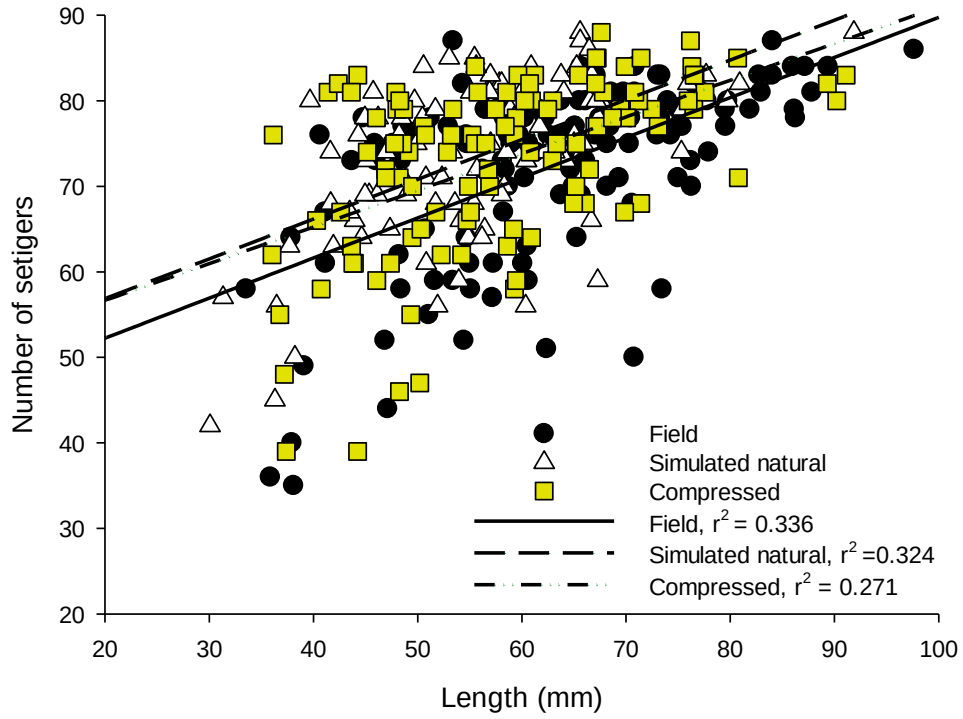


Figure 3.2 Correlation between number of setigers and length (mm) of the polychaetes. Pearson's correlation coefficient, *r* = 0.580 (field), r = 0.570 (simulated natural light treatment), *r* = 0.521 (compressed light treatment), and the correlation was significant for all groups ($p < 0.01$).

Figure 3.3 shows the relative growth rate of the polychaetes in the simulated natural light and the compressed light treatment plotted against experimental duration (%). The experiment duration (%) was calculated from the cumulative light hours in the experimental treatments, where 100 % was 295.3 light hours in the compressed light treatment and 746.5 light hours in the simulated natural light treatment and field. The experimental light cycle treatment started on the 28th of October, and this date was set as 0 % of the experiment duration time. The first sampling from field was taken the 23rd of October, but this sampling was set as 0 % to ensure that the model did not start at minus 4 %, which represents the 23rd of October. All the other

sampling days are calculated in percent from the start day for the light cycle experiment, which was the 28th of October.

Table 3.1 shows the mean initial and final weight of the polychaetes from each sampling in the simulated natural light and compressed light treatment groups, and the mean weight of the polychaetes sampled from the field. The Polychaetes sampled directly from field weighed generally less than the polychaetes from the two treatment groups (12.7 % less than simulated natural light treatment, and 17.2 % less than compressed light treatment). Figure 3.3 shows the relative growth rate throughout the experiment (%). The relative growth rate (formula in chapter 2.4.5) from field was calculated from the first sampling taken on the 23rd of October, and the final weights taken throughout the experimental period. For the experimental treatments the relative growth rate was calculated from the initial and final weight of each container sampled. It must be considered that the final weight was taken after the polychaetes were starved for approximately 20 hours. The polychaetes were not starved when the initial weight was taken on the 23rd of October. The regression lines in Figure 3.3 indicates that there was a negative relative growth rate for the compressed and the simulated natural light treatment throughout the experimental period.

Table 3.1 Mean initial and final polychaete wet weight (WW, milligram) throughout the experimental duration time for the simulated natural light and compressed light treatment groups. For field sampled polychaetes the mean weight is shown. The initial weight was taken the 23rd of October for the experimental treatment groups. For the polychaetes sampled from field there was only final weight from the 23rd of October. The polychaetes were weighed in bulks, and the mean weight was calculated from the whole sample (≤16 worms).

Experiment duration time (%)		**Field sampled**	**Simulated natural**		**Compressed**	
F & SN	**C**	**(WW mg)**	**Initial (WW mg)**	**Final (WW mg)**	**Initial (WW mg)**	**Final (WW mg)**
0	0	287				
11.9	10.9	284.8	268.1	285.3	290.6	314.3
24.6	23.9	262	317.5	306.8	310	292.5
36.5	37	216.9	311	300	250	260
50.8	50	209.4	278.7	262.4	295	292.6
60.1	60.9	237.4	305	307.1	300	330
73.7	73.9	219.4	300	293.1	261.8	274.3
86.4	87	294.7	288.7	277.3	317	340
100	100	261.6	293.7	244.1	275	295.7

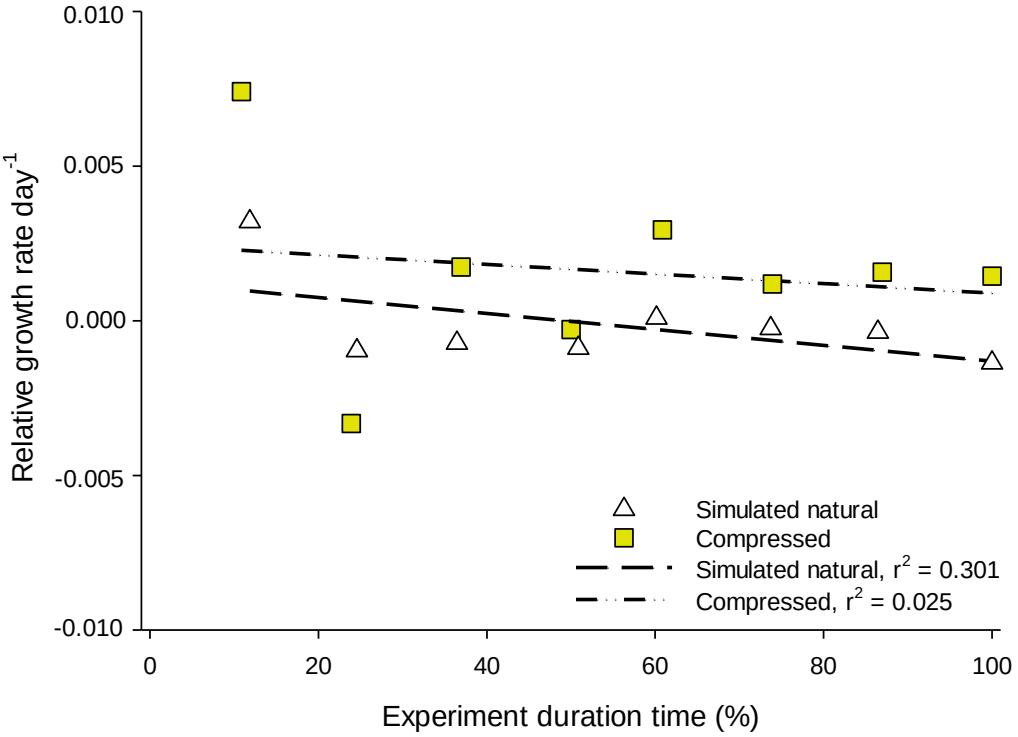


Figure 3.3 Relative growth rate per day throughout the experiment duration for the simulated natural light and compressed light treatment groups. The polychaetes were weighed in bulks, and the mean weight of each individual was calculated from the whole sample (≤16 worms). The initial weight for all treatments was taken the 23rd of October. The final weight was taken after the polychaetes were starved for approximately 20 hours.

3.3 Mortality

The mortality during the experiment in the two different treatment groups is shown in Figure 3.4. The figure includes dead polychaetes found above the sediment throughout the experimental period, and polychaetes that were not found in the containers at the different sampling times. The overall mortality throughout the experimental period was 25 % in simulated natural light treatment and 4.4 % in compressed light treatment. The percentage was calculated from the initial number of polychaetes in the different treatments (160 polychaetes in each). The higher mortality can be explained by the difference in durability for the two light cycle treatments (46 days in compressed light treatment, and 118 days in simulated natural light treatment). Total mortality in each container from the different treatment groups is shown in Appendix 2.

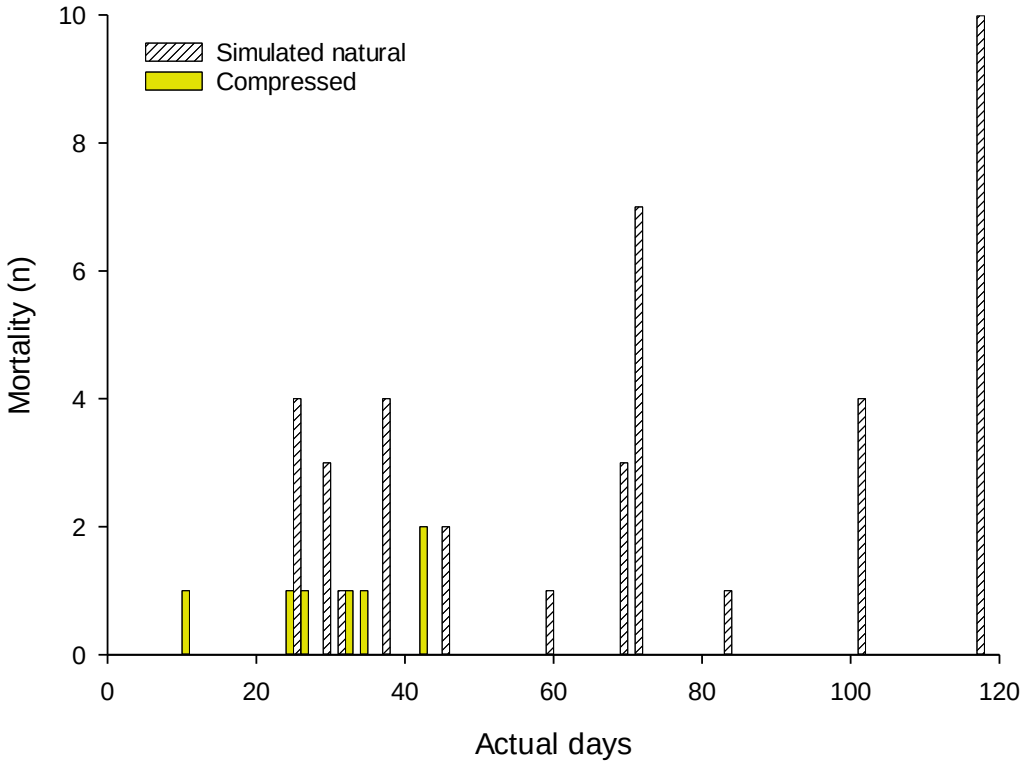


Figure 3.4 Mortality of individual polychaetes (n) in the two different experimental treatment groups (simulated natural and compressed) during the experimental period, in actual days. Both dead polychaetes found above the sediment throughout the experimental period, and dead polychaetes that were not found in the containers at the different sampling times, is included in the figure.

3.4 Sex ratio and number of females

In the histological samples for all treatments (198 polychaetes sectioned) it was found 5 % juveniles (no visible oocytes or sperm plates), and the sex ratio of the adults was 38 % males and 62 % females. This suggested that the largest individuals in the population at Leangbukta had a higher ratio of females, than males.

Figure 3.5 shows the number of reproductively competent females per sampling throughout the experiment. To ensure that only the females that had a valid chance of reproducing within the normality range of the population, females with small oocytes were taken out of the experimental results. The criteria for omitting immature females was set for the mean area measurements of the oocytes in the worms. Polychaetes with oocytes ≤ 40 % (mean oocyte size < 8000 μm^2) of the average oocyte area from the particular sampling day, in the different treatment groups, were omitted from the results. In the samples from field 10.4 % of the females were omitted, in the simulated natural light treatment 19.4 % were omitted, and in the compressed light treatment 21.8 % of the females were omitted. Figures of the omitted females from the different treatment groups are shown in Appendix 4.

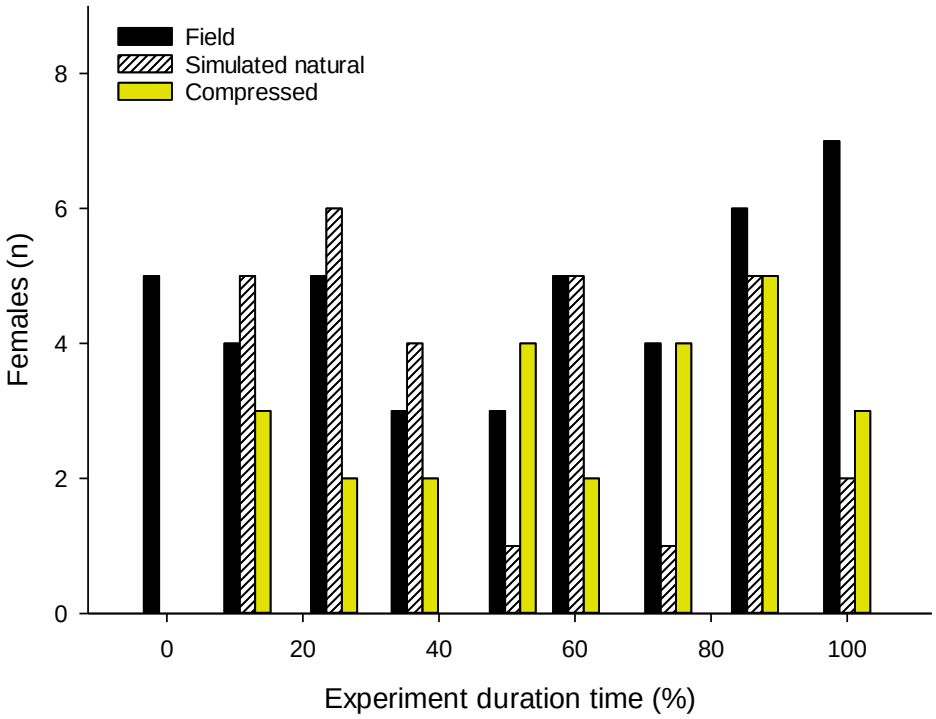


Figure 3.5 The number of reproductively competent females (n) sampled for histology from the different treatment groups throughout the experiment.

3.5 Structure of the oocytes

Figure 3.6 illustrates the structure of an oocyte with an area measurement of 30 000 µm^2. Morphological characteristics of the oocyte development of *H. diversicolor* are described in Table 3.2 and illustrated in Figure 3.7. The descriptions are based on samples taken directly from field. The oocytes in Figure 3.7 was selected based on size measurements. To find a representative oocyte for the individual polychaete, both mean area of the oocytes and the shape of the oocytes were considered. Oocytes that had an area measurement close to the mean value, and a shape that was representative for that individual, were selected.

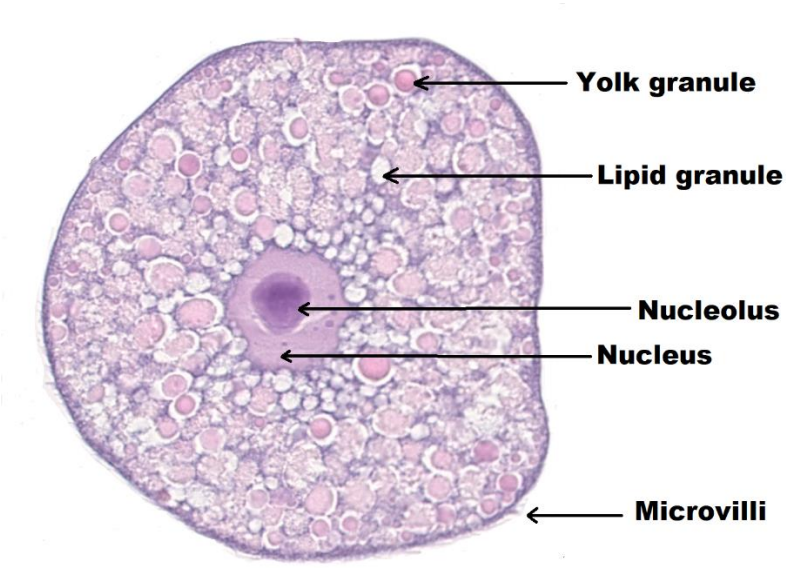


Figure 3.6 Structures in an oocyte of *H. diversicolor*. The area of the oocyte was 30 000 µm^2, and the calculated diameter was 195 µm. The picture is without a scale bar.

Table 3.2 Oocyte development of *H.diversicolor*. The table was based on the different size measurements conducted in the study. The table does not follow the oocytes chronologically in time. The descriptions are based on morphological observations/tendencies in the histological sections from the field samples (Leangbukta).

Figure 3.7	Oocyte area (μm^2) / diameter (μm)	Description
A	**1800 / 48**	The oocytes were irregular in shape. Nucleus was not centred in the oocyte. Oocytes distributed in the parapodia and in the coelom. Bundles of oocytes were present (arrow in Figure 3.7A). Parenchyma and/or coelomic corpuscles was present in the coelom.
B	**6000 / 87**	The oocyte was more irregular in shape (Figure 3.7B). Nucleus not centred. Bundles of oocytes were found in some of the polychaetes.
C	**15 000 / 138**	Irregular in shape (Figure 3.7C). Nucleus not centred. Oocytes has a hairy surface (microvilli). The composition of yolk in the egg seems to start changing. Bundles of oocytes were found in some polychaetes. Less parenchyma present in the coelom.
D	**20 000 / 160**	The oocytes were rounder in shape (Figure 3.7D). The nucleus was not centred. Microvilli present. Bundles of oocytes found in one polychaete.
E	**25 000 / 178**	The oocytes were rounder in shape (Figure 3.7E). The nucleus appeared more centred. The composition of yolk in the oocytes started to change. Lipid granules begin to appear in the oocytes. Microvilli present.
F	**30 000 / 195**	The oocytes were approaching a spherical shape (Figure 3.7F). The nucleus was centred in the oocytes. Larger lipid granules accumulated around the nucleus. Microvilli present. Thickening of the cell membrane. The parenchyma, in the coelom, was almost gone.

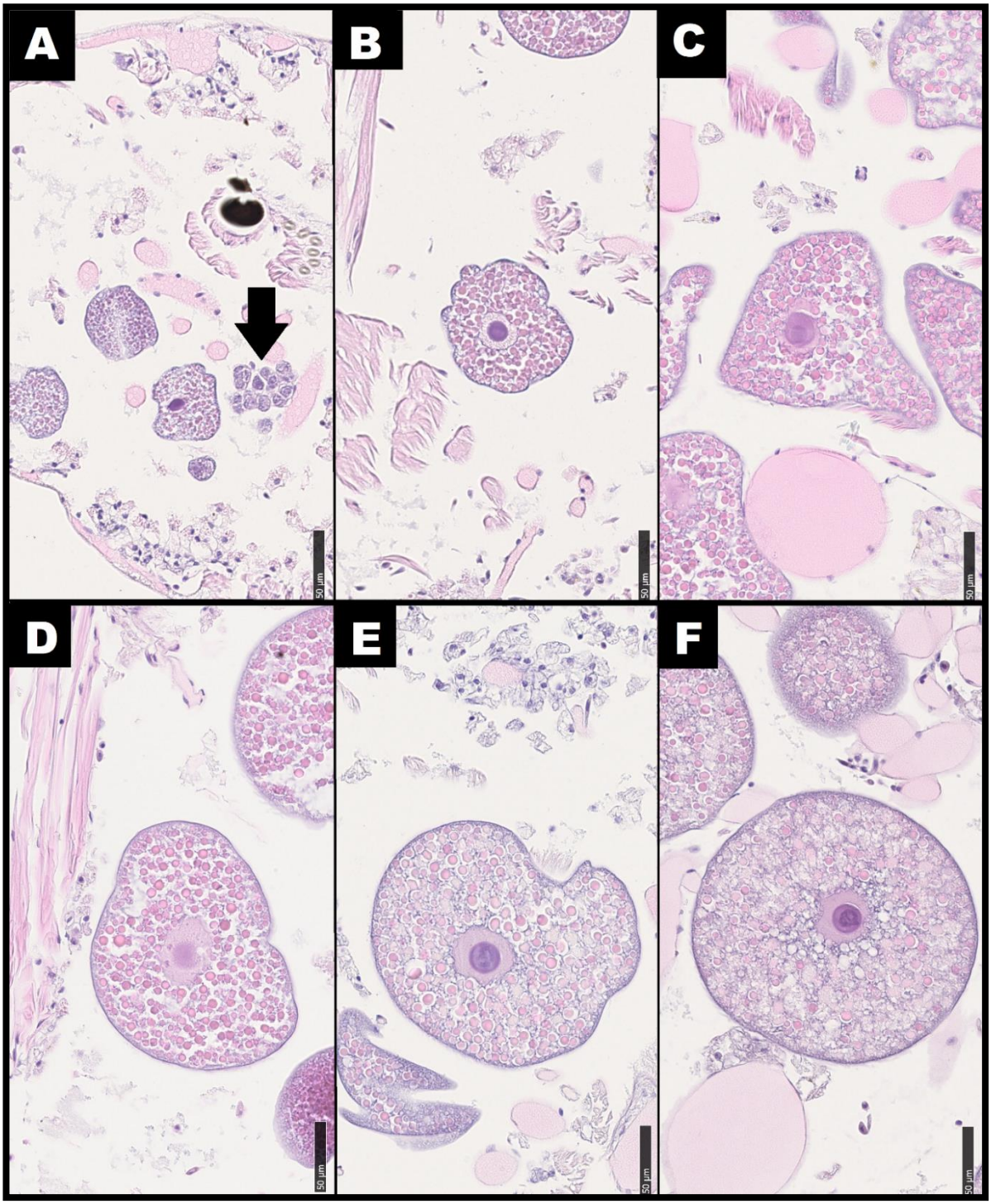


Figure 3.7 Illustration of the oocyte development observed in the polychaetes sampled directly from field. The selection of the oocytes was based on the average oocyte size measured in the individual polychaete. The oocyte size (and sampling date) was respectively A) 1800 µm^2 (23.10.2017), B) 6000 µm^2 (06.02.2018), C) 15 000 µm^2 (23.10.2017), D) 20 000 µm^2 (27.12.2017), E) 25 000 µm^2 (06.02.2018), F) 30 000 µm^2 (22.02.2018). Scale bar at 50 µm (NanoZoomer).

Figure 3.8 illustrates the oocyte stages observed in all the polychaetes from the last sampling day in the different treatment groups. In the samples from field (F1 to F7) some of the oocytes had started to accumulate lipid droplets around the nucleus (F3, F6 and F7). In some of the oocytes the composition of yolk was beginning to change (F3, F5-F7), but none of them were fully mature. In the oocytes from the simulated natural light treatment there was clearly two different developmental stages. Figure 3.8 S1 shows an oocyte were the yolk composition was beginning to change, while Figure 3.8 S2 shows a fully matured oocyte. Oocytes from the compressed light treatment had a more irregular shape than the other treatments. The composition of yolk was beginning to change in all the sampled polychaetes from the compressed light treatment, but none of the oocytes had a layer of accumulated lipid droplets around the membrane of the nucleus, as observed for the other treatment groups.

There was a variation in the number of polychaetes sampled from each treatment at the last sampling day (n = 2 to n = 7). This makes it difficult to draw a conclusion concerning the degree of development in each treatment group, due to a large variation between individuals within treatments. When comparing the structure of the oocytes from the different treatments, it seemed as if the simulated natural light treatment had developed furthest. The simulated natural light treatment group was the only treatment group where a mature (or nearly mature female) was found. Some of the oocytes from field seemed to be close to maturation, because of the apparent accumulation of lipids around the nucleus and the changes in yolk composition. The samples from field seemed to be a bit closer to maturation than the samples taken from the compressed light treatment, considering that the compressed light treatment had oocytes which were more irregular in shape and that there was no clear accumulation of lipids around the nucleus.

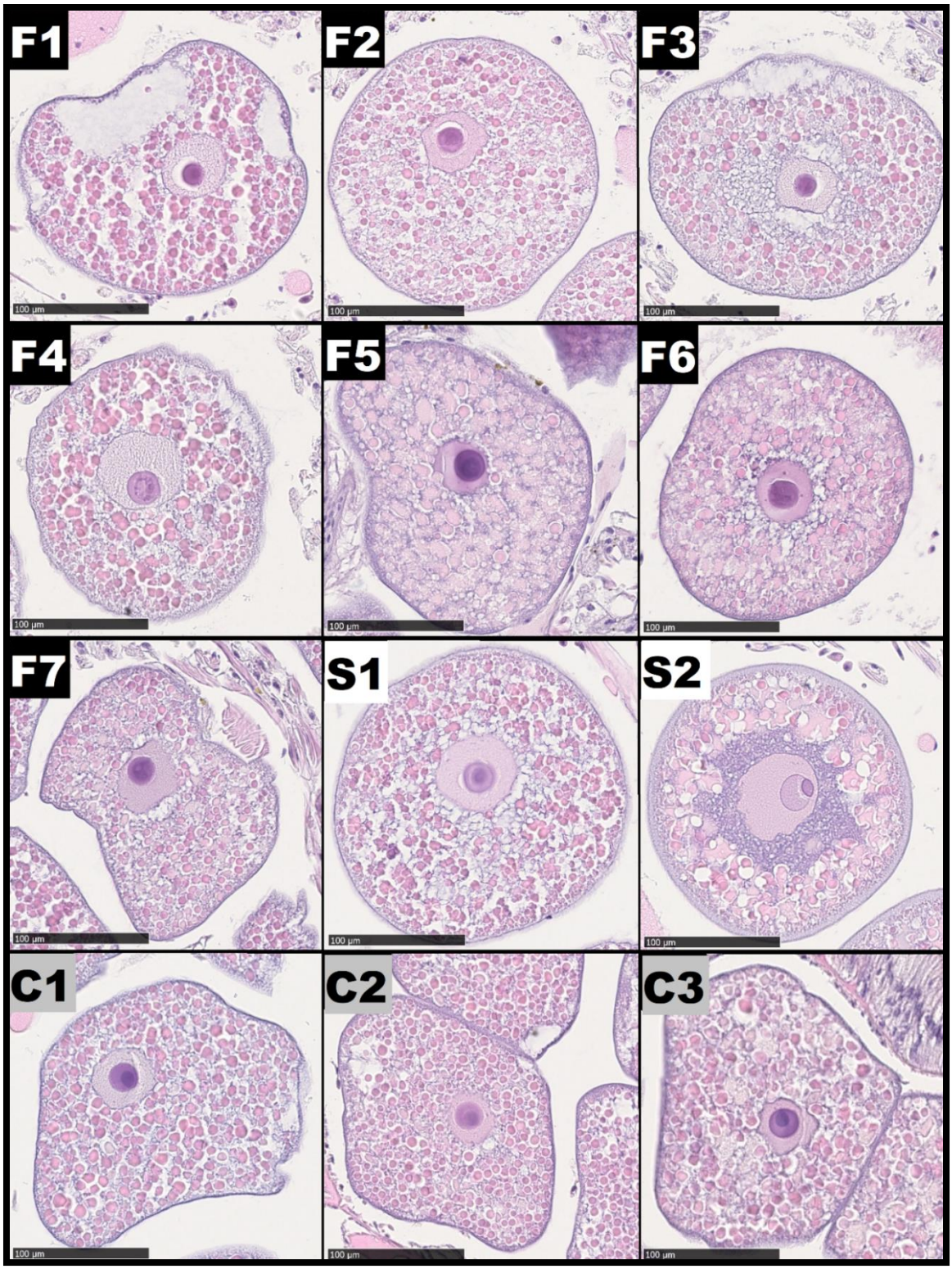


Figure 3.8 Illustration of the oocytes observed at the last sampling day (12th of December for compressed and 22nd of February for field and simulated natural) for the different treatment groups. Sampling from field; F1(28000 µm^2), F2 (29000 µm^2), F3 (30000µm^2), F4 (28000 µm^2), F5 (30000 µm^2), F6 (28000 µm^2), F7 (23000 µm^2). Sampling from simulated natural; S1 (36000 µm^2), S2 (29000 µm^2). Samples from compressed; C1 (28000 µm^2), C2 (23000 µm^2), C3 (27000 µm^2) The selection of oocytes was based on the average oocyte size measured in the individual polychaete. Scale bar at 100 µm (NanoZoomer).

3.6 Oocyte size

Figure 3.9 shows the mean oocyte area (μm^2) for *H. diversicolor* in the different treatment groups, expressed both as the experiment duration in percent of total time (Figure 3.9a) and as number of days (Figure 3.9b). In Figure 3.9a, all the oocytes in the different treatment groups had an increase in mean area during the experimental period (%). Oocytes from the simulated natural light treatment had the greatest increase in mean area. From the first field sampling (at 0 %) to the last sampling of the experiment (100 %) the mean oocyte area from the simulated natural light treatment had increased with 90.7 %, while the mean oocyte area from the field had increased with 68.8 % and the mean oocyte area from the compressed light treatment had increased with 52 %. Figures with the mean oocyte area for each polychaete in the different treatment groups are shown in Appendix 4.

There was an overall significant difference ($p < 0.05$) in the mean oocyte area between the simulated natural light treatment and the two other treatment groups (field and compressed light treatment) throughout the experiment duration time (p-values are shown in Table 3.7). There was no overall significant difference in oocyte area between samples from field and the compressed light treatment ($p = 0.065$) throughout the experimental duration time (%).

Figure 3.9b shows the mean oocyte area against the actual days in the experiment. The figure compares the compressed light treatment to the samples in the two other treatments taken around the same time period, but with different daylengths. There was no significant difference between the treatments up to day 46, when testing the difference with actual days as the covariate ($p > 0.05$, ANCOVA). Table 3.3 shows the different values for the regression lines in Figure 3.9.

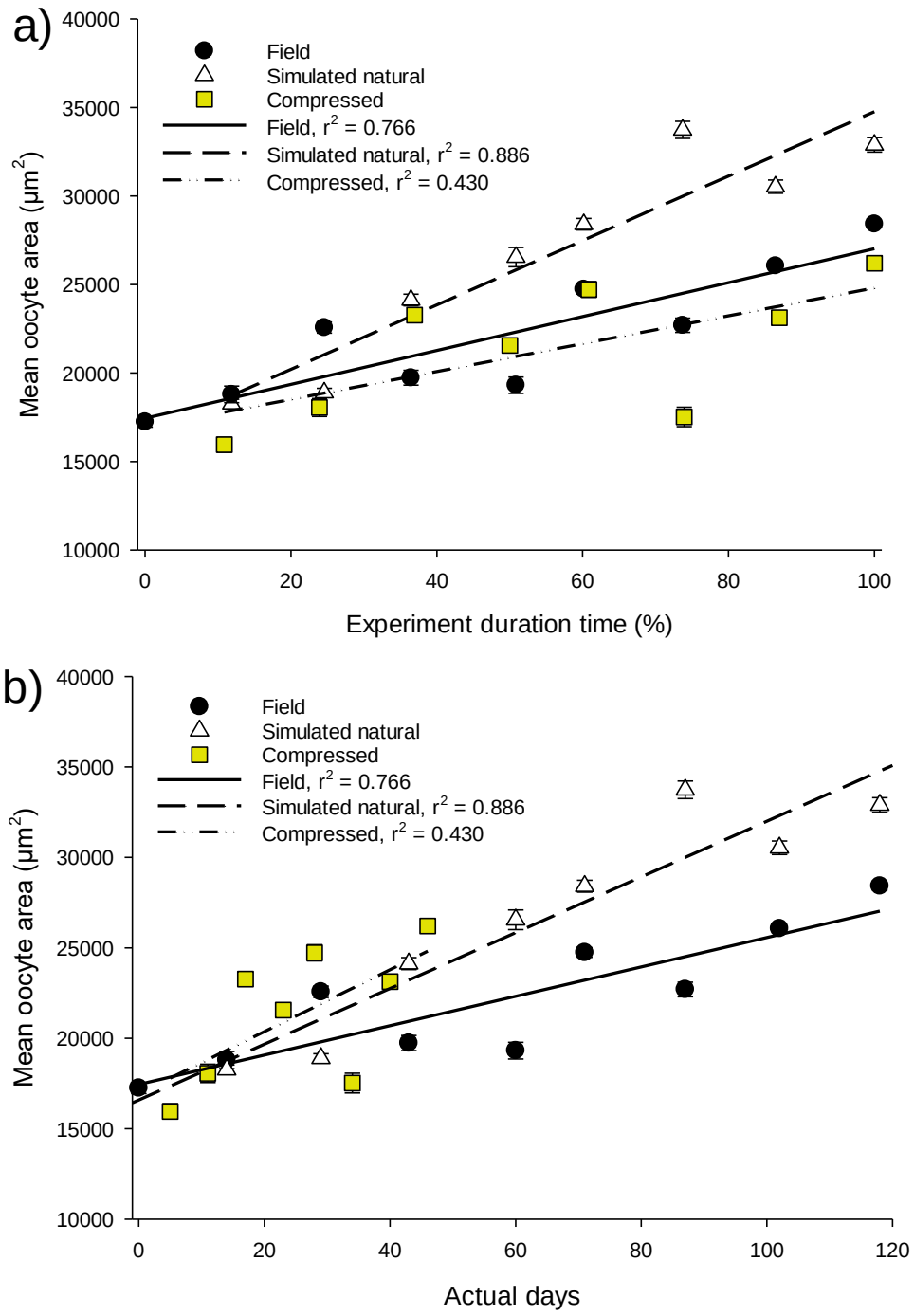


Figure 3.9 a) Mean area (µm^2) of oocytes for all treatments throughout the experimental period (in percent). **b)** Mean area (µm^2) of oocytes for all treatments for the actual sampling days. Sampling size varied from 1 to 7 polychaetes, with < 50 oocytes measured per polychaete. Standard error is represented by error bars (±). The data from the different treatments are separated into individual linear regression lines.

Table 3.3 Values for the regression lines of the different treatments shown in Figure 3.9 a (% time) and b (actual days). The r^2 was the same in Figure 3.9 a and b for all treatment groups.

Treatment	**Figure 3.9**	**y = ax + b**	**r^2**
Field	% time	y = 95.7x + 17449.7	0.766
	Actual days	y = 81x + 17449.3	
Simulated natural	% time	y = 181.9x + 16579.6	0.886
	Actual days	y = 154x + 16578.1	
Compressed	% time	y = 78.7x + 16940	0.430
	Actual days	y = 171x + 16939.5	

Figure 3.10 shows the mean oocyte diameter (µm), calculated from the measured area (μm^2) of the oocytes, for the experimental period (Figure 3.10a) in percent, and for the actual days (Figure 3.10b) of the experiment. Table 3.4 shows the values for the regression lines in Figure 3.10.

Table 3.4 Values for the regression lines of the different treatments shown in Figure 3.10 a and b. The r^2 was the same in Figure 3.10 a and b.

Treatment	**Figure 3.10**	**y = ax + b**	**r^2**
Field	% time	y = 0.37x + 147.7	0.766
	Actual days	y = 0.31x + 147.7	
Simulated natural	% time	y = 0.64x + 146.4	0.879
	Actual days	y = 0.55x + 146.4	
Compressed	% time	y = 0.31x + 145	0.386
	Actual days	y = 0.68x + 145	

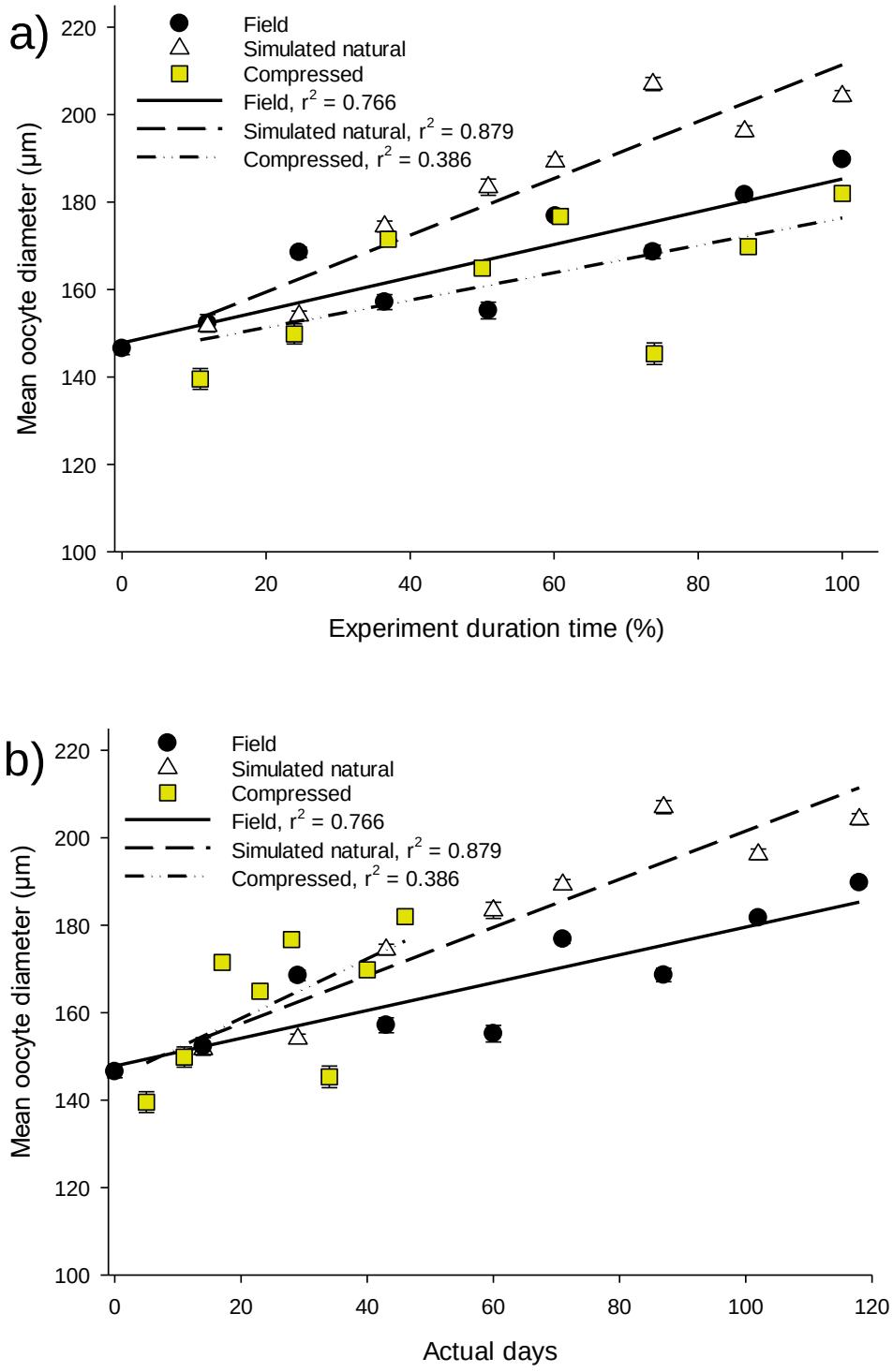


Figure 3.10 a) Mean oocyte diameter (µm) throughout the experimental duration time (%). **b)** Mean oocyte diameter (µm) for the actual days in the experiment. The oocyte diameter was calculated from the measured area of the oocytes in the different treatments. Standard error is represented by error bars (±). The data from the different treatments are separated into individual linear regression lines.

A comparison of the oocytes measured in the histological sections and the fixated oocytes from the polychaetes is shown in Figure 3.11. The figure shows that the oocyte diameter is generally larger for the extracted oocytes, in all treatments. Table 3.5 shows the values for the regression lines in Figure 3.11.

Table 3.5 Comparison between the values on the regression lines from diameter measured on fixated oocytes and calculated from histological sections in Figure 3.11.

Treatment	**Figure 3.11**	**y = ax + b**	**r^2**
Field	Histology	y = 0.37x + 147.7	0.766
	Fixated	y = 0.23x + 181.5	0.155
Simulated natural	Histology	y = 0.64x + 146.4	0.879
	Fixated	y = 0.31x + 192.1	0.510
Compressed	Histology	y = 0.31x + 145.0	0.386
	Fixated	y = 0.12x + 185.7	0.063

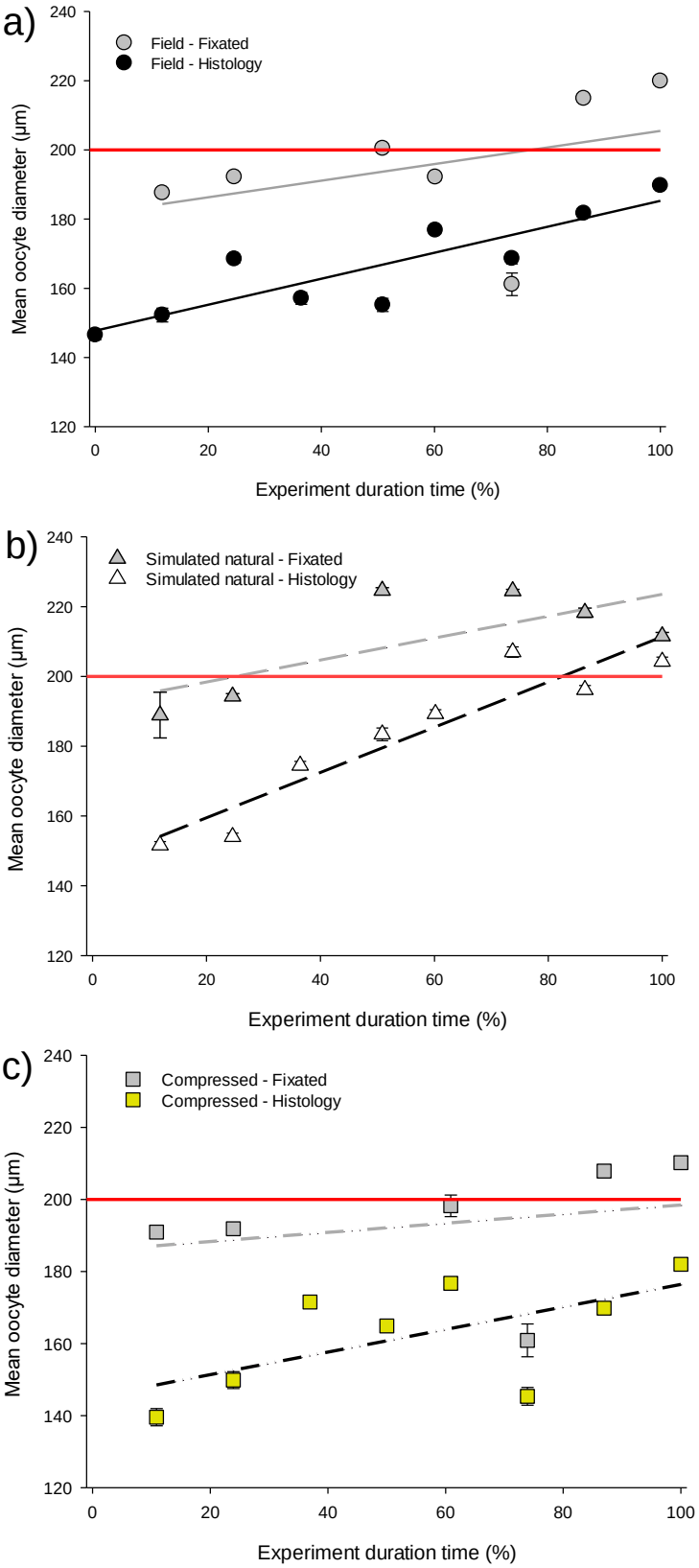


Figure 3.11 Comparison of the mean oocyte diameter (µm) for fixated oocytes and oocytes in histological sections in the different treatment groups; a) Field, b) Simulated natural, c) Compressed. The red line represents the threshold for when the oocytes are reckoned as mature (≥ 200 µm). Standard error is represented by error bars (±). The data from the different treatments are separated into individual linear regression lines.

3.7 Shape factor

The shape factor was calculated from the measured perimeter (µm) and area (µm^2) of the oocytes (see formula in chapter 2.4.4), where 1 equals a circle. The Pearson's correlation coefficient showed that there was significant positive correlation ($P < 0.01$) between mean oocyte area and mean oocyte shape factor in all treatment groups (Figure 3.12). This supports the theory that the oocytes become more spherical as the area of the oocytes increase.

Figure 3.13a shows the calculated shape factor for the oocytes in the different treatments for the experimental duration (%). There was a significant difference between the mean shape factor of the oocytes in the compressed light treatment and the oocytes in the two other treatment groups during the experimental duration (%). There was no significant difference between the shape factor of the oocytes in the simulated natural light treatment and in the field (p-values are shown in Table 3.7) The shape factor indicates that the simulated natural light treatment almost had spherical oocytes at the end of the experiment (mean shape factor = 0.96). In the field samples the mean shape factor of the oocytes were equal to 0.90 at the end of the experiment, while the oocyte in the compressed light treatment reached a mean shape factor of 0.84. The regression line for the different treatments clearly show that there was a development in the roundness of the oocytes during the experiment.

Figure 3.13b shows the calculated shape factor for the different treatments during the actual number of days in the experiment. There was no statistically significant difference in mean oocyte shape factor between the different treatment groups when accounting for actual days in the experiment (up to day 46, ANCOVA, p-values shown in Table 3.7). The values for the regression lines in Figure 3.13 is shown in Table 3.6.

Table 3.7 shows the p-values for both mean area and mean shape factor between the different treatment groups from the ANCOVA output.

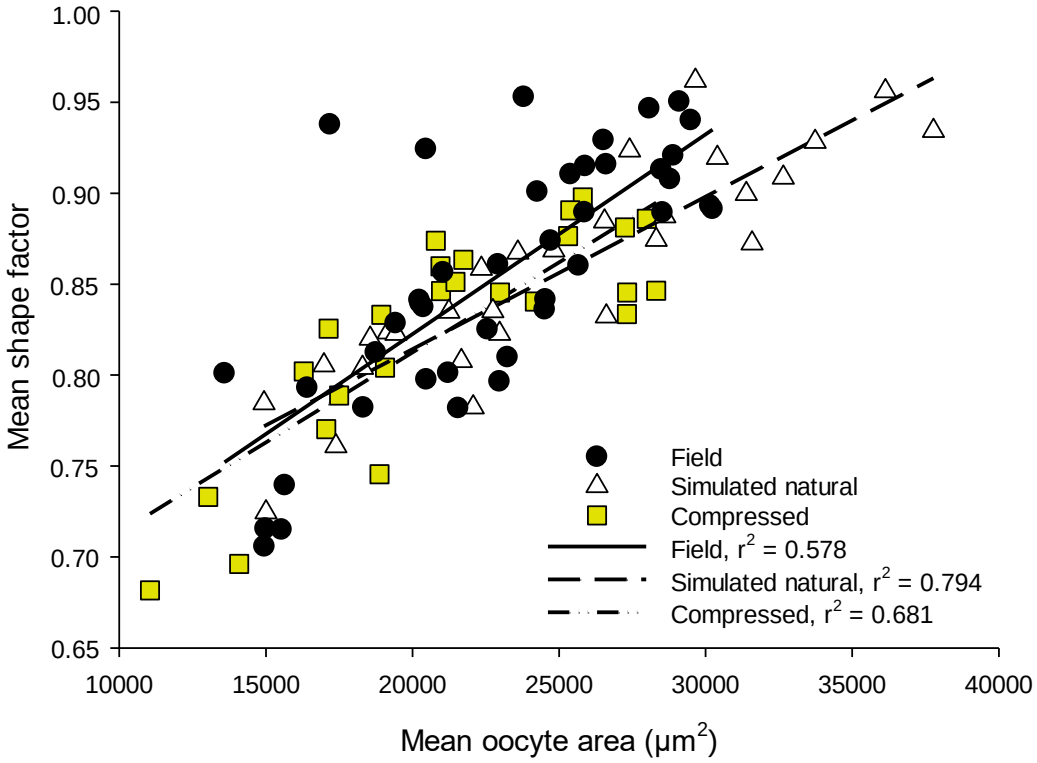


Figure 3.12 Correlation between the mean area and the calculated mean shape factor of each polychaete (≤ 50 measured oocytes per polychaete). The data from the different treatments are separated into individual linear regression lines. Pearson's correlation coefficient, r = 0.760 (field), r = 0.891 (simulated natural light cycle), r = 0.826 (compressed light cycle), and the correlation was significant for all groups ($p < 0.01$).

Table 3.6 Values for the regression lines of the different treatments shown in Figure 3.13 a and b.

Treatment	**Figure 3.13**	**y = ax + b**	**r^2**
Field	% time	y = 0.0013x + 0.781	0.656
	Actual days	y = 0.0011x + 0.781	
Simulated natural	% time	y = 0.0018x + 0.776	0.875
	Actual days	y = 0.0015x + 0.776	
Compressed	% time	y = 0.0005x + 0.799	0.094
	Actual days	y = 0.0011x + 0.799	

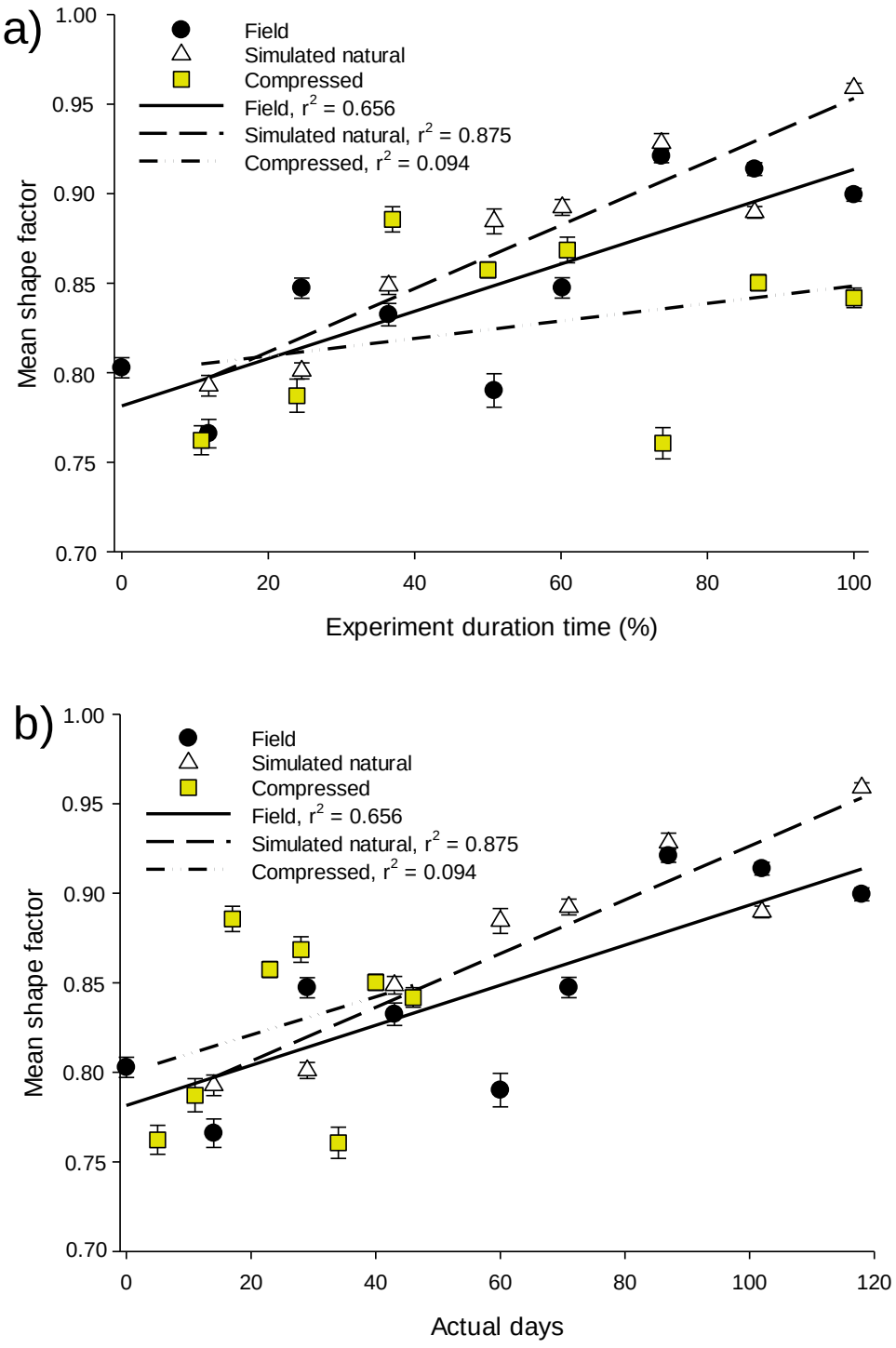


Figure 3.13 a) Shape factor for all treatments throughout the experimental period in percent. **b)** Shape factor for all the experimental treatments for the actual days in the experiment. The shape factor represents a scale from 0 to 1, were one is a circle. Sampling size varies from 1 to 7 polychaetes, with ≤ 50 oocytes measured per polychaete. Standard error (±) are represented with the error bars. The data from the different treatments are separated into individual linear regression lines.

Table 3.7 p-values from the ANCOVA for oocyte area (μm^2) and for shape factor. Comparison between the different treatment groups, for the experiment duration time (%) and for the actual days (up to day 46). Significant p-values ($p < 0.05$) are shown in red.

Comparison between treatments	**Area**		**Shape**	
	%	**Actual days**	**%**	**Actual days**
Field vs simulated natural	0.028	0.952	1.000	1.000
Field vs compressed	0.065	0.985	0.026	1.000
Simulated natural vs compressed	0.000	0.751	0.009	1.000

3.8 Fecundity

The number of oocytes per female was estimated for the fixated polychaetes, where the total number of oocytes was extracted. Figure 3.14 shows the relationship between the measured length of the female and the number of oocytes. The different treatment groups were combined to get a larger sample size for the comparison between the two variables. Pearson's correlation test shows that there is a significant positive linear relationship (r = 0.604, $p < 0.01$) between the length of the female and the number of oocytes. The mean number of oocytes varied from 1720 to 25400 per female.

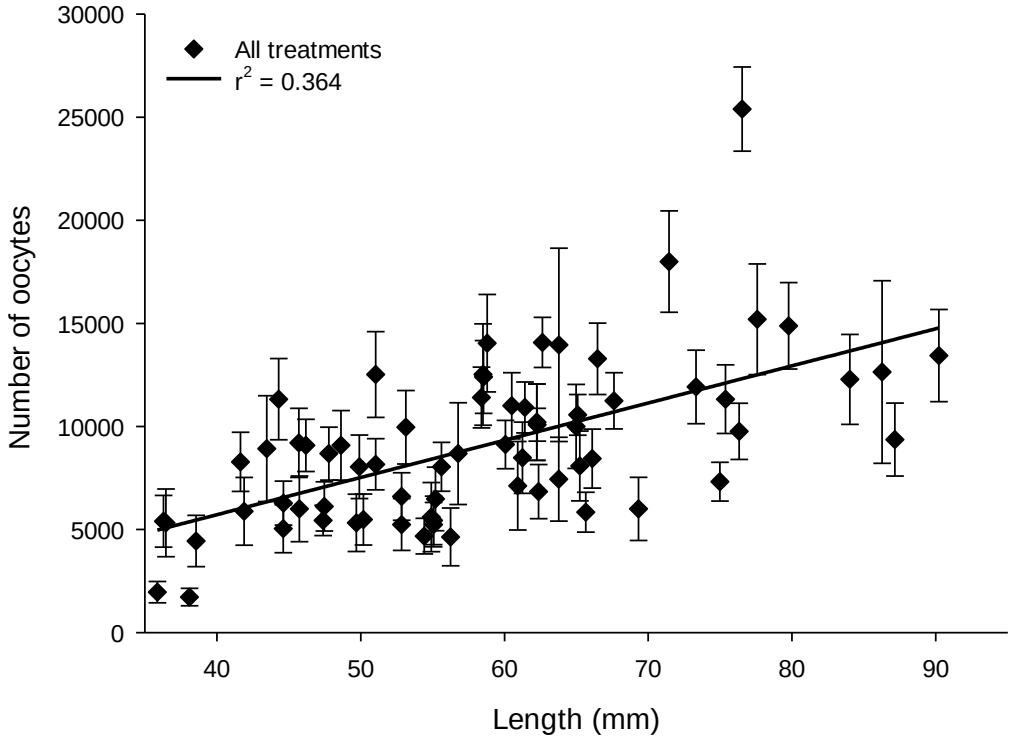


Figure 3.14 The number of oocytes per female against the measured length (mm) of the female. Pearson's correlation coefficient r = 0.604 and the correlation was significant ($p < 0.01$). The standard deviation is represented with the error bars (±).

3.9 Maturation of male *H. diversicolor*

The maturation of males was assessed by quantifying the presence of free spermatocytes, tetrades or free spermatozoans at the different sampling points. The males were divided into two categories: males with only spermplates in the coelom (Figure 3.15 A), and males with free spermatozoans in the coelom (Figure 3.15 B). The males were assessed as immature if they had only spermplates present in the coelom. Males with spermatocytes or free spermatozoans present in the coelom, was assessed as mature.

Figure 3.16 showed the number of mature and immature males in the different treatments. There were no mature males in the samples from field. In the simulated natural light treatment mature males were found at January 6th, February 6th and February 22nd (at 60.1 %, 86.4 % and 100 % completion of the experimental duration, respectively). In the compressed light treatment one mature male was found at the 6th of December (at 87 % duration of the experiment).

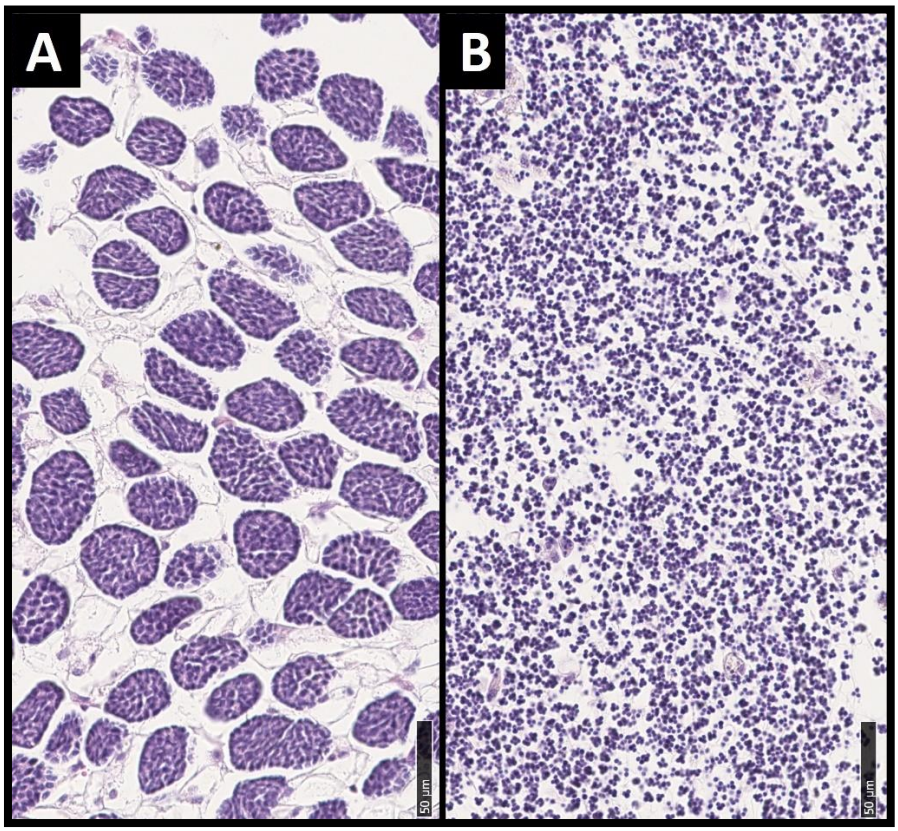


Figure 3.15 Immature and mature males of *H. diversicolor*. A) immature male with spermplates, B) mature male without spermplates. Scale bar at 50 µm (NanoZoomer).

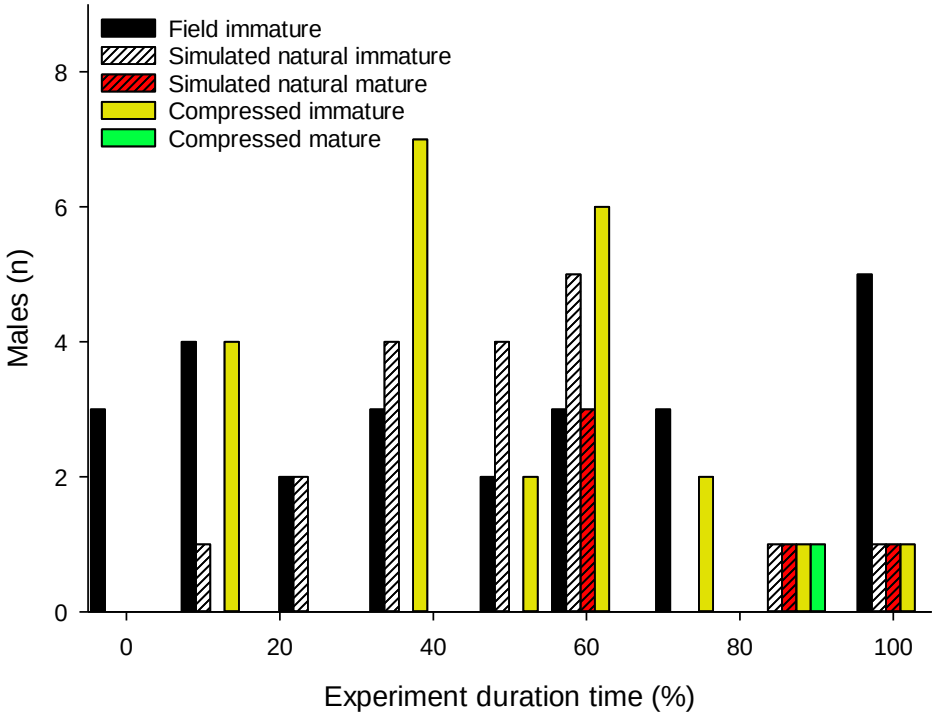


Figure 3.16 The number of immature and mature males (n = number of males) from the histological sectioning in the different treatments throughout the experiment duration time.

4 Discussion

In the present study the oocyte development in *H. diversicolor* was investigated and the possibility of controlling the maturation process by use of manipulated light cycles was investigated. Different light cycles as simulated natural light cycle and compressed light cycle were tested and compared to samples from the field.

4.1 Development of oocytes

Observations of the oocytes and a deduced shape factor showed that the oocytes got rounder in shape as they developed. There was a positive linear relationship between the mean area and the mean shape factor of the oocytes. This meant that the oocytes gradually got rounder in shape as they developed. There was observed structural changes in the yolk composition towards the end of maturation. A general description on oocyte development in polychaetes was provided by Eckelbarger (2005). Dales (1950) described the oocyte development of *H. diversicolor*. However, the present study presented a more detailed description of the oocyte development of *H. diversicolor*.

In the present study the size of the oocytes was measured by retracing the cell membrane of the oocytes in histological sections. This type of measurement was performed to ensure a precise measure of the oocytes, since their shape was quite irregular at certain times during the oocyte development. By performing this measurement both the area (μm^2) and the perimeter (µm) were measured for each oocyte, which made it possible to calculate a shape factor. The calculation of the shape factor was done based on observations in the present study, and the assumption that there was a possible pattern in the shape and the stage of the oocytes. Previous studies on the species had observed that the oocytes were more spherical towards the end of maturation (Möller, 1985, Olive & Garwood, 1981). There are no previous studies using shape factor to assess the maturation in this species. The shape factor is an important additional measurement that can be performed in future studies on this species, and on species with similar development of the shape of the oocytes. Previous studies conducted on this species have performed diameter measurements to assess the size of the oocytes (oocytes were extracted from the polychaete) (Chambers & Milne, 1975, Clark & Ruston, 1963, Dales, 1950, Kristensen, 1984, Olive & Garwood, 1981).

Comparisons between the oocyte diameter from the fixated oocytes to the diameter in the histological sections (Figure 3.11) showed that fixated oocytes had a much larger diameter. When tissue is processed for histological sectioning, it is well known that the tissue shrinks (Dorph-Petersen et al., 2001). The percentage of shrinkage is dependent on the tissue or organ in question, and the species from which it was retrieved (Dorph-Petersen et al., 2001). The shrinkage is caused mainly from dehydration and embedding, but some shrinkage can also be caused from fixation (Fox et al., 1985). The fixated oocytes in the present study were therefore closer to the real size of the oocytes than the oocytes from histological sectioning.

The graphs of the fixated oocytes clearly showed that the mean oocyte diameter was ≥ 200 µm at the last sampling day for all treatment groups. The largest oocytes found in the end of the experiment had a mean diameter of 220 µm. The oocytes of *H. diversicolor* are reckoned as mature when the diameter is ≥ 200 µm (the oocytes were not fixated in these studies) (Dales, 1950, Olive & Garwood, 1981). The diameter of mature oocytes in *H. diversicolor* from other areas (North-East England) can vary from 200 µm to 275 µm in diameter (200 µm to 275 µm (Dales, 1950), 200 µm to 225 µm (Olive & Garwood, 1981)).

The time of spawning for wild *H. diversicolor* was expected to be towards the end of February (based on previous unpublished observations at Leangbukta performed by Haiqing Wang). Although the mean diameter of the fixated oocytes was above 200 µm at the last sampling day from field, the oocytes had a mean shape factor and structure that suggested that the oocytes were not fully mature. Therefore, it was concluded that the spawning started later than the 22nd of February in Leangbukta. The exact time of when mature oocytes would be present, or when the spawning event would occur, was hard to pinpoint since the oocyte maturation depend on both internal and external influences (Olive et al., 2005).

4.2 Effect of manipulating light rhythm on maturation

When comparing the simulated natural light treatment group with the field collected specimens, the females from the simulated natural light treatment group had significantly larger mean oocyte area than field collected specimens throughout the experiment duration (%). The shape factor of the oocytes from the simulated natural light treatment group was not significantly different than the oocytes from field sampling. Furthermore, the structure of the oocytes from the last sampling day showed that oocytes from the simulated natural light treatment group were closer to maturation than the oocytes in the field. Only one polychaete had mature oocytes in the present study, and it was sampled from the simulated natural light treatment group. The mature oocytes were compared to a mature oocyte from *A. virens* (Figure 4.1). The oocytes were quite similar, but the oocyte from *A. virens* had a wider layer of cortical granules than *H. diversicolor*. This could suggest that the oocyte of *H. diversicolor* was not fully matured, or that the development was different between the species. There was not found any pictures of the structure of mature oocytes from *H. diversicolor*.

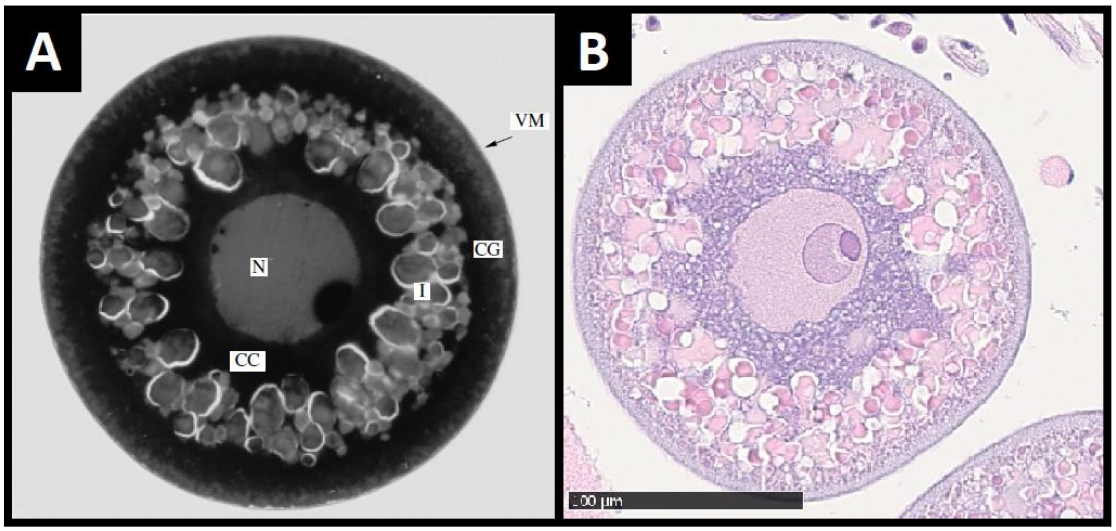


Figure 4.1 Comparison of mature oocytes. A) Mature oocyte from *A. virens* (stained with methylene blue, not to scale, from Kostyuchenko & Dondua (2000)). B) Mature oocyte from *H. diversicolor*, from the simulated natural light treatment at the last sampling day (scale bar 100 µm). CG = cortical granules, VM = vitelline membrane, I = zone of lipid and yolk inclusions, CC = clear cytoplasm, N = nucleus.

The oocytes in the simulated natural light treatment were further developed than the oocytes from field. The working hypothesis stating that the oocyte maturation in the simulated natural light treatment would coincide with samples taken from field can therefore be rejected. These two groups had different temperatures, the temperature in field ranged from 6.2 to -1.4 °C at 5 cm depth) and the temperature at the experimental treatments was 8 °C. The temperature

difference between field and the experimental treatments could have affected the oocyte maturation process. Temperature could be an environmental factor influencing the oocyte maturation. However, a previous study performed on *H. diversicolor* showed that when the species was kept in an experimental set-up with constant temperature (5, 10, 15 and 20 °C) and a constant light/dark cycle, the polychaetes in the different treatment groups matured at the same time (Olive & Garwood, 1983). This showed that when *H. diversicolor* was reared under different constant temperatures the worms maintained the same reproductive rhythmicity (Olive & Garwood, 1983). Further research should be performed to assess the influence of temperature during the last stages of oocyte maturation in *H. diversicolor*.

The oocytes from the compressed light treatment were less developed (smaller, lower shape factor, structure) compared to the oocytes from the simulated natural light treatment throughout the experiment. The differences in maturation were also reflected in differences in relative growth rate of the polychaetes. Both the compressed light treatment group and the simulated natural light treatment group had a negative relative growth rate of the polychaetes. The polychaetes from the simulated natural light treatment had a stronger weight decrease than the polychaetes from the compressed light treatment. Growth does not occur during the last stages of maturation (Golding, 1967), and the weight decrease could therefore be a result of the maturation process throughout the experiment.

When comparing the mean oocyte development (mean area and shape factor) from the two light treatment groups to actual days, there was no statistically significant difference between the treatments up to day 46. It can therefore be concluded that the oocytes in the compressed light treatment did not mature according to the light regime. The working hypothesis stating that the compressed light treatment would accelerate the oocyte development could therefore be rejected.

One possible reason why the compressed light treatment did not expedite the maturation process could be that the experiment duration time was too short for the polychaetes to respond to the light cycle. A more likely reason was that the increase in daylength did not have a strong influence on the oocyte maturation in *H. diversicolor*. In the present study, the increase in daylength did not seem to be a dominant environmental factor for *H. diversicolor*. The influence of increasing daylength could not be excluded for the species by the present study, but it could

be suggested that temperature also was an environmental factor affecting the maturation process. It is likely that the maturation process depends on an interaction between daylength, temperature and time, as shown in fish (Bromage et al., 2001). To exclude the influence of light cycles on oocyte maturation, an experiment in total darkness should have been conducted.

The effect of the environmental factors in determining the time of maturation varies in polychaetes (Last & Hendrick, 2014). A study on a species (*A. virens*) within the Nereididae family, showed that oocyte growth was strongly correlated with the photoperiod (Olive et al., 1998). The study showed that the oocytes grew more rapidly when the polychaetes were exposed to a fixed light/dark cycle with less than 13 hours of light per day (Olive et al., 1998). A fixed daylength has been proven to be the determining environmental factor for maturation in *A. virens* (Last & Olive, 2004, Olive et al., 1998, Rees & Olive, 1999), *Typosyllis porifera* (Franke, 1986) and *Kefersteinia cirrata* (Olive & Pillai, 1983). In *Harmothoe imbricata* (Clark, 1988, Garwood, 1980, Garwood & Olive, 1982) and *Neanthes limnicola* (Nereididae family) (Fong & Pearse, 1992), temperature is the dominant environmental factor for oocyte maturation.

4.3 What determines the time for sexual maturation?

The synchronicity of spawning is crucial for *H. diversicolor* since the worms only have one chance for reproducing before they die (Scaps, 2002). The environmental factors (daylength and temperature) affect the time of reproduction by regulating the physiological state up to the onset of maturation and determining the time when maturation begins (Olive et al., 1998). In natural populations of *H. diversicolor* the generation time can be from 1-3 years, which means that polychaetes can proceed passed one to two daylength and temperature periods that were favourable for spawning, in nature (illustrated in Figure 4.2) (Olive & Garwood, 1983). This means that the response to the environmental factors can be supressed, by feeding conditions and/or an endogenous rhythm, until the polychaetes are reproductively competent (Olive & Garwood, 1983).

Production of gametes outside the normal production time is not only dependent on environmental conditions for maturation (Olive, 1995, Olive & Garwood, 1983). Also the endogenous rhythm defines the periods where it is appropriate for gametogenesis to be induced (Olive & Garwood, 1983). A study performed on *H. diversicolor* found that a high level of

juvenile hormone inhibited rapid oocyte growth (Clark & Ruston, 1963). The study concluded that a combination of endogenous factors and environmental factors reduced the production of the juvenile hormone. The reduced level of juvenile hormone then resulted in a rapid growth of oocytes (Clark & Ruston, 1963).

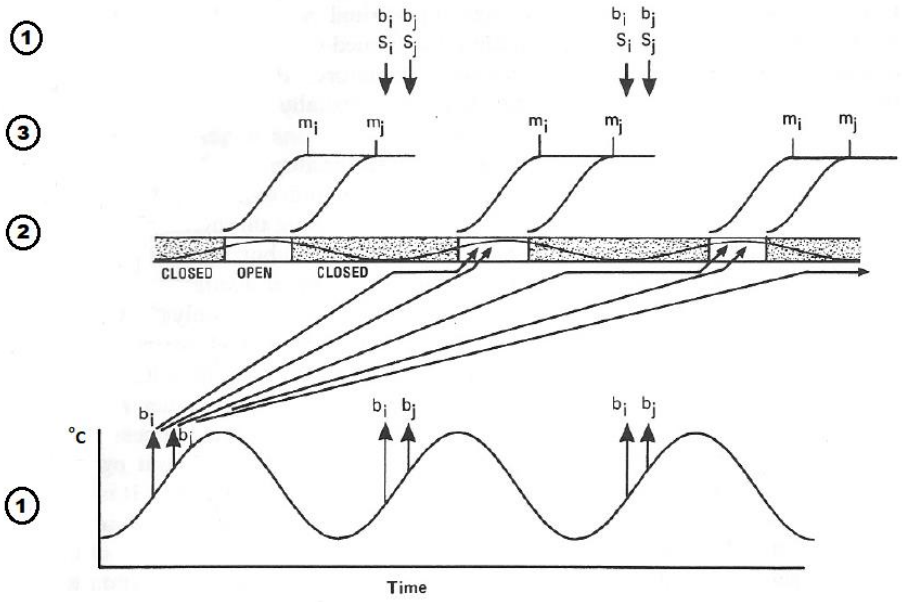


Figure 4.2 Model of sexual maturation rhythmicity of *H. diversicolor* (from Olive & Garwood (1983)). The components in the model are 1) an exogenous cycle (temperature) 2) an endogenous gated rhythm of circa annual periodicity, 3) intervals when the process of oogenesis is possible. Initiation and completion of oogenesis is a fixed relationship. Worms from the same year class may complete the oogenesis process at different gated periods. Worms from the same year class is shown to proceed through second and third gates after birth. b_{i-j}, range of possible birth dates, m_{i-j} interval when worms become sexually mature, s_{i-j} interval when spawning takes place.

H. diversicolor has an endogenous rhythm that defines the periods of critical stages when gametogenesis may or may not be initiated (a gated rhythm) (Olive & Garwood, 1983). Therefore, it is likely that maturation cannot be manipulated outside the time when vitellogenesis occurs, even if the polychaetes are kept with endogenous factors that normally would enhance maturation (Olive, 1995). Figure 4.2 illustrates that maturation of the species is affected by a congenital endogenous rhythm. The oocyte maturation in *H. diversicolor* is dependent on a relationship between mainly endogenous factors, but also exogenous factors when the time of maturation is imminent (Olive & Garwood, 1983). Even if temperature and/or photoperiod could affect the time of maturation, there would still be a fixed gated rhythm controlled by endogenous factors that would control the time when maturation can be initiated.

In order to establish a year-round production of polychaetes, it is crucial to be able to manipulate the endogenous rhythm of the species.

4.4 Quantity of females and fecundity

The gender distribution recorded in the present study during histological sampling was 38 % males and 62 % females. This distribution was not accurate to the population because we sampled only the biggest individuals. The natural gender distribution in different areas was found to be approximately 16 % males and 84 % females, hence more skewed in favour of the females (Abrantes et al., 1999, Clark & Ruston, 1963, Olive & Garwood, 1981, Smith, 1976). One study reported a percentage of males as low as 10 % (Dales, 1950). The number of females in the present study was therefore lower than expected.

There was a positive correlation between the fecundity and the length of the female in the present study. A previous study found that coelomic oocytes were present in females that were 7 cm long (Dales, 1950). The present study found that oocytes were present in the coelom when the females were above 3.5 cm long. The number of oocytes per female varied between 1720 to 25400, and the average was 8900 oocytes per female. Abrantes et al. (1999) found that the number of oocytes per female ranged from 40 to 15 000. The number of oocytes per female is related to size, number of setigers and age of the polychaete (Abrantes et al., 1999, Durou et al., 2007, Warren, 1976). The high fecundity in *H. diversicolor* suggests that the species has a high reproductive potential.

4.5 Methodological limitations

The intention of the present study was to investigate the effect of daylength on the reproductively capable individuals of the population, by selecting the largest individuals in the population. The study is therefore representative towards the most reproductively capable individuals of the population.

All polychaetes with a mean oocyte size of 40 % less than the mean oocyte size of the individual sampling days in the different treatment groups were omitted from the results and grouped as immature (non-maturing). The mean oocyte size of these omitted polychaetes ranged from 924 μm^2 to 7731 μm^2 (34 μm to 99 μm in diameter). Generally, it takes the oocytes a year to develop from their first appearance in the coelom until maturation (minimum 9 months (Clark & Ruston, 1963), maximum 18 months (Olive & Garwood, 1981)). The omitted polychaetes were most likely part of the next years spawning season (Olive & Garwood, 1981). Since these omitted females were not included in the experimental results the sample size was reduced, ranging from one to seven reproductively competent females per sample.

Orienting the polychaetes correctly in the blocks for the histological sectioning proved to be difficult. The specimens were often coiled, and the tissue was quite rigid which made it difficult to get the polychaetes to lie flat in the paraffin blocks. Consequently, more than half of the polychaetes had to be re-embedded. The problem was resolved by dividing the polychaetes into two to four pieces to get them to lie flat in the blocks. The angle of the sections was not perfectly longitudinal, but the sections were standardized by aiming for the digestive tract. Additionally, there were some problems with the sectioning of some of the polychaetes due to gravel in the digestive tract.

The size estimation of each individual polychaete proved to be difficult since the length of the polychaetes was quite flexible. The total body length was measured according to Olive (1971), and the number of setigers in each worm was counted. Almost half of the sampled polychaetes (48.5 %) had lost their last posterior setigers in the pygidium (tail) section. This made it difficult to estimate the size of the polychaetes based on body length and number of setigers. The posterior setigers can be lost due to predatory infauna (Zajac, 1985) or probably due to rough handling during sampling. In retrospect the size measurements could have been performed in the head region. Durou et al. (2007) used a size estimation named L3 length, which was the

combined length of prostomium (anterior presegmental region of the body), peristomium (presegmental region of the body surrounding the mouth) and the first setiger segment (Beesley et al., 2000). Olive & Garwood (1981) measured the total jaw length and compared it to the total body length to estimate the total body length of the polychaete. The L3 length measurement is recommended when the worms have lost the posterior section of the body (Díaz-Jaramillo et al., 2011).

5 Conclusion

A description and overview of the oocyte structure and development in wild caught *H. diversicolor* was provided in the present study. Observations from the present study showed that oocytes of *H. diversicolor* became more spherical in shape when spawning was imminent. A shape factor was therefore deduced. The shape factor proved to be a useful tool in assessing the maturation of *H. diversicolor* and can be used in future studies on this species, and on species with similar oocyte development.

The polychaetes in the simulated natural light treatment had oocytes that were more developed in mean area and structure than the field collected specimens. The two groups had different temperature conditions, being 6.2 to -1.4 °C at 5 cm depth in the field and 8 °C in the simulated natural light treatment. The temperature difference could probably have affected the oocyte maturation process.

The present study showed that the compressed light cycle did not accelerate the oocyte maturation in *H. diversicolor*. The oocytes in the compressed light treatment were significantly less mature than the oocytes in the simulated natural light treatment during the experimental duration (%). This suggested that light cycle was not a dominant environmental factor influencing the oocyte maturation in the present study.

6 Future perspectives

The results from the present study showed that female *H. diversicolor* did not mature earlier with a compressed light cycle and a constant temperature. Further studies with for example 50 % or 60 % compressed light cycles could be performed to investigate if the reduction of the light cycle in the present study was too much. Year-round spawning can possibly be achieved through other treatments than compressed light cycles. To establish a year-round production of *H. diversicolor*, further research on manipulating the sexual maturation must be conducted. The results from the present study suggested that temperature could influence the oocyte maturation during vitellogenesis, therefore an experiment with a constant light treatment and a changing temperature could be conducted in future studies. A study on the effect of a compressed temperature cycle could be conducted to investigate if artificial temperature rhythms can expedite the maturation process. Additionally, the effect of the endogenous rhythm on the oocyte maturation and the possibility of manipulating the endogenous rhythm must be investigated.

7 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.

BAERT, J.-L. 1986. Evidence for vitellin maturation within oocytes of Perinereis cultrifera (Polychaete Annelid). *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry,* 83**,** 847-853.

BAERT, J.-L. & SLOMIANNY, M. C. 1987. Heterosynthetic origin of the major yolk protein, vitellin, in a nereid, Perinereis cultrifera (polychaete annelid). *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry,* 88**,** 1191-1199.

BARRINGTON, K., CHOPIN, T. & ROBINSON, S. 2009. Integrated multi-trophic aquaculture (IMTA) in marine temperate waters. *Integrated mariculture: a global review. FAO Fisheries and Aquaculture Technical Paper,* 529**,** 7-46.

BARTELS-HARDEGE, H. & ZEECK, E. 1990. Reproductive behaviour of Nereis diversicolor (Annelida: Polychaeta). *Marine Biology,* 106**,** 409-412.

BATISTA, F., E COSTA, P. F., RAMOS, A., PASSOS, A., FERREIRA, P. P. & DA FONSECA, L. C. 2003. Production of the ragworm Nereis diversicolor (OF Muller, 1776), fed with a diet for gilthead seabream Sparus auratus L., 1758: survival, growth, feed utilization and oogenesis. *BOLETIN-INSTITUTO ESPANOL DE OCEANOGRAFIA,* 19**,** 447.

BEESLEY, P. L., ROSS, G. J. B. & GLASBY, C. J. 2000. *Polychaetes & Allies: the Southern Synthesis*, Australian Biological Resources Study/CSIRO Publishing.

BERNTSEN, F. H. 2018. *Growth of the Polychaete Hediste diversicolor (O. F. Müller, 1776) fed on Smolt Sludge and Biogas Residues - Evaluation of RNA/DNA Ratio as growth indicator* NTNU.

BOOLOOTIAN, R. A. & GIESE, A. C. 1958. Coelomic Corpuscles of Echinoderms. *Biological Bulletin,* 115**,** 53-63.

BROMAGE, N., PORTER, M. & RANDALL, C. 2001. The environmental regulation of maturation in farmed finfish with special reference to the role of photoperiod and melatonin. *Reproductive Biotechnology in Finfish Aquaculture.* Elsevier.

BUDD, G. C. 2008. *Hediste diversicolor Ragworm* [Online]. Plymouth: Marine Biological Association of the United Kingdom. Available: https://www.marlin.ac.uk/species/detail/1426 [Accessed 20. March, 2018].

CARVALHO, A. & SANTOS, P. 2013. Sustainability of bait fishing harvesting in estuarine ecosystems-Case study in the Local Natural Reserve of Douro Estuary, Portugal.

CHAMBERS, M. & MILNE, H. 1975. Life cycle and production of Nereis diversicolor OF Müller in the Ythan Estuary, Scotland. *Estuarine and Coastal Marine Science,* 3**,** 133-144.

CLARK, R. B. & RUSTON, R. J. G. 1963. The influence of brain extirpation on oogenesis in the polychaete Nereis diversicolor. *General and comparative endocrinology,* 3**,** 529-541.

CLARK, S. 1988. A two phase photoperiodic response controlling the annual gametogenic cycle in Harmothoe imbricata (L.)(Polychaeta: Polynoidae). *International journal of invertebrate reproduction and development,* 14**,** 245-265.

COSTA, P. F., OLIVEIRA, R. F. & FONSECA, L. C. D. 2006. Feeding ecology of Nereis diversicolor (OF Müller)(Annelida, Polychaeta) on estuarine and lagoon environments in the southwest coast of Portugal. *Pan-American Journal of Aquatic Sciences***,** 104-113.

DALES, R. P. 1950. The reproduction and larval development of Nereis diversicolor OF Müller. *Journal of the Marine Biological Association of the United Kingdom,* 29**,** 321-360.

DALES, R. P. & KENNEDY, G. 1954. On the diverse colours of Nereis diversicolor. *Journal of the Marine Biological Association of the United Kingdom,* 33**,** 699-708.

DELTA FARMS. 2018. *Delta Farms* [Online]. Available: https://www.deltafarms.nl/# [Accessed 25. November, 2018].

DÍAZ-JARAMILLO, M., DA ROCHA, A. M., GOMES, V., BIANCHINI, A., MONSERRAT, J. M., SÁEZ, K. & BARRA, R. 2011. Multibiomarker approach at different organization levels in the estuarine Perinereis gualpensis (Polychaeta; Nereididae) under chronic and acute pollution conditions. *Science of the Total Environment,* 410**,** 126-135.

DORPH-PETERSEN, K. A., NYENGAARD, J. & GUNDERSEN, H. 2001. Tissue shrinkage and unbiased stereological estimation of particle number and size. *Journal of microscopy,* 204**,** 232-246.

DUARTE, C. M., HOLMER, M., OLSEN, Y., SOTO, D., MARBÀ, N., GUIU, J., BLACK, K. & KARAKASSIS, I. 2009. Will the oceans help feed humanity? *BioScience,* 59**,** 967-976.

DUROU, C., SMITH, B. D., ROMÉO, M., RAINBOW, P. S., MOUNEYRAC, C., MOULOUD, M., GNASSIA-BARELLI, M., GILLET, P., DEUTSCH, B. & AMIARD-TRIQUET, C. 2007. From biomarkers to population responses in Nereis diversicolor: assessment of stress in estuarine ecosystems. *Ecotoxicology and environmental safety,* 66**,** 402-411.

ECKELBARGER, K. J. 1988. Oogenesis and female gametes. *The Ultrastructure of Polychaeta, Microfauna Mar,* 4**,** 281-307.

ECKELBARGER, K. J. 2005. Oogenesis and oocytes. *Hydrobiologia,* 535**,** 179-198.

ESSELINK, P. & ZWARTS, L. 1989. Seasonal trend in burrow depth and tidal variation in feeding activity of Nereis diversicolor. *Marine Ecology Progress Series***,** 243-254.

FAO 2016. *The State of World Fisheries and Aquaculture 2016. Contributing to food security and nutrition for all*, Rome.

FAUCHALD, K. 1977. *The Polychaete Worms: Definitions and Keys to the Orders, Families, and Genera*, Natural History Museum of Los Angeles County.

FISCHER, A. 1975. The structure of symplasmic early oocytes and their enveloping sheath cells in the polychaete, Platynereis dumerilii. *Cell and tissue research,* 160**,** 327-343.

FISCHER, A., DORRESTEIJN, A. & HOEGER, U. 2003. Metabolism of oocyte construction and the generation of histospecificity in the cleaving egg. Lessons from nereid annelids. *International Journal of Developmental Biology,* 40**,** 421-430.

FISCHER, A. H., JACOBSON, K. A., ROSE, J. & ZELLER, R. 2008. Hematoxylin and eosin staining of tissue and cell sections. *Cold Spring Harbor Protocols,* 2008**,** pdb. prot4986.

FONG, P. P. & PEARSE, J. S. 1992. Evidence for a programmed circannual life cycle modulated by increasing daylengths in Neanthes limnicola (Polychaeta: Nereidae) from central California. *The Biological Bulletin,* 182**,** 289-297.

FOX, C. H., JOHNSON, F. B., WHITING, J. & ROLLER, P. P. 1985. Formaldehyde fixation. *Journal of Histochemistry & Cytochemistry,* 33**,** 845-853.

FRANKE, H.-D. 1986. The role of light and endogenous factors in the timing of the reproductive cycle of Typosyllis prolifera and some other polychaetes. *American Zoologist,* 26**,** 433-445.

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. *Memoires de la Musee nationale d'Histoire naturelle,* 162**,** 593-603.

GARWOOD, P. 1980. The role of temperature and daylength in the control of the reproductive cycle of Harmothoe imbricata (L.)(Polychaeta: Polynoidae). *Journal of Experimental Marine Biology and Ecology,* 47**,** 35-53.

GARWOOD, P. R. & OLIVE, P. J. W. 1982. The influence of photoperiod on oocyte growth and its role in the control of the reproductive cycle of the polychaete Harmothoe imbricata (L.). *International Journal of Invertebrate Reproduction,* 5**,** 161-165.

GOLDING, D. 1967. Regeneration and growth control in Nereis: I. Growth and regeneration. *Development,* 18**,** 67-77.

GOOGLE MAPS. 2018. *Leangenbukta, Trondheim* [Online]. Google Maps. Available: https://www.google.no/maps/@63.4399421,10.4098373,13z?dcr=0 [Accessed 7. March, 2018].

GRIMMEL, J., DORRESTEIJN, A. W. & FRÖBIUS, A. C. 2016. Formation of body appendages during caudal regeneration in Platynereis dumerilii: adaptation of conserved molecular toolsets. *EvoDevo,* 7**,** 10.

JORDAN, E. L. & VERMA, P. S. 2001. *Invertibrate Zoology*, S. Chand & Company LTD.
KOSTYUCHENKO, R. & DONDUA, A. 2000. Ooplasmic segregation and axis formation in the polychaeteNereis virens embryo. *Russian Journal of Developmental Biology,* 31**,** 95-105.
KRISTENSEN, E. 1981. Direct measurement of ventilation and oxygen uptake in three species of tubicolous polychaetes (Nereis spp.). *Journal of comparative physiology,* 145**,** 45-50.
KRISTENSEN, E. 1983. Ventilation and oxygen uptake by three species of Neries(Annelida: Polychaeta). II. Effects of temperature and salinity changes. *Marine ecology progress series. Oldendorf,* 12**,** 299-306.
KRISTENSEN, E. 1984. Life cycle, growth and production in estuarine populations of the polychaetes Nereis virens and N. diversicolor. *Ecography,* 7**,** 249-250.
KÅSS, E. 2018. *Retroperitoneal* [Online]. Store medisinske leksikon. Available: https://sml.snl.no/retroperitoneal [Accessed 4. November, 2018].
LAST, K. S. & HENDRICK, V. J. 2014. The Clock-Work Worms: Diversity and Function of Clock Expression in Marine Polychaete Worms. *Annual, Lunar, and Tidal Clocks.* Springer.
LAST, K. S. & OLIVE, P. J. W. 2004. Interaction between photoperiod and an endogenous seasonal factor in influencing the diel locomotor activity of the benthic polychaete Nereis virens Sars. *The Biological Bulletin,* 206**,** 103-112.
LUIS, O. J. & PASSOS, A. M. 1995. Seasonal changes in lipid content and composition of the polychaete Nereis (Hediste) diversicolor. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* 111**,** 579-586.
MARTINS, C. I. M., EDING, E. H., VERDEGEM, M. C. J., HEINSBROEK, L. T. N., SCHNEIDER, O., BLANCHETON, J. P., D'ORBCASTEL, E. R. & VERRETH, J. A. J. 2010. New developments in recirculating aquaculture systems in Europe: A perspective on environmental sustainability. *Aquacultural Engineering,* 43**,** 83-93.
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.
MOEN, F. E. & SVENSEN, E. 2014. *Dyreliv i havet: nordeuropeisk marin fauna*, Kom.
MÜLLER, O. F. 1776. *Zoologiae Danicae prodromus: seu Animalium Daniae et Norvegiae indigenarum characteres, nomina, et synonyma imprimis popularium*, typis Hallageriis.
MÖLLER, P. 1985. Production and abundance of juvenile Nereis diversicolor, and oogenic cycle of adults in shallow waters of western Sweden. *Journal of the Marine Biological Association of the United Kingdom,* 65**,** 603-616.
NESTO, N., SIMONINI, R., PREVEDELLI, D. & DA ROS, L. 2012. Effects of diet and density on growth, survival and gametogenesis of Hediste diversicolor (OF Müller, 1776)(Nereididae, Polychaeta). *Aquaculture,* 362**,** 1-9.
OGLESBY, L. C. 1970. Studies on the salt and water balance of Nereis diversicolor—I. Steady-state parameters. *Comparative biochemistry and physiology,* 36**,** 449-466.
OLIVE, P. J. W. 1971. Ovary structure and oogenesis in Cirratulus cirratus (Polychaeta: Cirratulidae). *Marine Biology,* 8**,** 243-259.
OLIVE, P. J. W. 1995. Annual breeding cycles in marine invertebrates and environmental temperature: probing the proximate and ultimate causes of reproductive synchrony. *Journal of thermal biology,* 20**,** 79-90.
OLIVE, P. J. W. 1999. Polychaete aquaculture and polychaete science: a mutual synergism. *Reproductive Strategies and Developmental Patterns in Annelids.* Springer.
OLIVE, P. J. W. & GARWOOD, P. R. G. 1981. Gametogenic cycle and population structure of Nereis (Hediste) diversicolor and Nereis (Nereis) pelagica from north-east England. *Journal of the Marine Biological Association of the United Kingdom,* 61**,** 193-213.
OLIVE, P. J. W. & GARWOOD, P. R. G. 1983. The importance of long term endogenous rhythms in the maintenance of reproductive cycles of marine invertebrates: a reappraisal. *International journal of invertebrate reproduction,* 6**,** 339-347.

OLIVE, P. J. W., KYRIACOU, C. P., LAST, K. S., KRAMER, C., BAILHACHE, T. & ROSATO, E. 2005. Dancing to the rhythms of geological time: the biorhythm capabilities of Polychaeta in a geological context. *Invertebrate Reproduction & Development,* 48**,** 197-206.

OLIVE, P. J. W. & PILLAI, G. 1983. Reproductive biology of the polychaete Kefersteinia cirrata Keferstein (Hesionidae). II. The gametogenic cycle and evidence for photoperiodic control of oogenesis. *International journal of invertebrate reproduction,* 6**,** 307-315.

OLIVE, P. J. W., REES, S. W. & DJUNAEDI, A. 1998. Influence of photoperiod and temperature on oocyte growth in the semelparous polychaete Nereis (Neanthes) virens. *Marine Ecology Progress Series,* 172**,** 169-183.

PAJAND, Z. O., SOLTANI, M., BAHMANI, M. & KAMALI, A. 2017. The role of polychaete Nereis diversicolor in bioremediation of wastewater and its growth performance and fatty acid composition in an integrated culture system with Huso huso (Linnaeus, 1758). *Aquaculture Research,* 48**,** 5271-5279.

PORCHET, M., BAERT, J. & DHAINAUT, A. 1989. Evolution of the concepts of vitellogenesis in polychaete annelids. *Invertebrate reproduction & development,* 16**,** 53-61.

PURSCHKE, G. 2006. *Morphology, molecules, evolution and phylogeny in Polychaeta and related taxa*, Springer Science & Business Media.

REECE, J. B., URRY, L. A., CAIN, M. L., WASSERMAN, S. A. M., PETER V. & JACKSON, R. B. 2011. *Campbell Biology*, Benjamin Cummings / Pearson.

REES, S. W. & OLIVE, P. J. W. 1999. Photoperiodic changes influence the incorporation of vitellin yolk protein by oocytes of the semelparous polychaete Nereis (Neanthes) virens. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* 123**,** 213-220.

ROSS, M. H. & PAWLINA, W. 2016. *Histology: a text and atlas*, Lippincott Williams & Wilkins.

SANTOS, A., GRANADA, L., BAPTISTA, T., ANJOS, C., SIMÕES, T., TECELÃO, C., E COSTA, P. F., COSTA, J. L. & POMBO, A. 2016. Effect of three diets on the growth and fatty acid profile of the common ragworm Hediste diversicolor (OF Müller, 1776). *Aquaculture,* 465**,** 37-42.

SARS, M. 1835. *Beskrivelser og Iagttagelser over nogle mærkelige eller nye i Havet ved den Bergenske Kyst levende Dyr af Polypernes, Acalephernes, Radiaternes, Annalidernes, og Molluskernes Classer. Med en kort Oversigt over de hidtil af Forfatteren... fundne Arter, etc*.

SCAPS, P. 2002. A review of the biology, ecology and potential use of the common ragworm Hediste diversicolor (OF Müller)(Annelida: Polychaeta). *Hydrobiologia,* 470**,** 203-218.

SCHENK, S. & HOEGER, U. 2010. Lipid accumulation and metabolism in polychaete spermatogenesis: Role of the large discoidal lipoprotein. *Molecular reproduction and development,* 77**,** 710-719.

SEEKAMP, I. 2017. *Utilization of excess nutrients from land-based aquaculture facilities by Hediste diversicolor (OF Müller, 1776)-Production of polychaete biomass and its potential use in fish feed.* master thesis, NTNU.

SHORELINE POLYCHAETES FARMS LLP. 2018. *Seabait from shoreline* [Online]. Available: https://www.seabait.com/about_us.html [Accessed 25. November, 2018].

SMITH, R. I. Further observations on the reproduction of Nereis diversicolor (Polychaeta) near Tvärminne, Finland, and Kristineberg, Sweden. Annales Zoologici Fennici, 1976. JSTOR, 179-184.

STRICKER, S. A., TAKEDA, N. & DEGUCHI, R. 2015. Calcium signals and oocyte maturation in marine invertebrates. *International Journal of Developmental Biology,* 59**,** 271-280.

SUSTAINABLE FEEDS LTD. 2018. *Sustainable Feeds Ltd.* [Online]. Available: https://www.sustainablefeeds.com/copy-of-sustainable-feeds-5-ecru9 [Accessed 25. November, 2018].

TIMEANDDATE.NO. 2018. *Trondheim, Norge — Soloppgang, solnedgang og dagens lengde* [Online]. ©Time and Date AS 1995-2017. Available: https://www.timeanddate.no/astronomi/sol/norge/trondheim [Accessed 11. October, 2018].

VEDEL, A., ANDERSEN, B. B. & RIISGÅRD, H. U. 1994. Field investigations of pumping activity of the facultatively filter-feeding polychaete Nereis diversicolor using an improved infrared phototransducer system. *Marine Ecology Progress Series*, 91-101.

WANG, X., OLSEN, L. M., REITAN, K. I. & OLSEN, Y. 2012. Discharge of nutrient wastes from salmon farms: environmental effects, and potential for integrated multi-trophic aquaculture. *Aquaculture Environment Interactions,* 2, 267-283.

WARREN, L. M. 1976. A population study of the polychaete Capitella capitata at Plymouth. *Marine Biology,* 38, 209-216.

WATSON, G. J., MURRAY, J. M., SCHAEFER, M. & BONNER, A. 2017. Bait worms: A valuable and important fishery with implications for fisheries and conservation management. *Fish and Fisheries,* 18, 374-388.

WOUTERS, R., LAVENS, P., NIETO, J. & SORGELOOS, P. 2001. Penaeid shrimp broodstock nutrition: an updated review on research and development. *Aquaculture,* 202, 1-21.

YOUNSI, M., DAAS, T., DAAS, O. & SCAPS, P. 2010. Polychaetes of commercial interest from the Mediterranean East Coast of Algeria. *Mediterranean Marine Science,* 11, 185-188.

ZAJAC, R. N. 1985. The effects of sublethal predation on reproduction in the spionid polychaete Polydora ligni Webster. *Journal of Experimental Marine Biology and Ecology,* 88, 1-19.

8 Appendices

Appendix 1. Temperature and salinity from field sampling

Table 8.1 Temperature and salinity from field sampling. The temperature measurement was taken from two different locations within the sampling area.

Date	Depth (cm)	Temperature (location 1)	Temperature (location 2)	Salinity
23.okt	15	8,2		30
23.okt	10	7,4		
23.okt	5	6,2		
23.okt	0			
10.nov	20	5	4,8	
10.nov	15	4,4	4,5	
10.nov	10	3,9	4,2	
10.nov	5	3,6	3,8	
10.nov	0	3,1	3,1	
25.nov	20			
25.nov	15			
25.nov	10			
25.nov	5			
25.nov	0			
09.dec	20	5	4,8	
09.dec	15	4,4	4,5	
09.dec	10	3,9	4,2	
09.dec	5	3,6	3,8	
09.dec	0	3,1	3,1	
26.dec	25	3,3	3,4	
26.dec	20	3,1	3,1	
26.dec	15	2,7	2,7	
26.dec	10	2,4	2,6	
26.dec	5	2	2,1	
26.dec	0	1	1,4	
06.jan	20	2,8	2,7	30
06.jan	15	2,1	1,7	
06.jan	10	0,4	0,7	
06.jan	5	-0,5	-0,8	
06.jan	0	-0,1	-1,1	
22.jan	20	0	0,3	
22.jan	15	-0,5	-0,4	
22.jan	10	-0,9	-0,6	
22.jan	5	-1,5	-1,3	
22.jan	0	-4,3	-3,9	
06.feb	25	2,2	2	
06.feb	20	2,1	1,8	
06.feb	15	2	1,8	

06.feb	10	1,8	1,6
06.feb	5	1,4	1,2
06.feb	0	0,2	0,3
22.feb	25	1,7	1,7
22.feb	20	1,6	1,6
22.feb	15	1,6	1,6
22.feb	10	1,7	1,6
22.feb	5	1,9	1,8
22.feb	0	2,1	2

Appendix 2. Mortality in the experimental treatments

Table 8.2 Mortality in the given tanks throughout the experiment for the two experimental treatments. The initial number of polychaetes per tank was eight (in total 160 polychaetes per treatment).

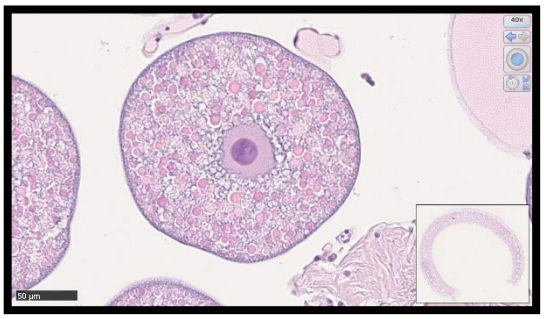
Tank	Natural	Speed-up
1	2	1
2	1	
3		
4		1
5	1	2
6		
7		
8	2	
9	8	1
10	8	1
11	2	
12		
13	8	
14		
15		
16		1
17		
18	8	
19		
20		
Total	40	7

Appendix 3. Hematoxylin and Eosin staining protocol

- Place slides containing paraffin sections in a slide holder
- Deparaffinise and rehydrate sections

TissueClear		5 min	
TissueClear		5 min	
TissueClear		5 min	
100 % ethanol		2 min	
100% ethanol		2 min	
96 % ethanol		2 min	
70 % ethanol		2-3 min	
Distilled H_2O		5 min	
Mayers Hematoxylin		3 min	(2-5 min)
Tap water	running	3 min	(to allow stain to develop)
1% HCl in 70 % ethanol		Dip 5x (fast)	(to destain)
(acid alcohol 4 ml 6M HCl + 200 ml 70 % ethanol)			
Tap water	running	3 min	
0,5% eosin		2 min	
Tap water		dip	
Distilled H_2O		dip	

Air dry or proceed to dehydration.



<u>Dehydration:</u>

70 % ethanol	dip
100 % ethanol	30 sek
100 % ethanol	2 x 2 min
Tissue Clear	5 min
Tissue Clear	5 min
Tissue Clear	5 min

- Coverslip slides using Neo-Mount® (Merck KGaA, Darmstadt, Germany)
- Angle the coverslip and let it fall gently onto the side. Allow the mounting medium to spread beneath the coverslip, covering all tissue.

Coloration:

- Nucleus – blue
- Cytoplasm, connective tissue, muscle etc – varying shades of pink

Appendix 4. Omitted females

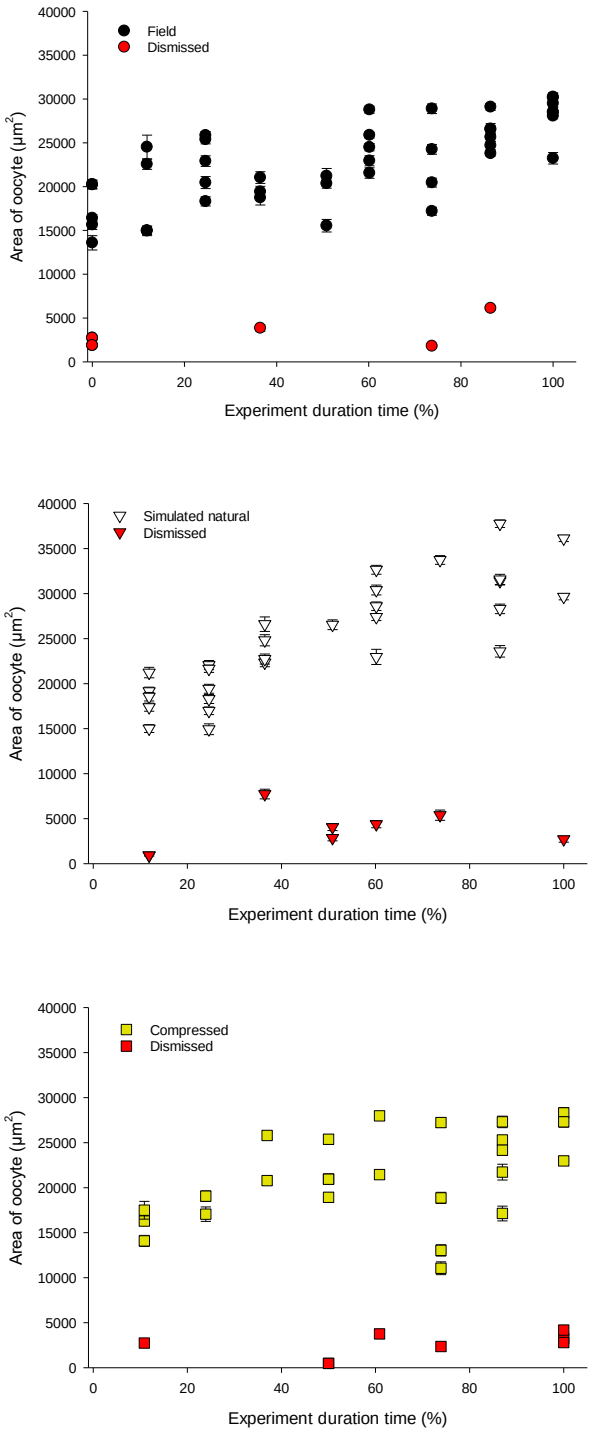


Figure 8.1 Mean area of the oocytes per individual for the three different treatment groups (field, simulated natural and compressed). Sampling size varies from 1 to 7 polychaetes, with ≤ 50 oocytes measured per polychaete. The red dots mark the individuals that had a mean oocyte size of 40 % less than the average oocyte size from the individual sampling days in the different treatment groups. All the polychaetes marked with red dots were taken out of the experiment. Standard error was represented by error bars (±).