Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biology

Tora Lillebjerka

Investigating development, incubation and dietary effects in egg and veliger larvae of *Littorina littorea* (Linnaeus, 1758)

Master's thesis in Ocean reources
Supervisor: Elin Kjørsvik
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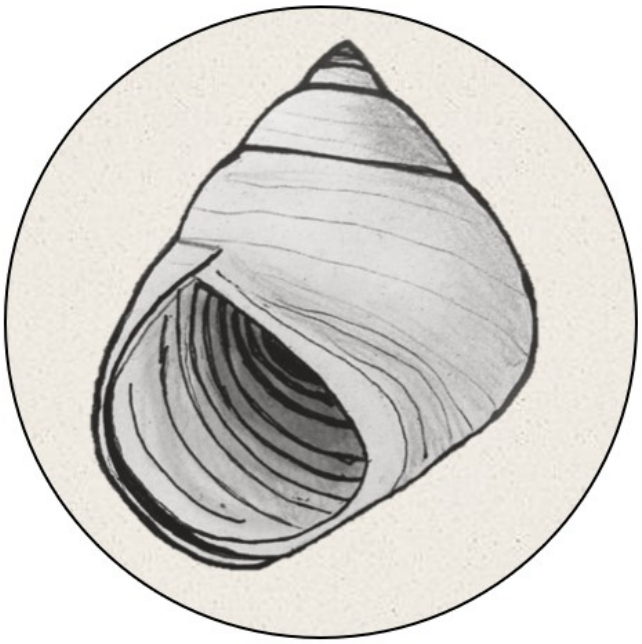
November 2022



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ABSTRACT

Despite being a mostly unrecognized delicacy in the Norwegian market, common periwinkles (*Littorina littorea*) are handpicked, exported, and consumed in many European countries. The present study has demonstrated some of the previously unknown factors affecting the early life stages of *L. littorea*, with a focus on incubation of eggs and nutrition in veliger larvae. The embryonal development increased with higher temperatures and the incubation temperature influences the size and development of the hatching embryos, as abnormal development was observed below 9 ℃ and the largest shell lengths found in hatching larva incubated at 11.3-16.5℃. An effect of salinity was found, as embryos in 20 ppt had significantly lower hatching success than embryos in salinities 25-40 ppt. There was not found any interaction between salinity and temperature when it comes to hatching success, but it is clear that both factors influence the development. The dietary preferences of the larvae were the two live algae *Rhodomonas baltica* and *Dunaliella tertiolecta*, with which the larvae were able to develop fully into settlement. These results are promising for the future cultivation of the species.

SAMMENDRAG

Vanlig Strandsnegl (*Littorina littorea*) er en lite utnyttet delikatesse i Norge, men arten sankes, eksporteres og konsumeres i mange europeiske land. Denne studien har undersøkt noen av de tidligere ukjente faktorene som påvirker de tidlige livsstadiene til *L. littorea*, med fokus på inkubasjon av egg og ernæring i veligerlarver. Den embryoniske utviklingen akselererte ved høyere temperaturer, og inkubasjonstemperaturen påvirket både størrelse og utvikling av embryoene. Irregulær utvikling ble observert ved temperaturer under 9°C og de lengste skall-lengdene ble funnet i embryo inkubert i 11,3-16,5°C. Salinitet hadde også en effekt og embryo i 20 ppt hadde signifikant lavere klekkerate enn embryo i saliniteter 25-40 ppt. Det ble ikke funnet en interaksjon mellom salinitet og temperatur for klekkerate, men det er tydelig at begge faktorene påvirker utvikling hos sneglene. Startfôring av larvene var en suksess med de to levende algene *Rhodomonas baltica* og *Dunaliella tertiolecta*, hvor larvene vokste til bunnslåing. Resultatene fra dette forsøket er lovende for fremtidig kultivering av arten.

CONTENT

1 INTRODUCTION

1.1 AN INTRODUCTION TO SNAIL AQUACULTURE

The human population is growing, and consequently the dependence on production of ocean-derived food increase. In 2020 the global production of aquatic animals (both aquaculture and fisheries) was estimated at 178 million tonnes and is expected to expand to over 200 million tonnes by 2030 (FAO, 2022). Norwegian fisheries and aquaculture yielded 4 million tonnes of aquatic animals in 2018, of which most is exported worldwide (OECD, 2021). The Norwegian aquaculture today is heavily dominated by salmonids, but new species are constantly evaluated (Havforskningsinstituttet, 2018; NIVA, 2019). Lower trophic species of marine algae, crustaceans and molluscs could be an important step in increasing future aquaculture (FAO, 2022). Despite making up 14.5% of the global aquatic production (FAO, 2022), molluscs have a low production in Norway with a yearly production of around 2200 tonnes and blue mussels being the dominating species (Fiskeridirektoratet, 2021a, 2021b; Havforskningsinstituttet, 2018).

The periwinkle species *L. littorea* has a cultural significance in several areas around the world (Cummins, 2002; Ghosh et al., 2018), including being an important food source in early Norwegian times (Hood & Melsæther, 2016). France is an important importer of periwinkles with sales peaking at Christmas due to French meal traditions (Jon Eirik Brennvall, personal conversation, June 15. 2021)(Cummins, 2002). Today, periwinkles are handpicked from the littoral zone before being exported live (Cummins, 2002). Snail farming is not a common practice in Norway today, however it is not a new idea on the global marked as both aquatic and terrestrial snails are recognized as a healthy food source in various areas across the world (Ghosh et al., 2016; Ghosh et al., 2018, 2022; Venugopal & Gopakumar, 2017; Yildirim et al., 2004; Zarai et al., 2011). As the snails are a low trophic species, this also opens the door for IMTA-RAS (Integrated multi-trophic aquaculture, Recirculating aquaculture systems) production of the species, potentially tied to the Norwegian salmon industry (Vlottes, 2022).

L. littorea is native to the north-eastern Atlantic (Blakeslee et al., 2021), where it is recognized as a key stone species in the marine coastal ecosystem (Lubchenco, 1978). It is an important algal grazer in the intertidal sone (Castro & Huber, 2016) which influences the coastal plant community and subsequently other animal species who relies on algae for food, shelter and/or substrate(Johnson & Mcdermott, 2018; Lubchenco, 1978). The species is also an important intermediate host for several parasitic trematodes (Granovitch, 2017; Stunkard, 1930) and are involved in interactions with other epibionts and polychaeta (Thieltges & Buschbaum, 2007). Although *L. littorea* has a varied diet the species show a strong preference for the two green algae *Ulva lactuca* (L.) and *Enteromorpha intestinalis* (L.) (Peckol et al., 2017; Watson & Norton, 1985).

1.2 BIOLOGY OF LITTORINA LITTOREA

Snails make up the largest part of the Gastropoda class, which also consist of limpets, abalones and nudibranchs (Castro & Huber, 2008). They have a coiled mass of organs which is enclosed by a , usually coiled, dorsal shell (Figure 1A) (Castro & Huber, 2008). They are all ectotherm (Segal, 1961) and have a foot and produce a mucus trail which they use to move around (Iwamoto et al., 2014). Most gastropods, including *L. littorea*, feed on algae scraped from rocks using their radula, while others are deposit feeders feeding on soft bottoms or carnivores preying on shellfish or small fish (Castro & Huber, 2008). The Littorina genus is diverse and globally distributed to all climactic sones (Reid, 1989) and *L. littorea* is one of eight species found in northern Europe (Sneli & Van Marion, 1979).

L. littorea is found in the littoral sone with a preference to rocky shores and stable substrates (Figure 1B)(Fretter & Graham, 1980), and due to living in the intertidal zone, the species is well adapted to both freezing temperatures and anoxic conditions (English & Storey, 2003; Miller & Denny, 2011) They prefer semi-exposed to sheltered coasts, but are also tolerant to estuarine conditions and great exposure (Petraitis, 2002). The snails can reach up to 53 mm in shell height and 20 years of age and mature when the shell height is approximately 11 mm or around 12-18 months after settlement (D. & C.A., 1998; Fish, 1972; Fretter & Graham, 1980; Williams, 1964). Egg production is related to the size of the snail, as larger individuals produce more eggs (Chase & Thomas, 1995). The snails have internal fertilization, and naturally spawn from January until June (Moore, 1937; Williams, 1964). They are rhythmical spawners and spawn several times each season, with a total egg number estimated to over 100 000 per female (Chase & Thomas, 1995; Grahame, 1975), and the spawning is known to be influenced by lunar cycles (Alifierakis & Berry, 1980; Grahame, 1975) and temperature (Chase & Thomas, 1995).

Figure 1: A) Adult *L. littorea* showing the coiled shell, head, antenna, and foot (Nozères, 2012). B) Cluster of *L. littorea* attached to a rocky substrate (Hiscock, 2008).

1.3 EGG AND LARVAE DEVELOPMENT

1.3.1 Descriptive development of L. littorea

While embryonic development in marine gastropods have been described for over a century, most available information on *L. littorea* is old and contains few specifics. The snails spawn individual pelagic capsules (Reid, 1989) which commonly contains one to three eggs (Thorson, 1946) but can contain up to nine (Lebour, 1935; Linke, 1933). The outer capsule is about 1 mm and the egg envelope is 205 µm (Lebour, 1937). Molluscs embryos commonly develop into a trochophore (Kawano et al., 2004) before developing into veliger larvae, which have velums (often referred to as lobes) used for swimming and collecting food particles (Lebour, 1937; Moran, 1999). The velum has long cilia along the edges, and the larvae of *L. littorea* is known for their distinctive purple spots on the velum (Lebour, 1935; Lebour, 1937).

Tactics for egg and larvae nutrition can differ between species. In *L. littorea* the larvae are planktotrophic, meaning they have little yolk and are dependent on feeding on plankton to develop to metamorphosis (Jablonski & Lutz, 1983; Mileikovsky, 1971; Thorson, 1950). Producing eggs with little yolk have a low parental cost, and the snails have an r-selected parental strategy where they produce a high number of eggs to combat the high larval mortality (>99%) (Jablonski & Lutz, 1983; Mileikovsky, 1971; Thorson, 1950). As the larvae are not dependent on a limited yolk supply, they are also able to have a longer larval period and can disperse over large areas using the ocean currents (Jablonski & Lutz, 1983; Thorson, 1950). The larvae differs from the adults by having large velums used for swimming, collection of feed particles (Riisgård & Larsen, 2010) and even encapsulated feeding when inside the egg (Moran, 1999) which devolve during metamorphosis to adult snails (Branco et al., 2014)

1.3.2 Temperature, salinity, and development time

The exact time until hatch in *L. littorea* is not known, but earlier research have estimated six to seven weeks (Cummins, 2002). However, as snails are ectotherm the embryonic development is temperature dependent and should be expressed as a function of both time and temperature (Formula 1).

$$Daydegree = t_{days} * \text{ °C} \qquad \text{[Formula 1]}$$

Similar temperature patterns are observed in other mollusc species, where the time until hatch decreases with increasing temperatures (Bashevkin & Pechenik, 2015; Costello et al., 1957; Doxa et al., 2021; García de Severeyn et al., 2000; Hamzah et al., 2021). Whether the increased growth rates affect the embryonal size or number of deformations in *L. littorea* is not known, and previous studies on gastropods show conflicting results. Collin

& Salazar (2010) found decreasing size of larvae with increased temperature, while other studies showed decreased size and/or increased deformation rates in cold water (Doxa et al., 2021). Many of these species are produced in warmer water (>20°C) and may not provide a sufficient model for a cold-water species like *L. littorea* from Norwegian populations. Salinity is also known to be an important abiotic factor which influences the development of marine embryos. It is generally known that a lower salinity down to a threshold might increase growth in marine organisms, due to less osmotic stress. However, a lot of variation is seen between species and populations when it comes to optimum salinities for growth. Interactions between temperature and salinity are also important, as increased stress from too high or too low temperatures can lower an individual's tolerance for non-optimal salinities and vice-versa. Including salinity in hatching experiments will thus give a larger picture when it comes to optimalisation for aquacultural production.

1.4 FEEDING AND KEEPING VELIGER LARVAE

There is little information regarding the natural diet of invertebrate larvae available, but many planktonic larvae might be omnivores, grazing on both phytoplankton and small heterotrophic plankton (Vargas et al., 2006). The larvae uses their cilia to transport the food to the food grove and into the mouth while swimming in the water column (Riisgård & Larsen, 2010; Romero et al., 2010), possible using chemoreceptors to detect edibility (Tamburri & Zimmer‐Faust, 1996). Despite having a naturally varied diet in nature most invertebrates are reared using microalgae diet in research or aquaculture setting. Microalgae are good sources for long chain polyunsaturated fatty acids (LC-PUFA) (Khozin-Goldberg et al., 2011; Patil et al., 2005; Santos-Sanchez et al., 2016), which is important for marine molluscs (Pernet & Tremblay, 2004), especially EPA and DHA (Aarab et al., 2013). The bacterial cultures in the water may also contribute to larval nutrition, possibly reducing the effect of a non-optimal diet (Robert & Trintignac, 1997). However, as most bacteria does not contain polyunsaturated fatty acids or sterols, which are considered very important in bivalve (molluscan) diets, microalgae should be considered the most important portion of the diet (Robert & Trintignac, 1997). Several different algal species have been tested as a diet for a variation of gastropods (Aldana-Aranda & Suarez, 1998; Dang et al., 2011; Mai et al., 1996; Robert & Trintignac, 1997) but little is known about the feed preference in *L. littorea* larvae.

Rhodomonas baltica is an alga commonly used in aquaculture, with its balanced nutritional profile as its rich in EPA and DHA (Klein Breteler et al., 1999; Yamamoto et al., 2015). The algae is used in larval production in other marine species, and have shown good growth for instance in bivalves (Aldana-Aranda & Suarez, 1998; Gagne et al., 2010), sea urchins (Araujo et al., 2020), sea cucumbers (Yamamoto et al., 2015), copepods (Arndt & Sommer, 2014; Koski & Breteler, 2003) and artemia (Albuquerque et al., 2005). The alga is not commonly used in production of gastropod larvae but may have good potential due to its balanced nutritional profile. The cells are ~7.5 µm and have an oval shape

(Støttrup & Jensen, 1990) and the cells contain approximately 36 pg C cell^{-1} (Koski & Breteler, 2003; Støttrup & Jensen, 1990; Thomas et al., 1985).

Dunaliella tertiolecta is also often used in aquaculture (Patil et al., 2005). However, it has a less balanced nutritional profile, and lacks both EPA and DHA, two important fatty acids for marine life (Klein Breteler et al., 1999; Nevejan et al., 2003). It does however contain very high quantities of the PUFA 18:3(ω3) and adequate amounts of amino acids (Klein Breteler et al., 1999). This nutrient balance makes the algae less desirable for rearing animals which have high demands for PUFA lipids in their diet. However, the demand for these nutrients varies between species, and is not known for *L. littorea*. While species of conch (Aldana-Aranda & Suarez, 1998; Pillsbury, 1985) and copepods (Koski & Breteler, 2003) show little growth and high mortality from D*. tertiolecta*, other species of copepod (Koski & Breteler, 2003), sea cucumber (Tan et al., 2017) and gastropods perform well (Pechenik & Tyrell, 2015). The cells are 7 µm (Støttrup & Jensen, 1990), and publications on carbon content vary between 14.9 pg C cell^{-1} (Støttrup & Jensen, 1990) and 27 pg C cell^{-1} (Koski & Breteler, 2003).

Nannochloropsis is a large group of algae commonly used in rearing live feed for fish production. The cell size is smaller than *R. baltica* and *D. tertiolecta* at around 3-5 µm (Baroni et al., 2019; Hibberd, 1980; Nell & O'Connor, 1991; Rodríguez‐Pesantes et al., 2020). The alga is rich on EPA but does not contain any DHA (Lubzens et al., 1995; Ma et al., 2016; Rodríguez‐Pesantes et al., 2020; Sukenik et al., 1993). Several species of *Nannochloropsis* are used successfully as a diet when rearing rotifers and have also been used in other mollusc larvae both as the main diet and in combination other algae (Aranda et al., 2020; Aranda et al., 2021; Noble et al., 2015). However, as it lacks essential fatty acids like DHA, it might not be suitable for all aquatic larvae and performed poorly as a diet for rock oysters (Rodríguez‐Pesantes et al., 2020).

To create a more balanced nutrient profile of algae, many producers choose to use a combination of multiple alga types. One example is Rotifer diet ™, which is a combination of *Nannochloropsis* and *Tetraselmis* algae. *Tetraselmis* is a common algae used in mollusc aquaculture, with a cell size around 9 µm (Rodríguez‐Pesantes et al., 2020) but a poor fatty acid profile, with no DHA and little to no EPA (González‐Araya & Robert, 2018; Rodríguez‐Pesantes et al., 2020) *Tetraselmis* sp. has been used in rearing conch (Brito-Manzano & Aranda, 2004), but the use of a single species produced high mortality and mediocre growth in bivalves (oysters)(González‐Araya & Robert, 2018) and sea cucumbers (Tan et al., 2017). Therefore, marine larvae might benefit from being paired with another algal group, which we find in the commercial algae paste Rotifer diet from Instant algae.

Using live microalgae as a feed source in aquaculture require a lot of resources as they need to be continuously cultured (Coutteau & Sorgeloos, 1992). Algal pastes may be used as a cheaper option, as they allow for nutritious algae to be stored for long periods (Robert

& Trintignac, 1997). Production of microalgal pastes often influence the nutrients in the algae cells, both lipid content and amino acids (Chini Zittelli et al., 1999; Nell & O'Connor, 1991; Rayner & Hansen, 2019). However, the pastes are still a viable alternative to live algae, and have shown good results when being used for food organisms like rotifers (de la Cruz-Huervana et al., 2022) and reasonable results in copepods (Rayner & Hansen, 2019). Algal paste has been tested on bivalves (Heasman et al., 2000; Nell & O'Connor, 1991; Ponis et al., 2003) with mixed results, and is also used in green water techniques (Sales et al., 2016). However, the product has not been extensively tested in gastropod veliger larvae, and not on *L. littorea*.

1.4.1 Briefly on settlement

As the larvae grow towards the end of their larvae stage, they metamorphose and settle on the bottom. While younger larvae crowd at the surface, older larvae in many species gather near the bottom (Thorson, 1946). The larvae might also use turbulence to transport them down to the bottom substrate (Fuchs et al., 2004). While the mechanisms behind the settlement is mostly unknown, larvae have been observed reluctant to settle until presented with subtidal rocks and associated biota (Strathmann & Strathmann, 2007)It is also theorized that the settlement and early juvenile phase that have the highest mortality rates in the young snails (Jablonski & Lutz, 1983).

1.5 PLAUSIBILITY OF STARTING AQUACULTURE AND CURRENT KNOWLEDGE GAPS

Despite the ecological importance of this species, there is limited regulation on harvesting making estimation of the harvested biomass difficult (Johnson & Mcdermott, 2018). The populations of *L. littorea* in northern Europe are generally considered sustainable, and little focus have been put on its conservation and stock monitoring (Johnson & Mcdermott, 2018). As the species have a long lifespan, and increased fertility with increased age (Chaparro et al., 2019; Hohenlohe, 2002) the current harvesting with focus on larger individuals makes the species more prone to over-exploitation and possibly selection towards smaller individuals over time (Doyle et al., 2022; O'Dea et al., 2014). *L. littorea* lives in shallow waters along the coast, which is a very accessible habitat for collection, making harvesting of the species relatively easy. Other threats to the populations have appeared as well as they have proven sensitive to certain chemical releases (Rank, 2009; Van den Broeck et al., 2009) and may bioaccumulate both heavy metals (De Wolf et al., 2000; Howard & Nickless, 1978) and microplastics (Gutow et al., 2019). The species may also be sensitive to temperature, thus is also threatened by climate change and rising sea temperatures (Cardoso et al., 2017). The increase of aquaculture of other marine species (e.g. salmonids) along the Norwegian coast might

also increase parasitic pressure on the species from parasites with an indirect life cycle, using both *L. littorea* and fish as intermediate hosts (Kristoffersen, 1991).

To relieve pressure on the wild stocks of *L. littorea* and create a long-term sustainable industry it is important to investigate the potential of aquaculture on the species. While some is known regarding the diet preference, habitat, and growth in adult snails there is still lack of knowledge surrounding the reproduction and early life stages of the snail. In order to develop large-scale production of *L. littorea* it is necessary to increase the knowledge surrounding egg incubation, temperature, salinity, and larvae nutrition which can be common bottlenecks in marine aquaculture.

1.6 AIM OF THE STUDY

The main purpose of this study was to increase knowledge on the egg and larvae stages of the common periwinkle *L. littorea*, as little is known about the early life stages of the snail. To achieve this the main purpose was divided into two aims:

1. Provide a description of embryonic development in *L. littorea* and evaluate the influence of temperature and salinity during development.
2. Evaluate dietary effects on growth in veliger larvae fed different diets until settlement.

Eggs were spawned and reared until hatch in the laboratory, using a temperature apparatus to create a temperature gradient. Embryo were also incubated in different temperature-salinity combinations to provide data on hatching rates and produce optimal ranges for incubation. Hatched larvae were fed four different alga types until settlement/starvation and growth effects were determined using shell length (SL).

The following was hypothesized:

1. Embryonal developmental rates will increase with higher temperatures but result in more developmental defects.
2. Salinity, and salinity in interaction with temperature, will affect the embryonal development.
3. Developmental rates/growth rates are dependent on nutritional quality of larval diet

2 MATERIALS AND METHODS

The experiments for this thesis were conducted at SINTEF Ocean (SeaLab) during spring 2020, due to the snails natural spawning period from January to June (Moore, 1937; Williams, 1964). The outbreak of Covid-19 caused the experimental period to be postponed and it lasted from 15.05.20 to 01.08.20. During this period, five experiments were conducted to investigate the egg and larvae life stages of the snails (Table 1).

Table 1: General overview of the different experimental sections conducted. All experiments were conducted in 2020, between week 20 and 32.

Experiment		**Duration** (week number)	**Treatments**
1A	General description of the embryonic development and egg morphometrics	2 weeks (20-22)	10 different temperatures for egg incubation with one replica per temperature. Sampled every 15 daydegrees.
1B	Effect of temperature on time until hatch and larvae size	2 weeks (20-22)	10 different temperatures with three replicas for egg incubation. Sampled after hatch at 110 daydegrees.
2	Effect of temperature and salinity on hatching rates	2 weeks (24-26)	Five salinities and five temperatures for egg incubation combined to create 25 treatments. Daily monitoring.
3	Effect of microalgal feed on growth and development of veliger larvae	3 weeks (21-24)	Newly hatched larvae reared on four different feed treatments to investigate growth given by shell length.
4	Larval preference for settlement substrate	6 weeks (25-32)	Settling larvae kept in tanks containing different substrates. Substrates were monitored daily but low observations led to the experiment being cancelled.

2.1 KEEPING THE ADULTS

The snails were gathered in Ørland county in shallow coastal water during early May 2020. During transportation to the lab the snails were kept out of water before being added to large flow-through tanks with aeration keeping approximately 10° C and a light regime of 8/16 (light/dark) upon arrival. All the seawater used in the experiment, including to keep the adults from the Trondheim fjord (collected at 70 m) which were

filtered through a sand filter (20 µm) and a protein skimmer. Snails were continuously fed using a mix of kelp types collected at the same time, including sugar kelp (*Saccharina lattissima*), egg wrack (*Ascophyllum nodosum*), and sea lettuce (*Ulva lactuca*). The shell height was measured using callipers (Figure 2) and the shell height varied between 25 and 34 mm. To avoid potential influence of female size (Chaparro et al., 2019; Collin & Salazar, 2010) a smaller selection of females (26-31 mm) was used in the experiments. These snails served as a brooding stock and supplied eggs for the different experiments.

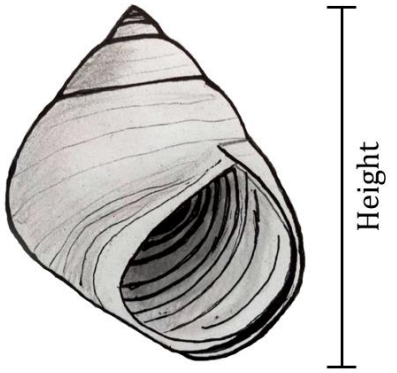

Figure 2: Measuring the height of snail shells was done from the top (apex) and to the lowest section of the aperture when held vertically. Illustration: Tora Lillebjerka

To produce eggs adult snails (~30 - 50) were moved to a smaller tank (10 l plastic bucket) with filtered seawater over night. Estimated time of fertilization and thus beginning of daydegrees were estimated to midnight for all experiments. The water temperature was ~16°C and 24 h light. It was assumed that all breeding containers had an even mix of males and females during the experiments. Egg collection was done four different times during the experimental period, with the only exemption being for Experiment 1B, where the adults were added directly into small glass beakers to spawn.

2.2 Experiment 1A: Effect of temperature on embryonic development and hatching success

Experimental design

To study the effect of temperature on snail juvenile development, eggs were incubated at 10 different temperatures until hatch and monitored every 15 daydegrees. Newly spawned eggs were retrieved from the adult population (2.1 above) and distributed into 10 glass beakers (750 mL) which were placed at 10 different temperatures in a

temperature gradient apparatus (Thomas et al., 1963) (Figure 3) with a density of 10 – 15 egg/mL. Initial temperatures of the apparatus spanned from 7.7 ± 0.0 to 15.4 ± 0.1 and the temperatures were then adjusted gradually over 24 hours to allow the eggs to acclimatize. The final temperatures were 4.8 (± 0.4), 6.9 (±0.3), 9.1 (±0.3), 11.3 (±0.2), 13.7 (± 0.2), 16.0 (±0.2), 17.3 (±0.2), 19.1 (± 0.2), 20.9 (± 0.2) and 22.8 (±0.2) which was kept throughout the experiment. The beakers were sampled every 15 daydegree by gently stirring the water with a spoon to resuspend the sedimented eggs before removing 2 mL of water from the centre of the beaker. The eggs in the sample were counted, photographed, and prepared for fixation (Method described in 2.6 below). When approximately 50% of the eggs had hatched, a larger sample (100-300 eggs and larvae) were removed and fixated as well.

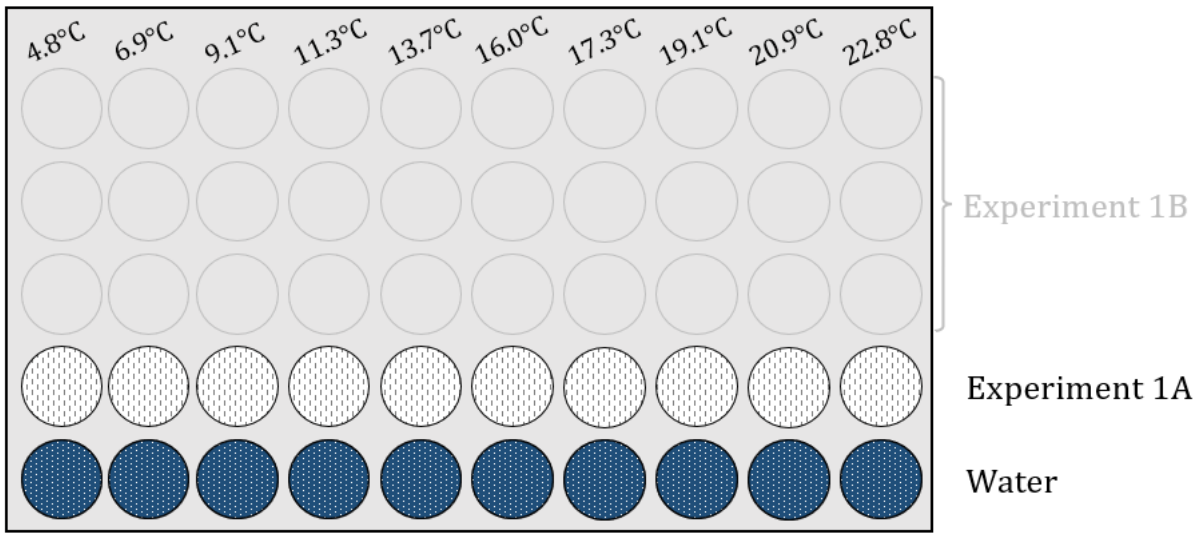

Figure 3: Temperature gradient apparatus shown from above. Coldest temperatures to the left, and warmest to the right, with a natural gradient created from the aluminium apparatus. Exact temperatures shown at the top row. The three rows at the top were used for Experiment 1B, and the second to bottom was used for in the present experiment. The bottom row was used for acclimating water for water exchange. Illustration: Tora Lillebjerka

Water conditions

In addition to temperature monitoring, salinity, pH, and oxygen were measured daily (YSI ProDSS Multi-Parameter Water Quality Meter) (Appendix 1). Due to an error during water exchange, the salinity in the 9.1 °C treatment was decreased to 23.99 on day five of the experiment. This was noticed on day six, and the water exchange was increased the following days to allow the salinity to normalize without shocking the eggs. The salinity was increased above 30 ppt in 48 hours and were normal (>34 ppt) after 72 hours.

2.3 EXPERIMENT 1B: EFFECT OF TEMPERATURE ON LARVAE SIZE

Experimental design

To evaluate the effect of incubation temperature during spawning, eggs were spawned and incubated in beakers in the temperature gradient apparatus (Figure 3). The experiment used 10 different temperatures and three replicas per temperature, totalling in 30 glass beakers (750 mL). Each beaker was filled with filtered seawater and six adult snails. The snails were also *fed U. lactuca* to avoid food availability affecting spawning. The adult snails were added directly into the water, which kept around 10 °C. The apparatus was then started and changed the water temperatures to range between 6.6 and 19.5 °C after a couple hours. The snails were then left over night to spawn. After approximately 24 hours the snails were removed from the beakers, and a small water sample was removed from each beaker to check for eggs. As all the beakers contained eggs, the largest pieced of debris was removed from the water while the eggs remained for incubation. The temperature was readjusted were 4.8 (± 0.4), 6.9 (±0.3), 9.1 (±0.3), 11.3 (±0.2), 13.7 (± 0.2), 16.0 (±0.2), 17.3 (±0.2), 19.1 (± 0.2), 20.9 (± 0.2) and 22.8 (±0.2) which were kept throughout the experiment. The eggs were incubated for 110-120 daydegrees until the majority of the larvae had hatched, when the water was sieved, and a large sample of larvae and eggs (50-500) were removed using a spatula. The eggs and larvae were then fixated in PFA (Method described in 2.6 below).

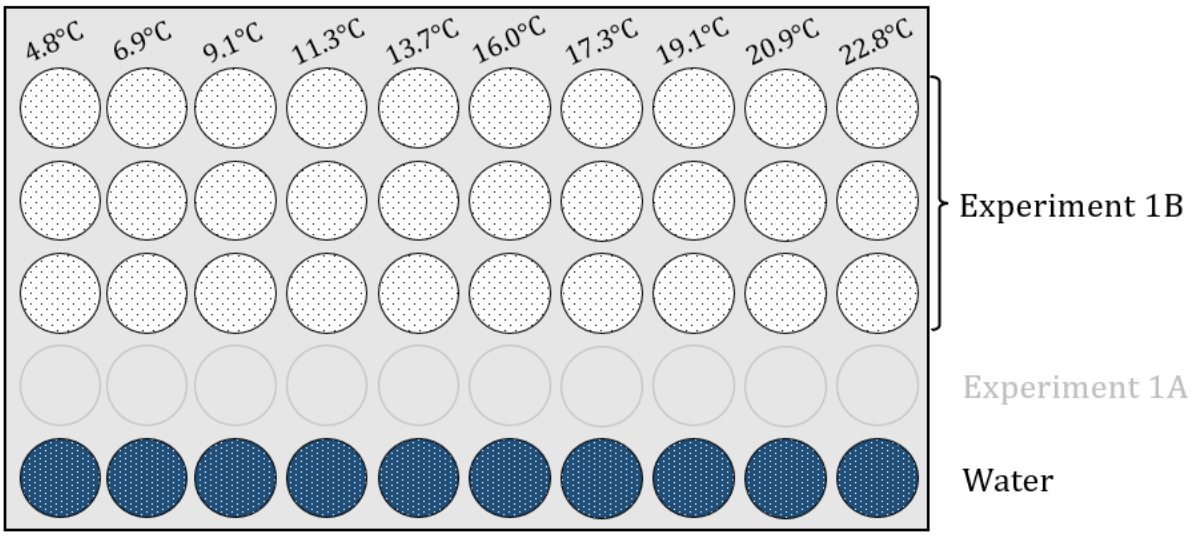

Figure 4: Temperature gradient apparatus from above. Coldest temperatures to the left, and warmest to the right, with a natural gradient created from the aluminium apparatus. Exact temperatures shown at the top row. The three rows at the top were used for Experiment 1A, and the bottom row was used for acclimating water for water exchange. Illustration: Tora Lillebjerka

Water conditions

Salinity, oxygen, temperature, and pH were monitored daily (Appendix 1), and approx. 25% of the water was replaced with tempered seawater. Due to an error during water exchange, the salinity in one of the T3 replicas was decreased to 29.99 on day 5 of the experiment. This was noticed on day 6, and the water exchange was increased the following days to allow the salinity to normalize without shocking the eggs. Salinity was 33 ppt after 48 hours and returned to normal (>34 ppt) after 72 hours.

Oxygen was not measured until day 3 due to lack of equipment and when oxygen measurements were taken, they were lower than expected (>58.6 %), probably due to the respiration from the adult snails, as similar levels were not measured in the egg development experiment (2.2 above) Oxygen was increased with the daily water exchange and remained high (> 90%) for the rest of the experiment.

2.4 EXPERIMENT 2: EFFECT OF TEMPERATURE AND SALINITY ON HATCHING RATE

2.4.1 Experimental design

In addition to the development and temperature experiments, the effect of salinity and temperature on hatching rate were investigated. A salinity gradient containing five salinities (20, 25, 30, 35, 40 ppt) were created using sea salt from evaporated seawater, filtered seawater and distilled water. The water mixtures were aerated to ensure sufficient oxygen levels (>100%) before being added to 50 mL transparent cell culture flasks (Greiner bio-one, cellstar ® TC). For each salinity, five culture flasks were filled, creating a total of 25 culture flasks. Newly spawned eggs were retrieved from the adult population (2.1 above), and ~50 eggs were then counted and added into each culture flask (1 egg/mL). The 25 culture flasks were then distributed between five different temperatures on the temperature gradient table (Figure 5) and put into beakers filled with water. The flasks were monitored daily, and hatched larvae were counted and removed from the flask and fixated (Method described in 2.6 below). The experiment was ended at 110 daydegrees, when most of the eggs were expected hatched. The remaining eggs (ranging from 33-93%) were counted and fixated as well.

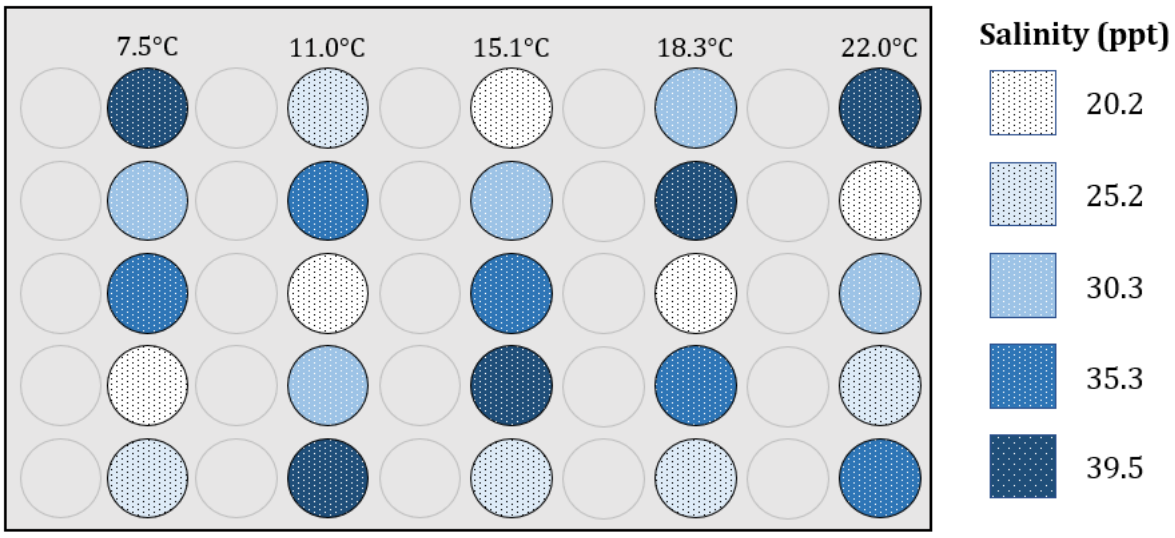

Figure 5: Temperature gradient apparatus seen from above showing the distribution the different salinities between flasks containing *L. littorea* eggs. The coldest temperatures are to the left and placement of flasks are randomised for each temperature interval. Illustration: Tora Lillebjerka.

Water conditions

Temperature was measured daily in the water surrounding the flasks. The water inside the flasks were only measured at the start and end of the experiment, as the flasks were too small for the measuring probe. It was assumed the respiration from 50 eggs would be too low to impact the oxygen content in the water significantly, and when the experiment was ended, the water from each temperature was pooled, and remeasured, to ensure oxygen levels did not sink significantly (>98%) (Appendix 3).

2.5 Experiment 3: Effect of microalgal feed on growth and development of veliger larvae

2.5.1 Experimental setup

Newly spawned eggs were retrieved from the adult population (2.1 above) and incubated until hatch to be used in the larval feeding experiment. The incubation was done in a bucket (10 l, semi-transparent plastic) containing aerated seawater (16° C, 34 ppt, 24h light) for six days (100 daydegrees) until hatch. A small sample was removed to confirm hatch, and then the water was sieved (64 µm) to collect the larvae. The larval density was counted, and the larvae were distributed among 24 50 mL transparent cell culture flasks (Greiner bio-one, CellStar® TC, model 690160) (approx. 500 larvae/flask). The flasks were randomly distributed into four feeding regimes with five replicates and starvation control with four replicates.

The water in the culture flasks were exchanged daily to keep an even availability of microalgal feed. During water exchange, the culture flasks were poured through a filter (initially 64 µm, increased to 90 µm, see below) to remove water and microalgae, while collecting the snail larvae. New clean culture flasks were filled ~30% with filtered seawater and algae feed were added. The larvae were rinsed from the sieve into the culture flask using a small glass funnel. Each culture flask was then filled completely with filtered seawater to avoid air bubbles affecting the turbulence in the water. To keep the algae and eggs from sedimentation, the culture flasks were distributed between two ICES incubators (HYDROBIOS, Kiel, Germany) keeping 18 ± 0.5°C and continuously spinning at a low speed (approximately 5-6 rpm) (Figure 6). The ICES incubators were kept in 24 h light. Five larvae from each flask were removed, photographed, and fixated every fourth day. The experiment lasted until the adults settled, which were defined as after the loss of the velum, despite the development of antennae and a foot at an earlier stage.

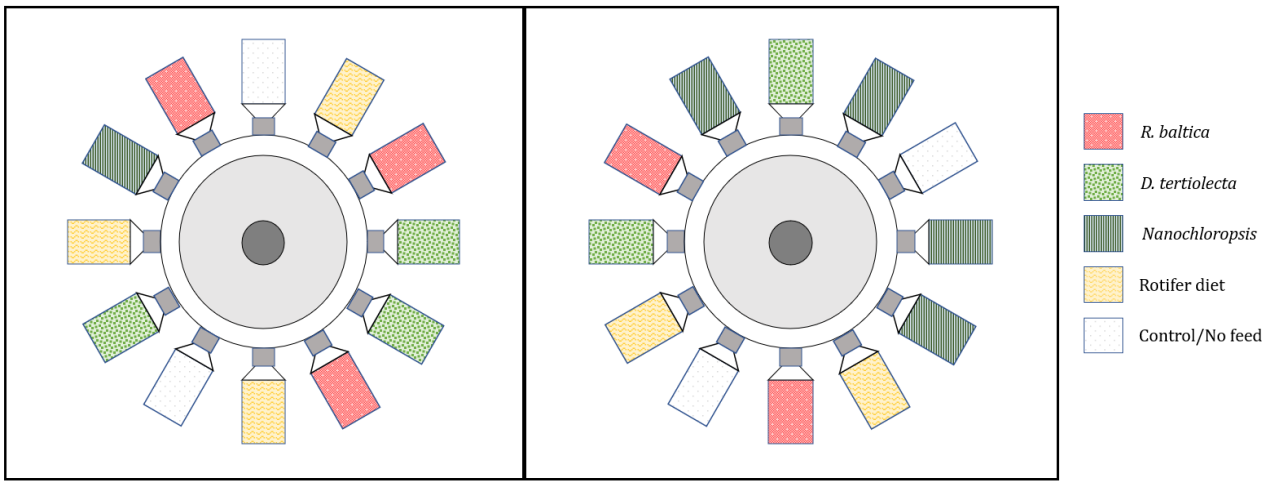

Figure 6: Random distribution of cell culture flasks containing the five different distributed between two ICES incubators.

2.5.2 Feed types and regimes

The first two feed types were *R. baltica* (CCAP 19/27) and *D. tertiolecta* (NIVA 5/91)) which are both live microalgae derived from stock cultures at NTNU and were cultured semi-continuously through the experiment (See 2.5.3). The third feed type was *Nannochloropsis* FrozenPaste (BlueBioTech Gmbh, Germany) which was thawed and kept at 4° C. The fourth feed type was Rotifer diet™ (Instant algae®) which is a mix of *Nannochloropsis* and *Tetraselmis* kept in the freezer (-20 °C).

Preparation of the algae for feeding varied between the treatments. The two live algae were continuously cultivated throughout the experiment. The *Nannochloropsis* paste feed

was prepared by adding a small amount (size of a grain of rice) of the frozen paste (kept at -20°C) to a plastic cup (~1 dl), adding filtered seawater and stirring until the paste dissolved. The final feed type was the Rotifer diet, which were kept in a fridge. The liquid was highly concentrated, and a small amount was added to a plastic cup before filling it up with filtered seawater. Diluted *Nannochloropsis* and rotifer diet pastes were kept in a fridge (4 °C) a maximum of 3 days before being replenished with a fresh mixture. All algae samples were measured using a Coulter counter (Multisizer ™). The samples were diluted (Beckman Coulter™ ISOTON™ II Diluent) 200-400 times to avoid clogging the aperture.

Feed density was adjusted Padilla et al. (2018) where food density was normally 100-200 000 cells/mL. To ensure an even nutrient availability the feed density was based in algal cell size (Table 2). Initial feeding densities were 100 000-200 000 cells/mL, and the density was lowered several times during the experiment (Appendix 2). The feeding density was halved on day 7 to 50 000 and 100 000, and then further reduced day 18, 22 and 23 for the two remaining live algae treatments. The final densities were 30 000 cells/mL for *R. baltica* and 20 000 cells/mL for *D. tertiolecta*.

Table 2: Overview of the cell size, initial density, and final density for the four alga types used in rearing *L. littorea* larvae.

Diet	**Size (µm)**	**Initial density (cells/mL)**	**Final density (cells/mL)**
R. baltica	7.5[a]	100 000	30 000
D. tertiolecta	7[a]	100 000	20 000
Nannochloropsis	3-5[b]	200 000	100 000
Rotifer diet (*Nannochloropsis* and *Tetraselmis)*	3-5[c]	200 000	100 000

a (Støttrup & Jensen, 1990)
b (Baroni et al., 2019; Hibberd, 1980; Nell & O'Connor, 1991; Rodríguez‐Pesantes et al., 2020)
c (Rodríguez‐Pesantes et al., 2020)

The reason for reduction in feeding density was the appearance of unwanted (possibly bacterial or fungal) growth on the snail shells (Figure 7a). The snail shells became covered in algae cells, and several shells could be observed with long, thin sticky threads (Figure 7c). This possibly made all the snails stick together in large lumps (Figure 7b) which could have caused a higher mortality. To avoid further problems the feed amount was reduced and the sieve size during water exchange was increased (from 64 to 90 um). The snail larvae were also thoroughly rinsed with filtered seawater which detached them, but reattachment occurred during the night.

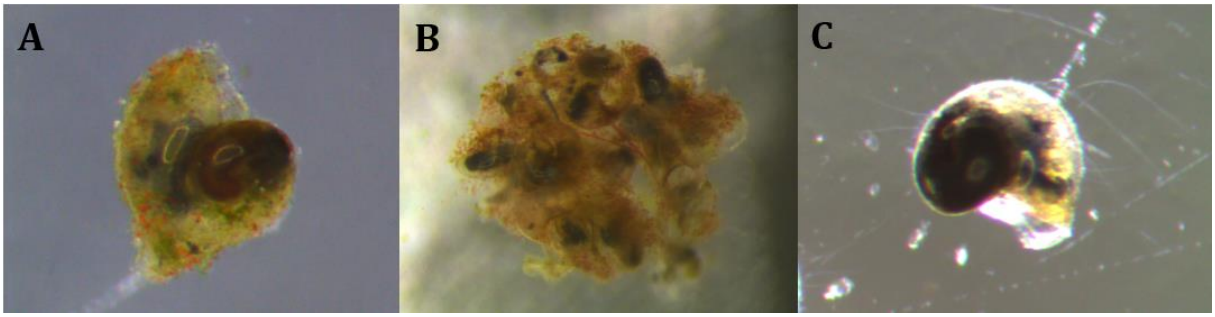

Figure 7: Issues with overfeeding or bacterial growth in the tank led to A) Algae growth on the snail shells, which led to B) clumping of dead and alive individuals together. In addition, there appeared to be some bacterial growth (C) which produced sticky threads. Photo: Tora Lillebjerka.

2.5.3 Cultivation of microalgae for L. Littorina larvae feed

The two algae *R. baltica* and *D. tertiolecta* were cultivated semi continuously in 20 litre glass bottles with a salinity of 34 ppt, a temperature between 20-22° and continuously illuminated (24:0, light intensity 250 µmol m^{-2} sec^{-1} measured between culture flasks). A sample was removed daily to monitor cell density, which was kept between 500 000 and 2 500 000 cells/mL (measured with Coulter counter (Multisizer ™)). A maximum of 50 mL was removed from the cultures daily to be used as feed, and when needed the algae cultured were further diluted by 30-60% to keep down the concentration. All seawater used for microalgae production was chlorinated for 24h using Sodium hypochlorite before being dechlorinated with sodium thiosulphate according to (Hoff & Snell, 1987). Dichlorination lasted for a minimum of five hours before the water was used. New water was added 1 mL of Conwy growth medium (Walne, 1979) per litre when added to the cultures. The cultures were continuously aerated with CO_2 enriched air (1% CO_2).

2.6 MORPHOMETRIC MEASUREMENTS

2.6.1 Preparation of samples and photographing

During all experiments, egg and larvae were fixated in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) with pH 7.4 for storage. The procedure for the eggs meant being transferred to a petri dish, where they were counted and photographed (microscope and camera). Using a pipette, the eggs were then transferred into a 2 mL cryotube were they sedimented and excess seawater were removed from the tube. The tube was then filled with PFA. The larvae were sedated using a 2 g/L mixture of tricaine methanesulfonate (MS-222) which the sample was pipetted directly into, creating an approximately 1:1 dilution of the sedative and water from the larvae flasks. They were then photographed and moved into a 2 mL cryotube using the same method as with the eggs.

Sampling for dry weight was additionally planned once a week but was not continued. To get precise weight reading, the salt needed to be removed from the snails, and they quickly disintegrated in contact with fresh water.

2.6.2 Laboratory work – larvae size from fixated material

Shell length for hatched larvae 110 daydegrees post spawn was measured from fixated material. The larvae were rinsed in phosphate buffer using a sieve (64 µm) and a 12-well cell culture plate to remove fixation fluid (PFA). After rinsing, the sieved material was flushed into a petri dish using phosphate buffer before visual examination in a stereo microscope (Leica M205C). The larvae were separated from other debris in the sample and 30 larvae from each replica were photographed, except for the temperature treatments 4.8 ℃, 6.9 ℃ and 13.7 ℃ where fewer larvae were found, in which case all larvae were used. The shell length was measured using the image processing software ImageJ (Schneider et al., 2012) as described in 2.6.4 below .

2.6.3 Calculation of specific growth rate (SGR)

Specific growth rate (SGR) was calculated using shell length for specific sampling intervals according to the following equation (Houde & Schekter, 1981):

$$SGR = \frac{\ln(L_2) + \ln(L_1)}{t_2 - t_1}$$

Where L_2 and L_1 are individual shell lengths at time t_2 and t_1.

2.6.4 Morphometric measurements for eggs and larvae

Three different lengths were measured on the eggs: The outer diameter, inner capsule and egg envelope diameter (Figure 8). For size measurements, 10-20 eggs from each temperature were measured at approx. 15 daydegree intervals. The eggs were chosen haphazardly, and only eggs with intact capsules and live embryos were measured. The number of eggs inside each capsule was also noted for all the eggs.

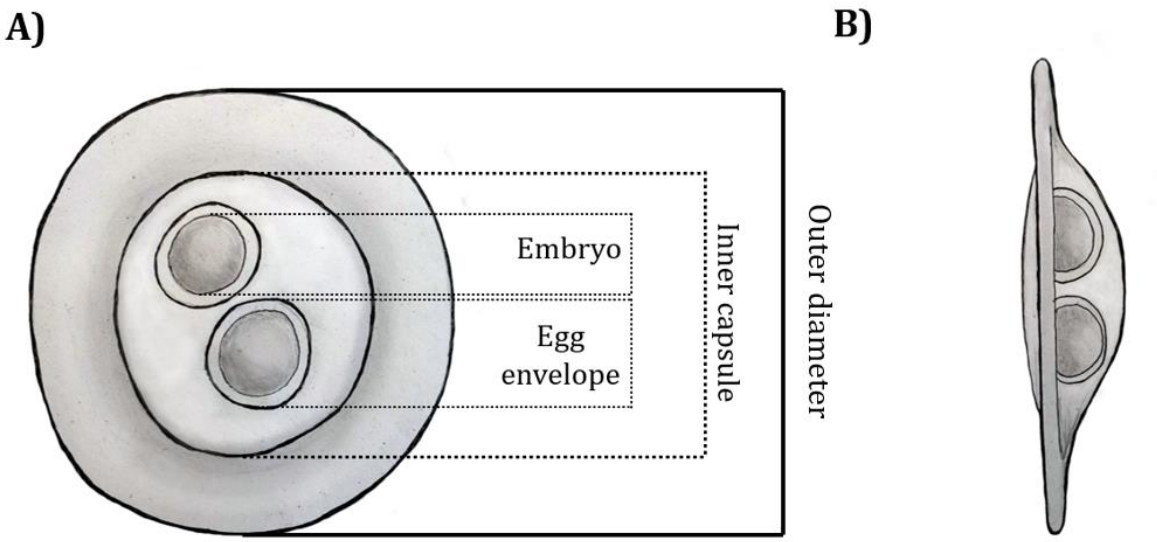

Figure 8: Drawing of *L. littorea* eggs and capsules from A) above and B) from the side. The capsules have a UFO shape, with a fringe at the edge, and an inner capsule cavity where the eggs are placed. Both capsule and egg envelopes are see-through. Illustration: Tora Lillebjerka

Shell length (SL) were used as the indicator of larval growth (Branco et al., 2014; Zehra & Perveen, 1991). Shell lengths was defined as the longest width of the larvae (Figure 9). Newly hatched larvae were measured from the tip of the shell opening (Figure 9a) but as the larvae grew they became more circular, and the widest section of the larvae were no longer from the shell opening tip (Figure 9b).

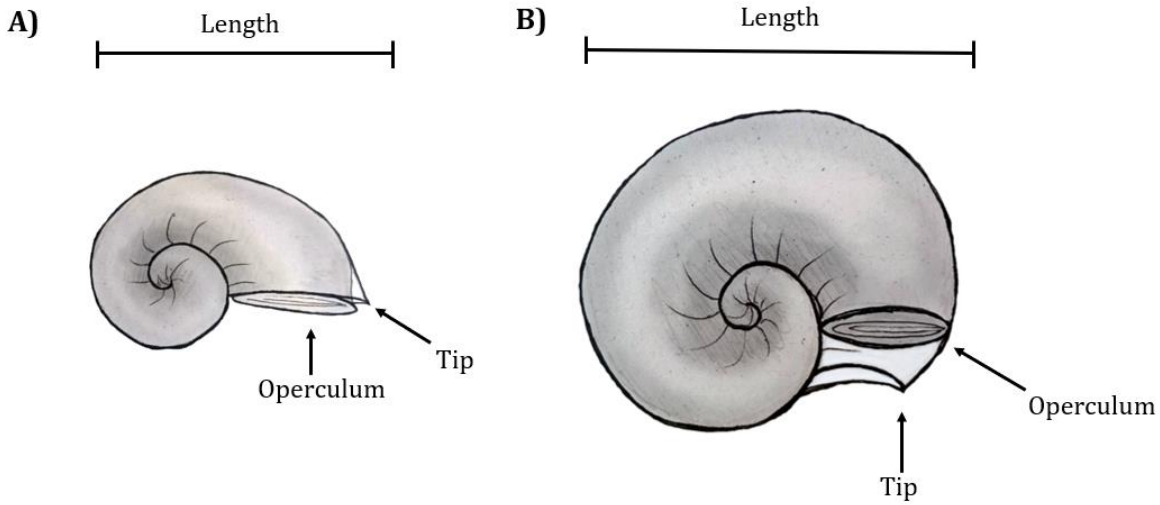

Figure 9: Simplified models of shell form in *L. littorea* larvae. A) Young larva with a pointy shell, and the shell opening covers approximately half the shell length. B) Mature larva with a more circular shell, and a shell opening covering less than half the shell length. During these life stages the shells are see-through and internal organs of larvae can be seen (not pictured). Illustration: Tora Lillebjerka

2.7 STATISTICAL ANALYSIS

All statistical analysis were done in IBM ® SPSS ® Statistics 27. Graphs were made using a combination of Microsoft office excel and rStudio, using the packages ggplot, grid and car. All tables were created using Microsoft word. Gimp 2.10.30 were used as an image editing program. A significance level of $\alpha = 0.05$ was used for all statistical tests and all means and graphs are presented with standard errors (± SE).

Normality in the datasets were tested using Shapiro Wilks test. However, as the test is more unreliable with higher number of datapoints, all datasets were $N > 50$ were also visually inspected using QQ-plots to determine normality, as ANOVA is relatively robust to variations in normality when sample sizes are large (Sawilowsky & Blair, 1992). However, if normality was violated, significant differences were tested using either Mann-Whitney U test or Kruskal-Wallis H test depending on if the dataset had two or more groups respectively. After normality test, the datasets were tested using Levene's test of equality of error variances to check for homogeneity. If the test failed, ANOVA or independent-samples-t-test was conducted, bit if homogeneity was detected a welch ANOVA or welch t-test were used instead.

3 RESULTS

3.1 EXPERIMENT 1A: DESCRIPTION OF EMBRYONAL DEVELOPMENT AND EGG MORPHOMETRICS

3.1.1 Characteristics of eggs and general developmental stages

The eggs of *L. littorea* were laid in capsules containing 1-3 embryos, which sedimented at the bottom. The capsules were transparent, soft, and appeared gelatinous, allowing the observation of the developing embryos inside the capsule cavity (Figure 10).

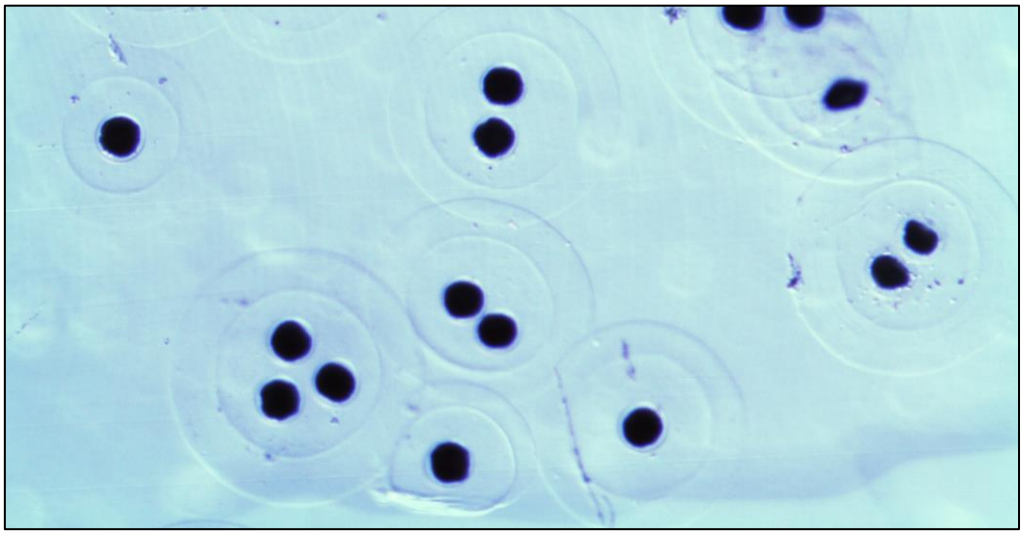

Figure 10: *L. littorea* egg capsules. The outer capsule edge and the capsule cavity are clearly visible containing 1-3 embryos (black) inside their egg envelope.

The embryonal development was described using external observations of embryos from 11.3, 13.7, 17.3 and 19.2 °C as these temperatures showed a similar development (Section 1.2). The embryos had a beige colour, and no nurse eggs or large yolk was observed. Early cell divisions were holoblastic and happened within the first 10 daydegrees (Figure 11a). At around 20 daydegrees the embryos were gastrulas with an invagination on one side of the embryo, and it was no longer possible to discriminate cells (Not pictured). After 30 daydegrees the embryos developed into trochophore larvae and ciliate movement were observed (Figure 11b). At this stage the embryos had a clear anteroposterior axis with a dark colouration in the posterior end becoming the shell-incapsulated organs, and a lighter coloration in the posterior end developing into the head and velums (Not pictured). At 40 daydegrees early shell formation was starting and rudimentary velum developed (Figure 11c). At this time the egg size also increased from 149 ± 9 µm to 172 ± 16 µm (3.1.2 Egg morphometrics). After 50 daydegrees the embryos developed began looking like veliger larvae and had eyespots, operculum and a

shell which covered their internal organs (Figure 11d). The embryos were also very active inside the egg envelope, moving their cilia and rotating inside the egg. After 60 daydegrees the embryos began to hatch. The larvae first broke through the egg envelope, swimming inside the capsule (Figure 11e) before breaking through to the free water masses (Figure 11f). Immediately after hatching the larvae were swimming using the cilia-covered velums, gathering in the top of the water column (Figure 11g).

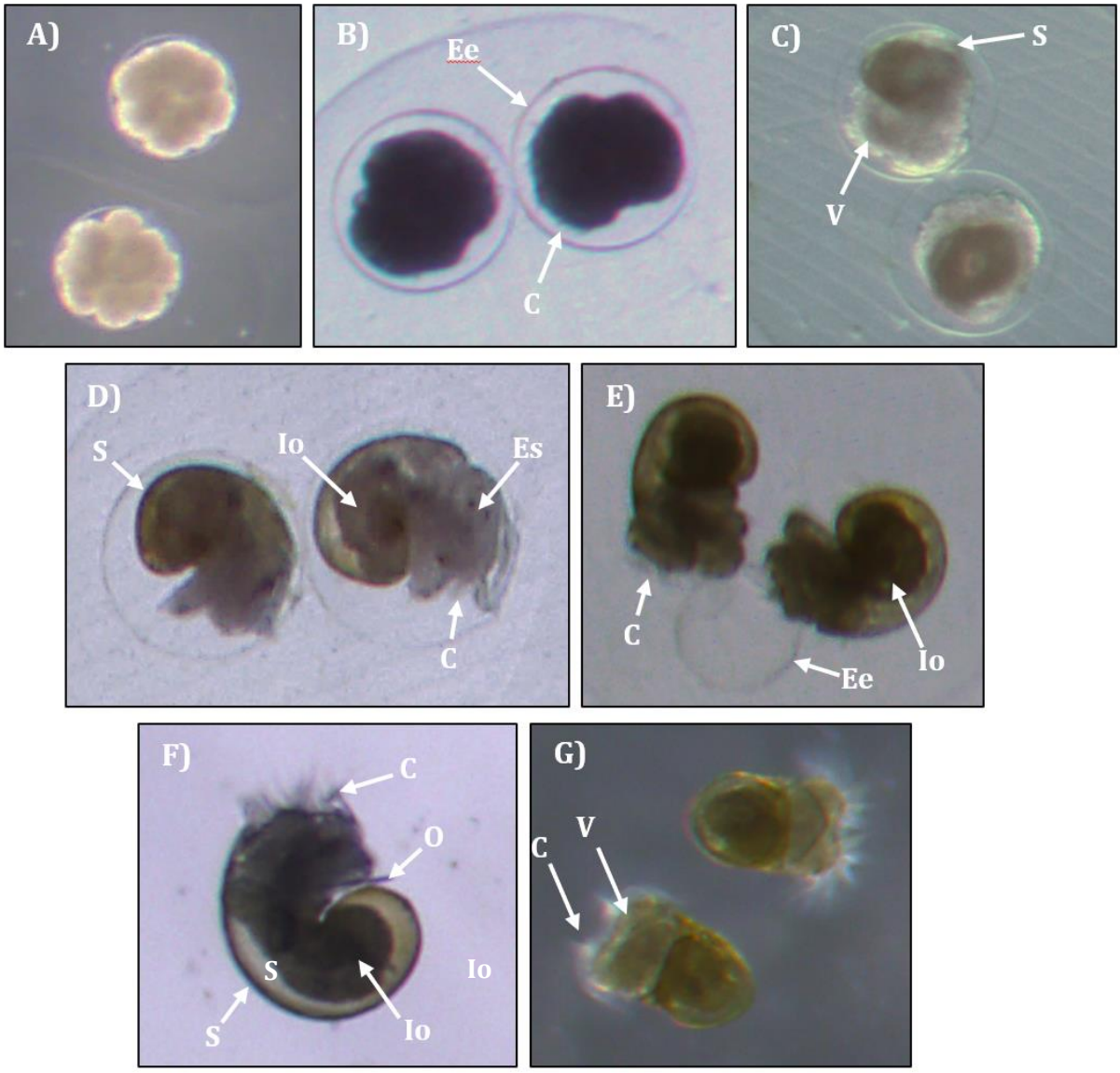

Figure 11: Developing embryos of *L. littorea.* A) Morula stage. B) Trochophore larvae with cilia (31 daydegrees). C) Early veliger with rudimentary velum and beginning to develop a shell (38 daydegrees). D) Veliger larvae with eyespots and shell (49 daydegrees). E) Veliger larvae that have hatched out from egg envelope, but still inside capsule (63 daydegrees). F) A fully hatched larvae seen from the side. G) Swimming veliger larvae observed from above. C = cilia, Es = Eyespot, Ee = Egg envelope, Io = Internal organs, O = Operculum, S = Shell, V= velum.

3.1.2 Egg morphometrics

Data for egg morphometrics were combined for all temperature treatments, as there was no significant difference between them. The overall average of egg size was 157 ± 0.83 (µm), but from visual inspection of the dataset an increase in egg size was observed starting at approximately 40 daydegrees (Figure 12).

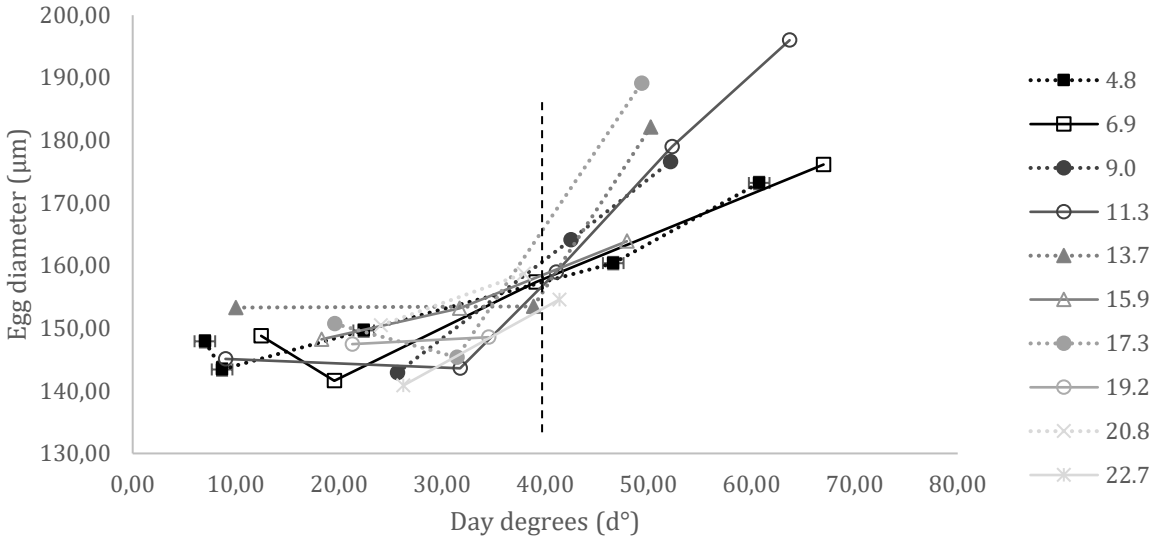

Figure 12: Egg diameter (µm) as a function of day degrees (daydegrees) in *L. littorea* from spawn until hatch. Each point represents the mean measured egg diameter from each temperature with 4-24 datapoints. (Total N = 412)

The newly spawned eggs had a diameter of 148 ± 0.57 µm which then significantly increased in eggs older than 40 daydegrees to 171 ± 1.31 µm ($p<0.000$) (Table 3).

Table 3: Egg size given as mean ± SE and range in size for early (<40 daydegrees), late (>40 daydegrees) and average (all daydegrees). The measurements are done on eggs from all temperature treatments, ranging from 4.8 to 22.7 °C.

Stage	Mean egg size (µm) ± SE	Range in egg size (µm)	N eggs
<40 daydegrees	148 ± 0.57	131-193	253
>40 daydegrees	171 ± 1.31	141-223	159
Average	157 ± 0.83	131-223	412

3.1.3 Capsule morphometrics

Eggs were laid in egg capsules with 1.6 ± 0.03 (N = 251 capsules) eggs per capsule. The diameter of the egg capsule varied between 400 and 824 µm with a mean of 632.7 ± 5.3 (N = 245) and the intracapsular cavity between 301 and 536 µm with a mean of 426,2 ± 3.2 (N = 244). There was a statistically significant, strong positive correlation between the capsule diameter and the inner capsule size, r(236) = 0.873 (p < 0.0005) (

Figure 13a). The variation of inner capsule size is explained by 76% by this model (r^2 = 0.76) and the estimated slope coefficient was 0.53 µm capsule cavity per µm capsule. Size differences in the capsule was influenced by the number off eggs it contained and the capsule size increase with a higher number of embryos inside (Figure 13b). Capsules containing one egg were 566 ± 7 µm (N=100), significantly smaller than capsules containing two eggs with a mean of 677 ± 5 µm (N=134) (p<0.001) and three eggs with a mean of 760 ± 22 µm (N=7) (p<0.001). The capsules containing three eggs were also significantly larger than the capsules with two eggs (p=0.022).

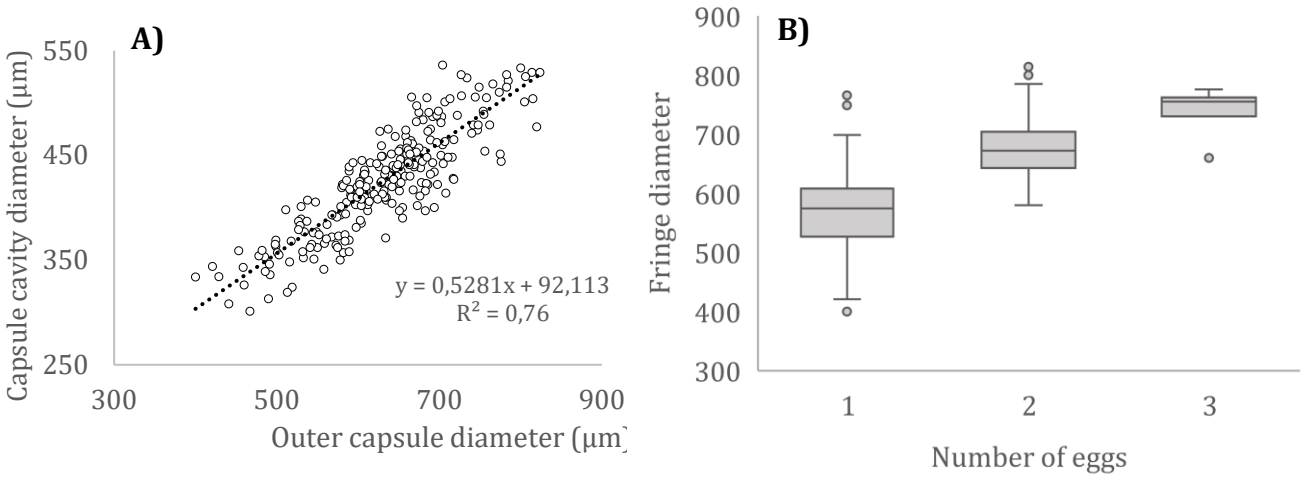

Figure 13: A) Diameter of intracapsular cavity as a function of outer capsule size (µm). The model is fitted with a linear regression line with the formula y = 0,5281x + 92,113 and R^2 = 0,76. B) Diameters of capsules containing 1-3 eggs. All capsular sizes are significantly different from each other.

3.2 EXPERIMENT 1B: EFFECT OF TEMPERATURE ON TIME UNTIL HATCH AND LARVAL DEVELOPMENT

Hatching time was defined as when hatched eggs in sample >50%. The time interval varied between 57 daydegrees (20.5 °C) and 72 daydegrees (4.5 °C) with an estimated slope coefficient of -0.47 daydegrees until hatch per increased °C in hatching temperature (Figure 14). This model explains 36.7% (r^2 = 0.3674) of the variation in time until hatching.

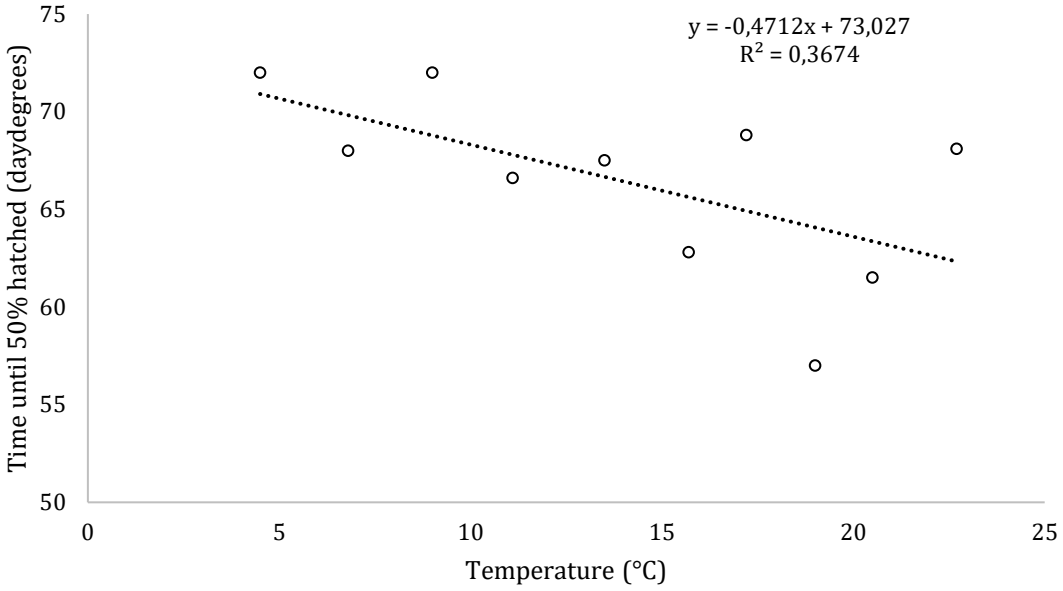

Figure 14: Time until hatched expressed by 10 different temperatures. Each datapoint represents a beaker monitored every 15 daydegrees from Experiment 1A. The model is fitted with a linear regression line: y = -0,4712x + 73,027 and R^2 = 0,3674.

The shell length of the hatched larvae varied between the 10 temperature treatments after incubation for 10 daydegrees, showing a bell shape, with the largest larvae in the intermediate temperatures (11.3-17.3 °C) (Figure 15). The average shell length for the four temperatures with the largest larvae averaged around 220 µm while the larvae at 19.1 °C were slightly smaller at 216 µm (Appendix 2). The shortest shell lengths were found in the outer edges of the temperature gradient at the temperatures 9.1 °C, 20.9 °C and 22.8 °C. The two coldest temperature treatments 4.8 and 6.9 °C had an abnormal development, resulting in no larval shell and are therefore omitted from the graph.

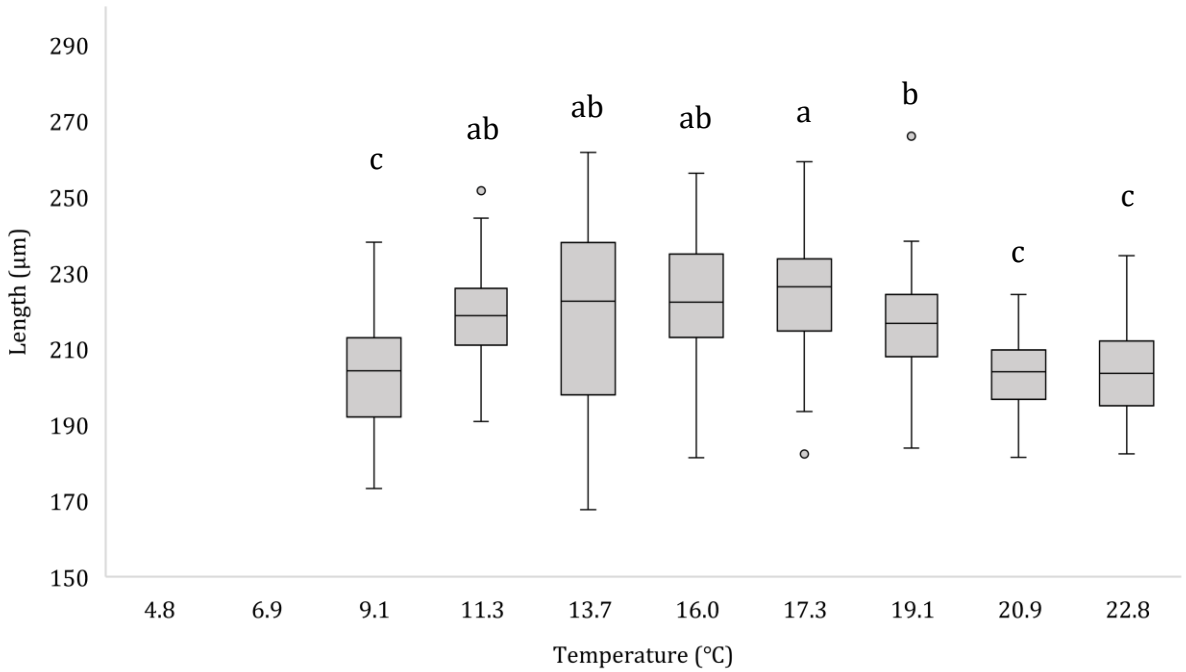

Figure 15: Shell length in *L. littorea* larvae 110 daydegrees after spawning. The temperature groups are divided into abc categories based on statistically significant differences between groups. The larvae from the two coldest temperatures are excluded from the graph as they were severely deformed and did not have a shell to measure.

The abnormal development seen in the larvae from the colder temperatures appeared to be temperature dependent, as the larvae was less developed in 4.8 °C than in 6.9 °C. This also affected all the larvae in both colder temperature treatments. The larvae incubated in 4.8 °C had an abnormal body shape, being very round like a trochophore larvae, without clearly differentiated swimming lobes (Figure 16). They also lacked a shell, and the internal organs appeared to be relatively unprotected. However, the larvae had cilia and several individuals were observed swimming around. The larvae kept in 6.9 °C were more developed than in the colder temperature of 4.8 °C, showing more developed swimming lobes and a more oblong body, covered by å larval shell (Figure 17). The larvae also had clearly visible eye spots and cilia covering the swimming lobes, but where not able to retract into their shell. Some individuals were observed with what looked like a rudimentary operculum but had still not been able to retract into the shell. The rest of the larvae kept at temperatures above 6.9 °C (9.1-22.8 °C) developed into normal veliger larvae. They had a fully developed shell, which they were able to retract completely into (Figure 18). As the shells were see-through the eyespots and cilia could be seen through their shell.

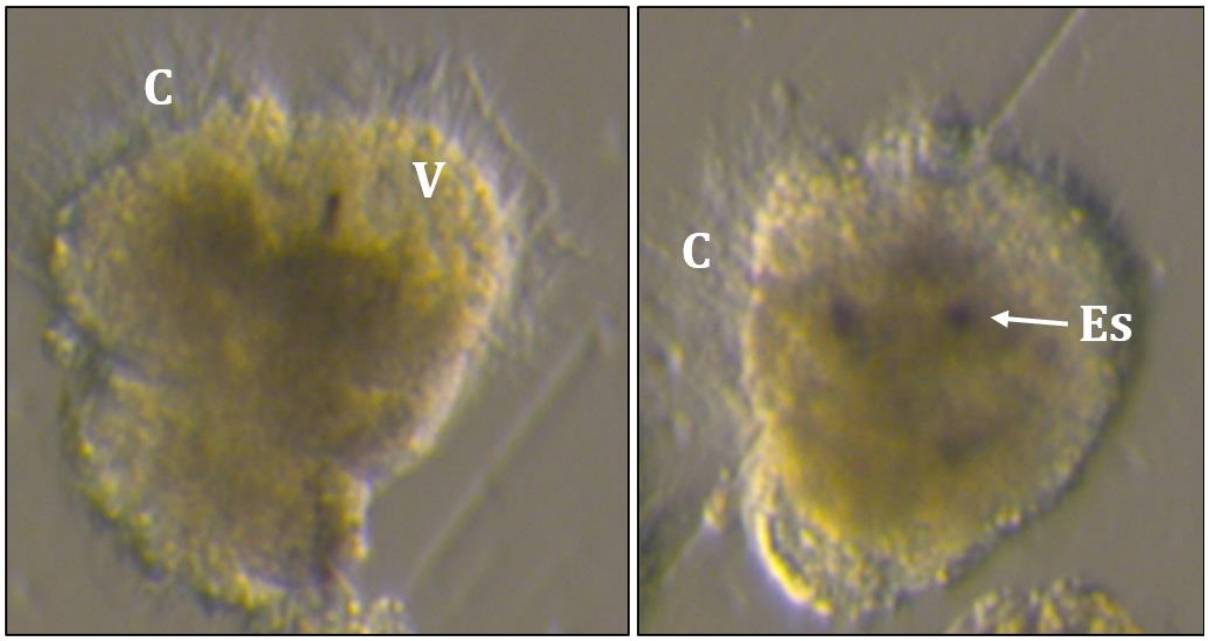

Figure 16: Two larvae having been incubated at 4.8 ℃, the lowest temperature treatment. Both larvae er oriented with eyes and swimming lobes at the top and the intestine (which should be contained within the shell) at the bottom. The larvae are very circular, and does not show normal development, including a lack of shell development. C = Cilia, E = Eye, L = Lobe.

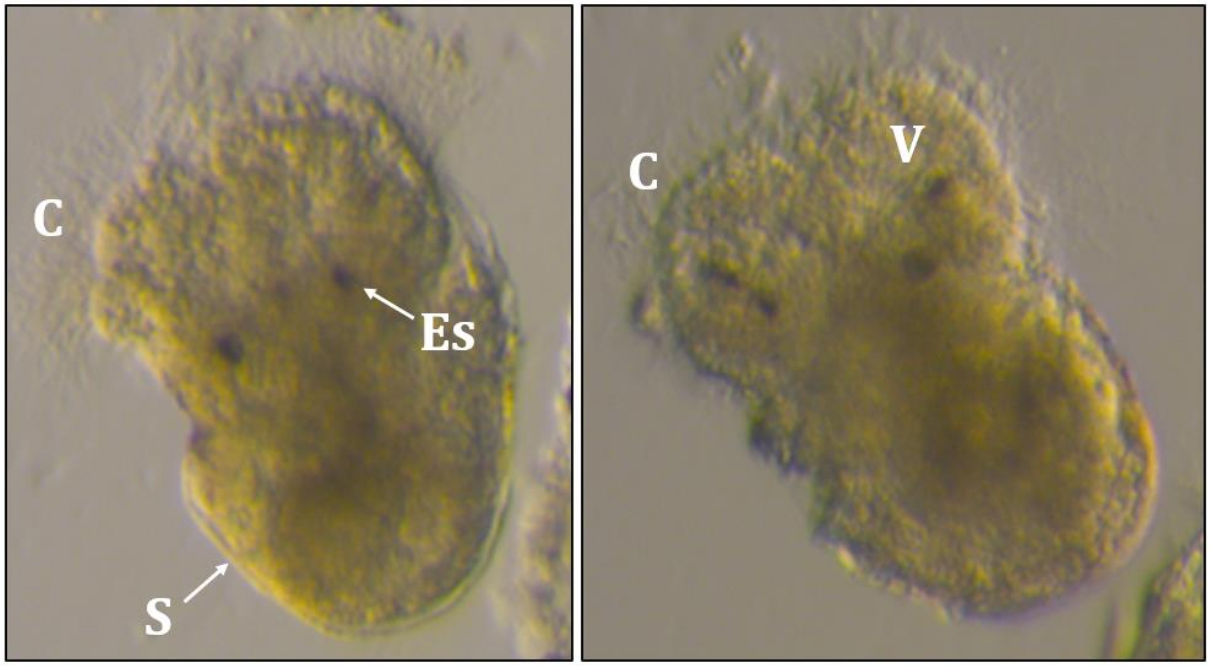

Figure 17: Two larvae from the temperature treatment keeping 6.9℃. Both larvae are seen from above, with swimming lobes and cilia at the top and the shell covered intestine at the bottom of the image. They have not developed a large shell they are able to retract into. C = Cilia, E = Eye, L = Lobe, S = Shell.

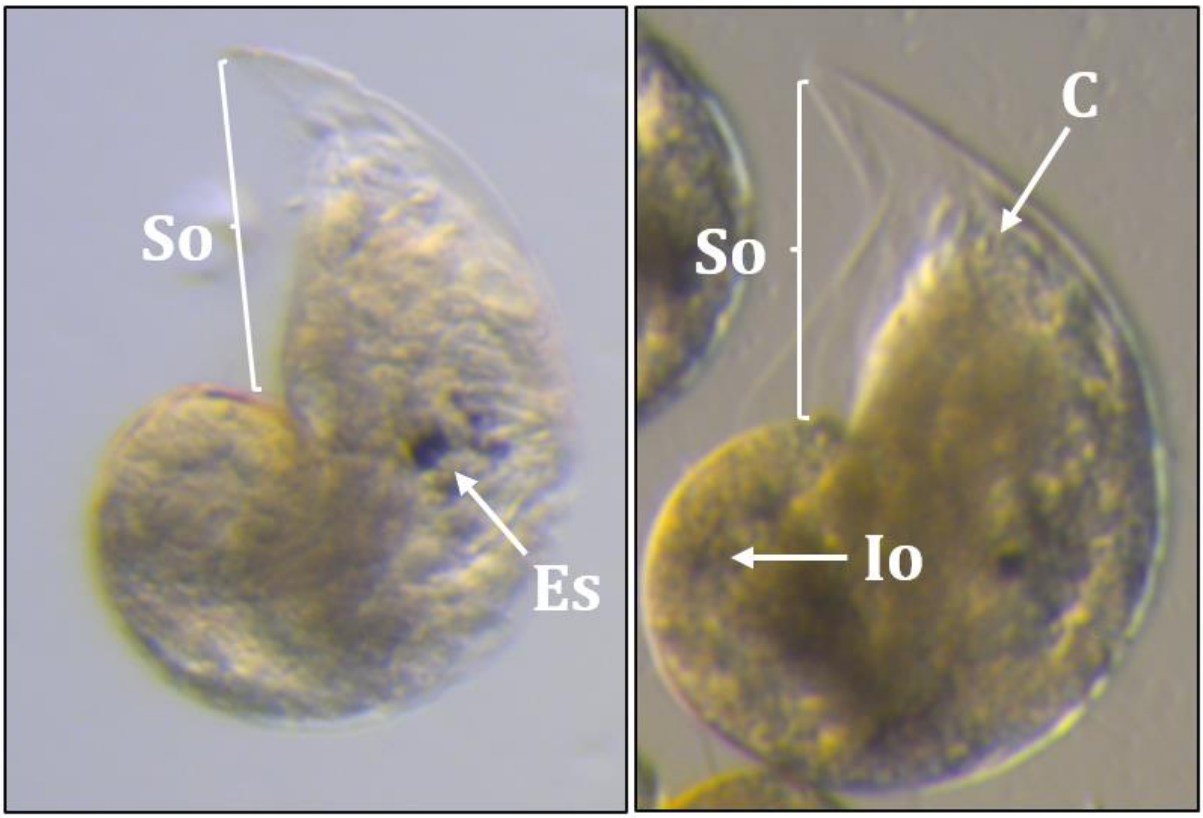

Figure 18: 20.9 °C (left) and 11.3 °C (right). The remaining temperatures (9.1-22.8°C) showed similar development of the larvae. The classical helical shape can be observed, and eyes are visible through the shell. The shells are large and able to contain the entire larvae and has a clear tip at the front. The cilia can also be seen to some degree through the shells. The larvae are placed on their side and thus observed in profile. C = Cilia, E = Eye, S = Shell, SO = Shell opening.

3.3 Experiment 2: Hatching rate as an effect of temperature and salinity

3.3.1 Assessing the effect of temperature and salinity on hatching

Hatching was observed in all temperature and salinity combinations during Experiment 2 (Figure 19). Salinity showed a significant influence on the number of hatched eggs (p = 0.00316, N = 25), with 20 ppt having a significantly lower hatching success than 25 ppt (p = 0.0442826), 30 (p =0.0025521) and 40 ppt (p = 0.0073767). There was not found a significant effect of temperature during the experiment.

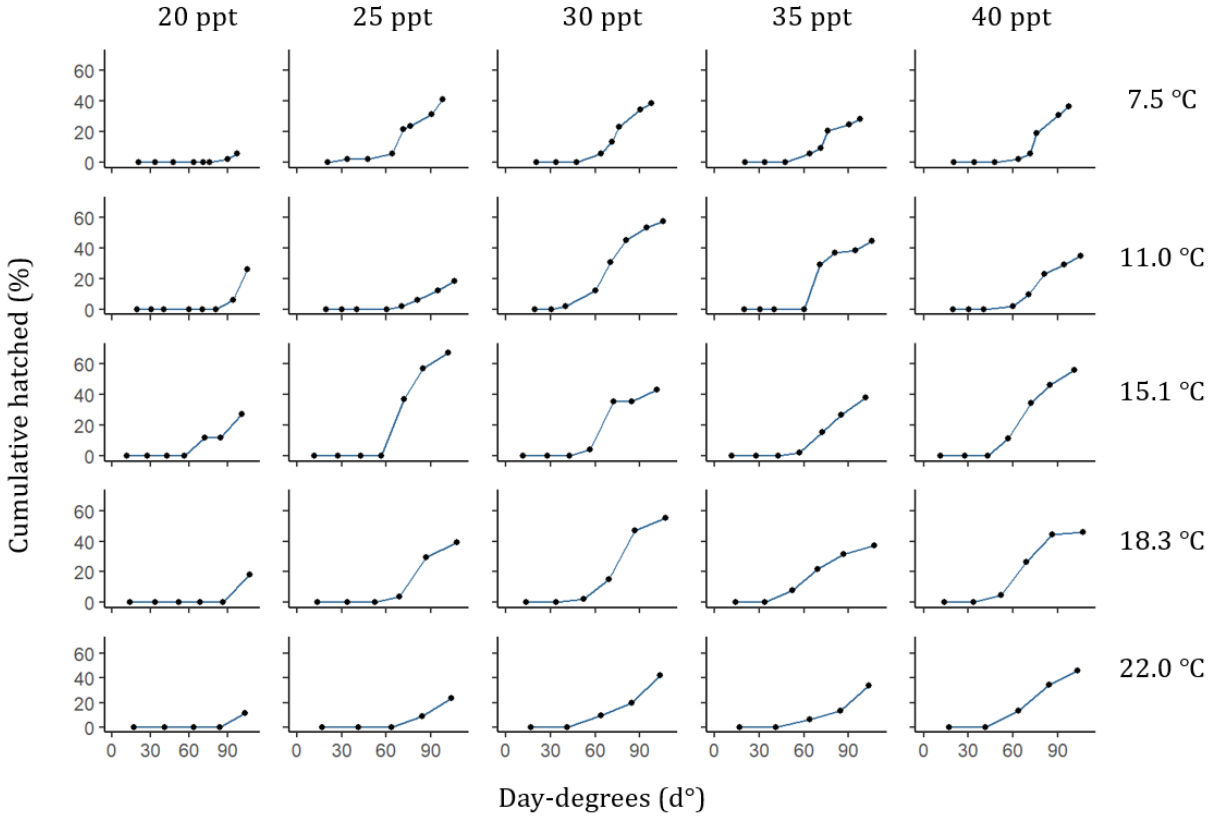

Figure 19:Total hatching in all 25 flasks as monitored approximately every 15 daydegrees until 100 daydegrees expressed in %. Each flask contained approximately 50 eggs. Graphs are organized into a grid by temperature (rows) and salinity (columns).

The overall hatching success during Experiment 2 was low compared to Experiment 1B with an average percentage hatched of 36,6 ± % with only three of the treatments producing a hatching percentage above 50% (15.1°C, 25 ppt; 15.1°C, 40 ppt; 18.3°C, 30 ppt). As a result, time until hatch was defined at 10% and not as 50% hatched as in Experiment 1B. A similar decline in daydegrees with higher temperatures was not

observed in this experiment (Figure 20a). When pooling together all treatments the majority of the hatching occurred between 60 and 105 daydegrees (Figure 20b).

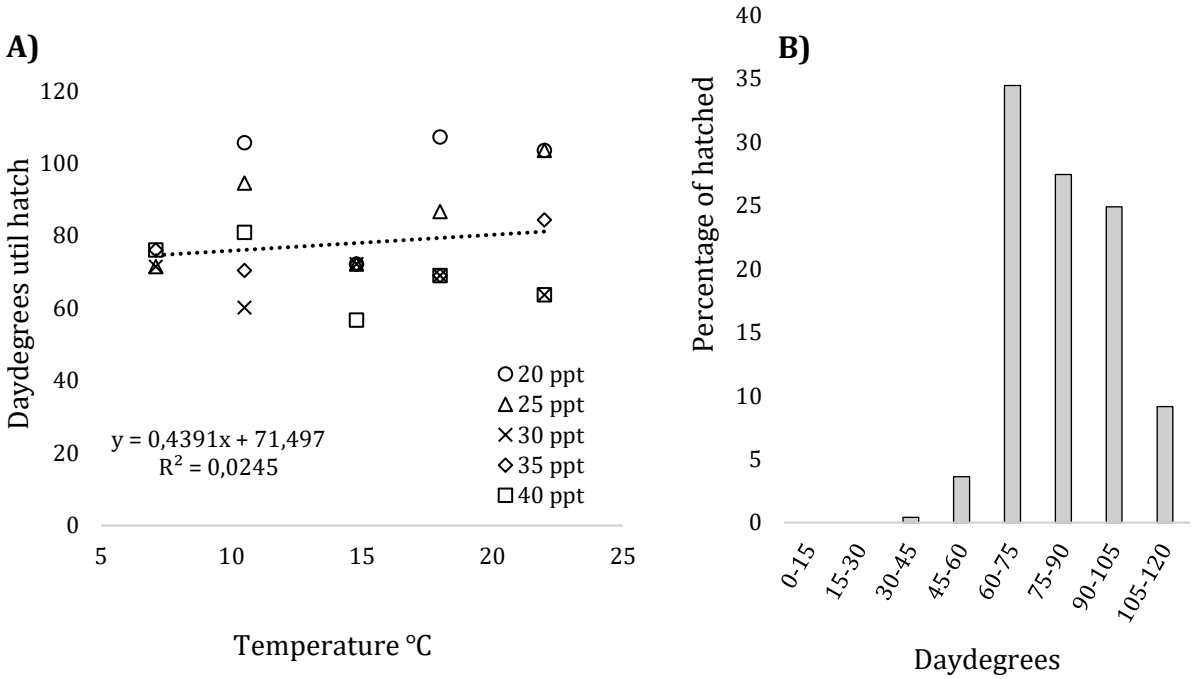

Figure 20: A) Time in daydegrees until 10% hatched larvae was observed. A) Combined data of all hatched larvae to find the hatching range. Data is presented as total percentage of hatched larvae divided into intervals of 15 daydegrees.

3.3.2 Finding salinity and temperature optimum

By combining the hatching success data from all temperatures and salinities, a surface chart could indicate the temperature and salinity optimum for hatching (Figure 19). The zone containing the highest hatching numbers are around 15.1 ℃ and 25 to 30 ppt salinity while the outer edges of the model have lower hatching numbers.

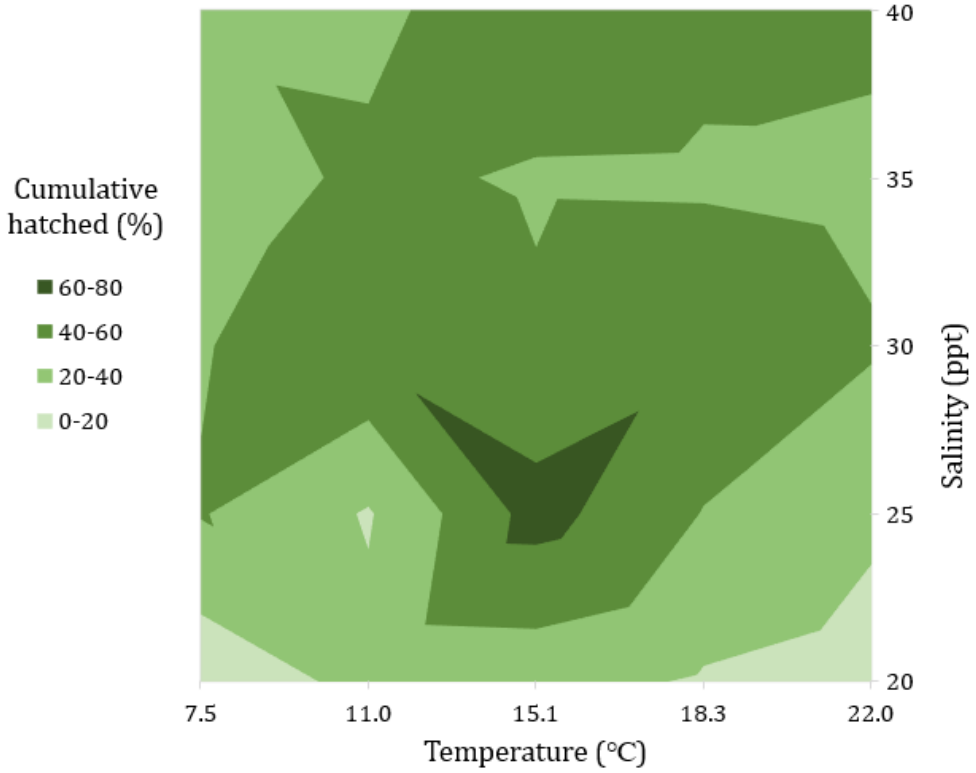

Figure 21: A surface chart showing the temperature and salinity where the highest numbers of hatch of eggs from *L. littorea* were found. The hatching optimum was around 15°C and 25-30 ppt, with lower hatch in the edges of the model.

3.4 EXPERIMENT 3: EFFECT OF DIFFERENT MICROALGAL DIETS ON GROWTH OF *L. LITTOREA* LARVAE

3.4.1 Growth measurements

Shell length for the newly hatched larvae used in this experiment were 182 ± 1 µm. The experiment lasted for 22 days, when the snails were ready to settle (Figure 22). After nine days the larvae fed *Nannochloropsis*, Rotifer diet and the starvation control died after showing little to no growth (Appendix 1). The live algae diets *R. baltica* and *D. tertiolecta* both led to larvae growth with very similar shell lengths, which were able to develop until metamorphose (Appendix 1). The final shell length was measured on day 22 and showed no significant differences between *R. baltica* and *D. tertiolecta*. Larvae fed *R. baltica* were on average 350 ± 3.8 µm (N=54) and larvae fed *D. tertiolecta* had a mean shell length of 340 ± 3.2 µm (N = 35).

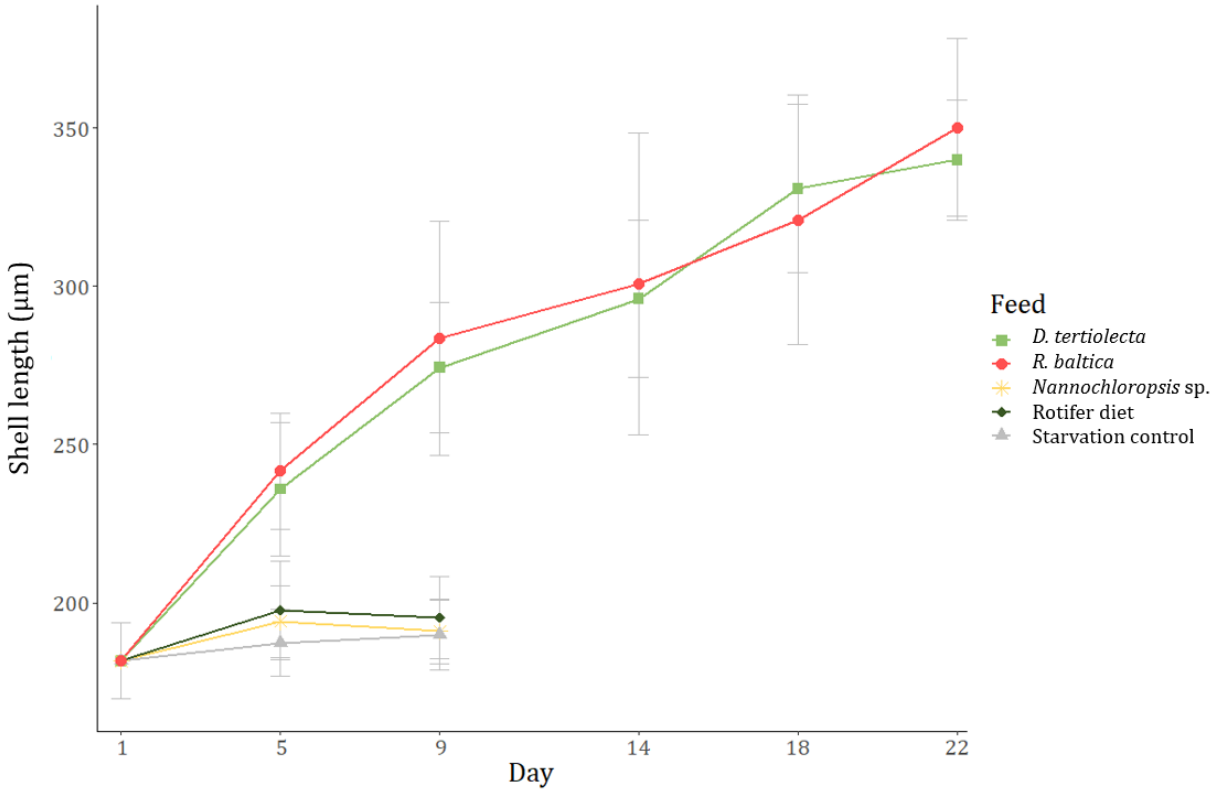

Figure 22: Average shell lengths (µm) of *L. littorea* larvae reared on four different types of microalgal food and a starvation control.

A model using logarithmic regression lines were made to explain the growth pattern of the veliger larvae for the two live diets where the larvae survived the entirety of the experiment. The formulae for larvae fed *R. baltica* indicate a slightly higher growth rate (49.461*ln(daydegrees)) than in larvae fed *D. tertiolecta* (46.674 * ln(daydegrees)). It should also be noted that there was a difference in variance between the *R. baltica* group and the *D. tertiolecta* group. On day 4 the two groups showed similar distributions of

length, but at day 9 *R. baltica* have more variations in size. The large variance in the distribution of the *R. baltica* group can be found for the remaining days of the experiment.

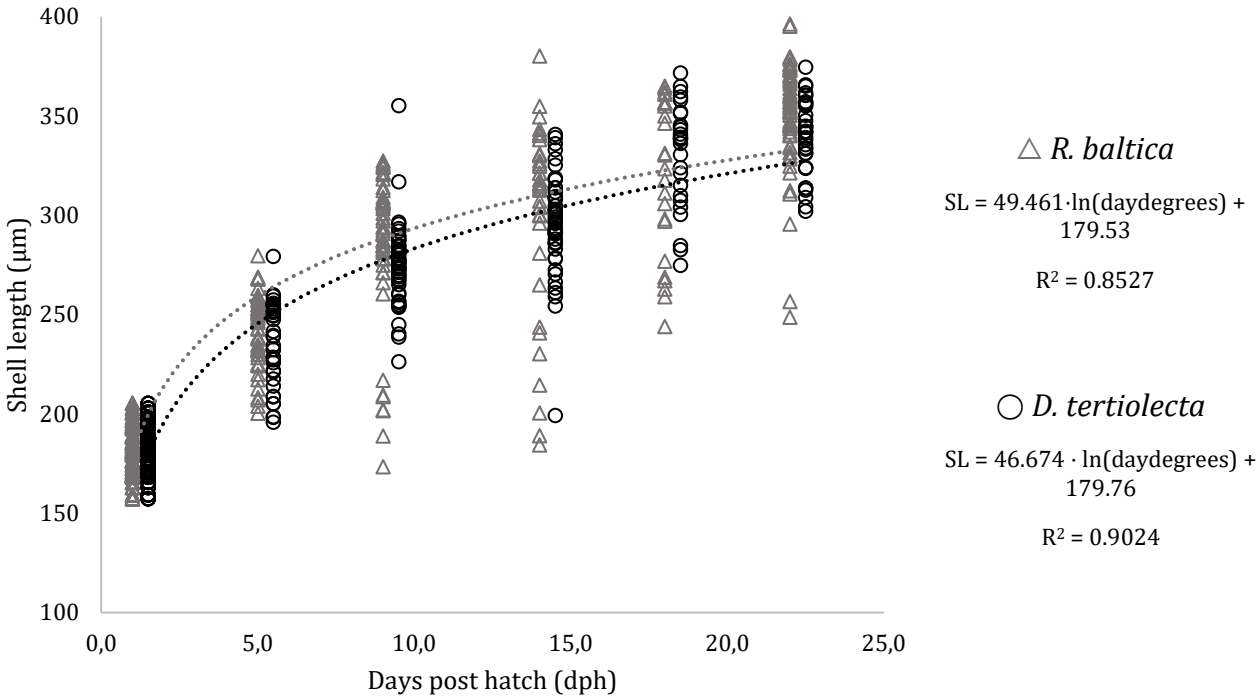

Figure 23: Model showing the distribution of shell length for larvae fed *R. baltica* and *D. tertiolecta*. The datasets are fitted with logarithmic regression lines. Datapoints for *D. tertiolecta* are offset with 0.5 days to avoid overlap in data between the two diets.

SGR was calculated for all treatments (Figure 24). The live algal diets *R. baltica* and *D. tertiolecta* gave the larvae an SGR of 0.0547 ± 0.00253 (N=53) and 0.0514 ± 0.00132 (N=48) respectively for days 1-9, while the remaining diets had hardly any growth and promptly died on day 9. Larvae fed *R. baltica* had an SGR of 0.0164 ± 0.00091 (N= 54) and *D. tertiolecta* had an SGR of 0.0170 ± 0.00073 (N=35) for day 9-22 and they did not significantly differ from each other. The SGR for the total experiment averaged around 3% growth in shell lengths per day (Appendix 1)

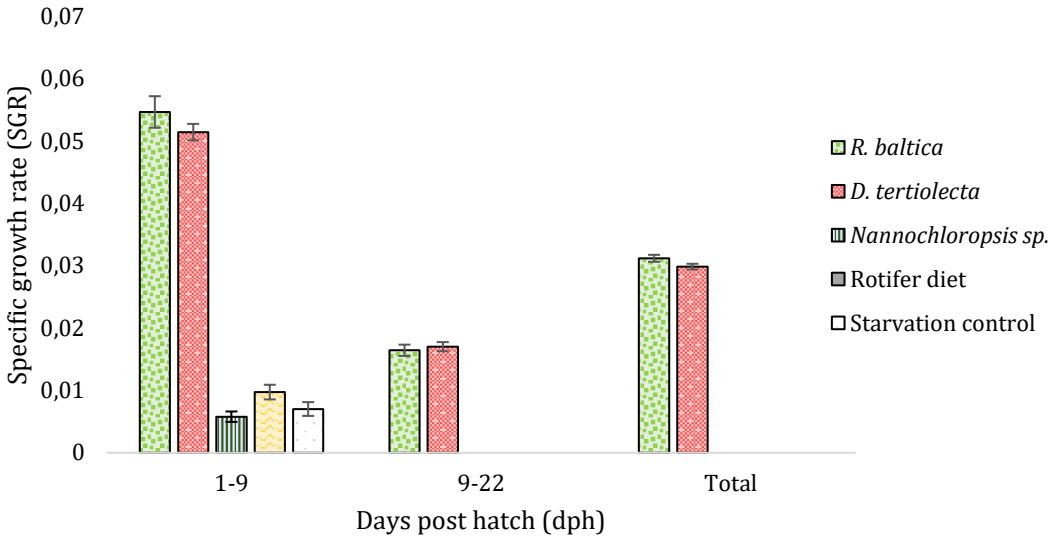

Figure 24: Specific growth rates (SGR) for *L. littorea* larvae reared on four different algal diets in and a starvation control. The growth data is divided into three categories; day 1-9, day 9-22 and Total, which is the average SGR for the entire period (1-22 days). As three of the diet groups died on day 9, complete data is only available for the *R. baltica* and *D. tertiolecta* groups of larvae.

3.4.2 Larvae morphology

As the larvae grew it was possible to see a clear difference in shape, as the shells become rounder and more three-dimensional due to coiling (Figure 25). The internal organs were somewhat visible through the shell, and coloration from consuming microalgae were visible in the intestine.

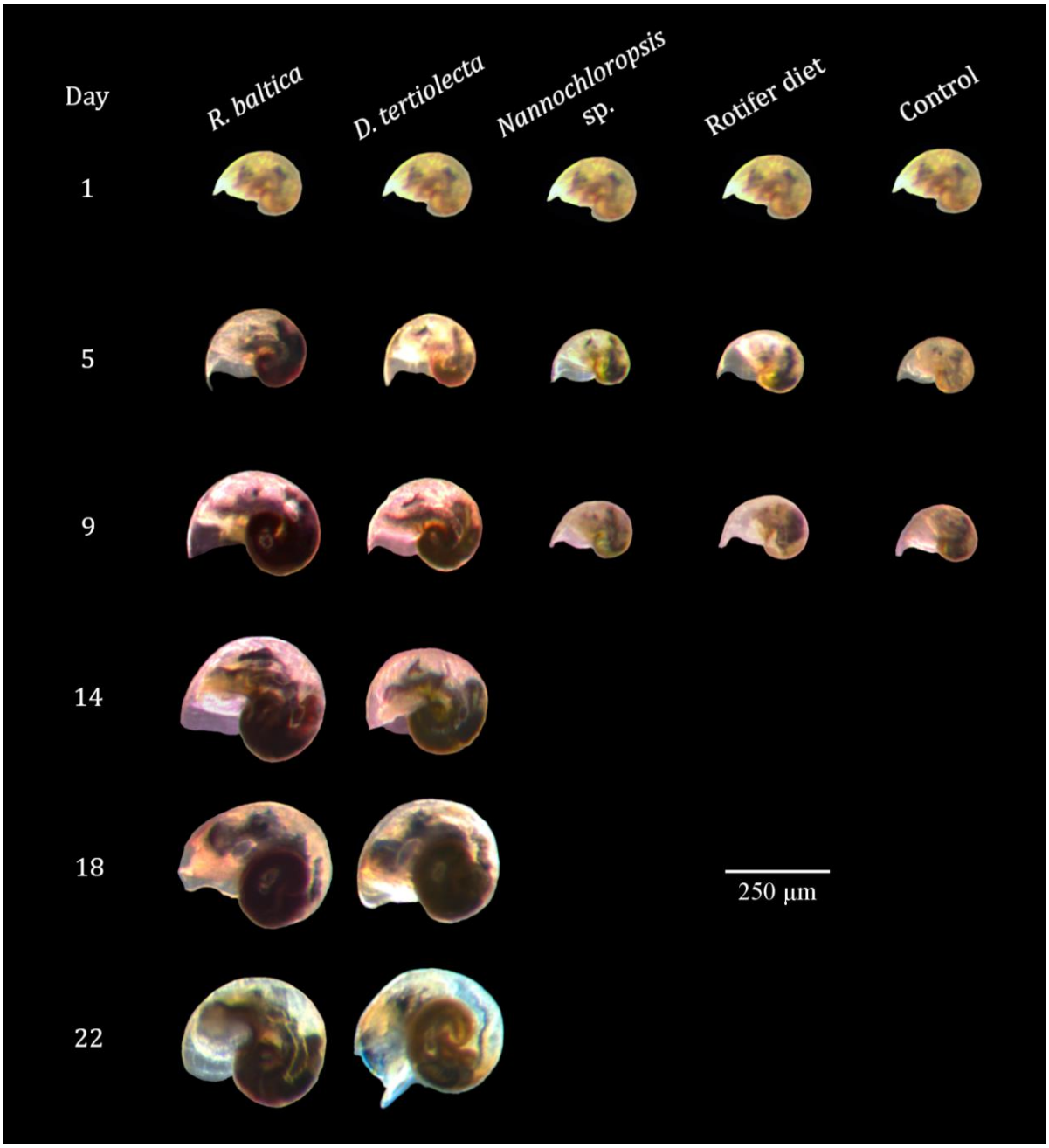

Figure 25: Photographs of *L. littorea* veliger larvae of during development from newly hatched (top row) until settlement (bottom row). Three of the diet groups died on day 9, thus lacking in the rest of the model. Consumed algae can be observed inside the larvae intestine and is either red or green/brown.

4 DISCUSSION

4.1 THE EGG AND LARVAE DEVELOPMENT OF *L. LITTOREA*

The embryonal development in *L. littorea* in the present study was similar to the development in many other gastropods (Kawano et al., 2004; Moran, 1999; Romero Bastías, 2014). The embryos had a holoblastic cell division, and an overall round shape during early embryogenesis. While not observed, it is assumed the snails had a spiral cleavage pattern as it is found in all gastropods (Nielsen, 2004). The length of the embryonal period in gastropods can vary greatly, from 24 hours (Romero Bastías, 2014) to several weeks (Cañete et al., 2012), both depending on species and abiotic factors like temperature, oxygen, salinity etc. In *L. littorea* the development took between 60 and 70 daydegrees, and the appearance of trochophore larvae happened at around 30 daydegrees. These larvae are a stage in the development of molluscs, and have a rounder shape, often accompanied by ciliate bands (Nielsen, 2004). After around 40-50 daydegrees the larvae began looking like veliger larvae, having velums covered in cilia and a developed shell. The timeline of the development share many similarities with *Littorina brevicula*, a different species in the *Littorina* genus (Son & Hong, 1998). While *L. brevicula* has a longer time until hatch (7 days in 11-13°C ≈ 70-80 daydegrees) the appearance of trochophore and veliger in this study happened at approximately the same time as in previous studies of *L. brevicula* (Son & Hong, 1998).

As soon as the larvae hatched from the egg capsules, they were able to retract fully into their shells, indicating that they had completed the larval torsion stage while still in the egg envelope (Ghiselin, 1966). The retraction serves as useful protection against predation (Bryan et al., 1997; Edgell & Miyashita, 2009; Vaughn, 2004), but is not seen in all gastropod larvae, as some hatch without a fully developed shell (Underwood, 1972). The larvae were also able to swim immediately, using their velum and cilia. The larvae swam upwards, a behaviour seen in other gastropod larvae, possibly regulated by phototaxis and geotaxis (Barile et al., 1994; Son & Hong, 1998). This behaviour could help larvae stay in the photic zone where they are able to graze on microalgae.

4.2 EMBRYONAL DEVELOPMENTAL RATES WILL INCREASE WITH HIGHER TEMPERATURES BUT RESULT IN MORE DEVELOPMENTAL DEFECTS.

The developing embryos of *L. littorea* showed an increase in developmental rates with higher temperatures, using 3 days (22.8°C) to 16 days (4.5°C) to hatch during Experiment 1B. When presenting the data in daydegrees this pattern was still present with a linear relationship showing decreased number of daydegrees with increasing temperatures. However, conflicting results was found in Experiment 2 with no decrease in daydegrees with increasing temperatures. A similar change in daydegrees as in Experiment 1 was

found in the eastern mud snail (*Nassarius obsoletus*) (Scheltema, 1967) where a non-linear relationship was found for the number of days needed for development, and the relationship increased more rapidly (2 days per 1°C) between colder temperatures (19.5-16.5 °C) than between warmer temperatures (19.5-28.0 °C) (0.25 days per 1°C) (Scheltema, 1967). Similarly, in the nudibranch *Cadlina luteomarginata,* daydegrees decreased from 430 daydegrees in 5°C to 350 daydegrees in 10°C (Dehnel & Kong, 1979). A possible explanation for this results is using daydegrees instead of effective daydegrees as it does not consider the temperature threshold of the species (Kamler, 2002). Despite temperature being the most important influencing factors on hatching time (Spight, 1975), there is still much to be known.

The embryos were able to develop to hatch in all temperatures, giving a hatching range from 5-23 °C. However, variations in size and abnormalities in the warmest and coldest temperature treatments respectively, indicates that the temperature optimum is smaller, ranging from 11-17°C. This is in accordance with the results from Experiment 2, where the highest hatching success was found near the middle values of both temperature and salinity, creating a hatching optimum at around 15°C and 25-30 ppt. A salinity-range for hatching success was determined, with hatching seen in all salinities (20-40 ppt). While no absolute thresholds for hatch was found in the experiment, a very low hatching success in 20 ppt suggests the salinity-range is 25-40 ppt. The same effect is seen in many other mollusc species, where eggs or larvae show increased mortality or higher frequency of abnormal development in low salinities with the exact salinity values varies between different species (Dos Santos & Nascimento, 1985; Génio et al., 2008; Roller & Stickle, 1989; Zhang et al., 2014).

The spawning period for *L. littorea* is from January to June (Moore, 1937; Williams, 1964). In the coldest months (January-April) the temperature in littoral waters in the Norwegian sea sinks to about 5°C (Institute of Marine Research, 2022). All the embryos incubated in water below 7°C had an abnormal development, resulting in deformed body shapes, velum, and shells. The negative effect of low incubation temperature is also seen in other gastropod species like the predatory snail *Charonia seguenzae* (Doxa et al., 2021). The eggs incubated at 17°C had high numbers (95%) of deformed larvae with small bodies, shortened shell or velums, or having a body detached from the shell. The snails spawn in fall and the larvae hatches early winter, when sea temperatures can drop to 15-16°C (Doxa et al., 2019; Doxa et al., 2021; World sea temperature, 2022). These results raise questions, as both species have a lower temperature tolerance than their natural environment should suggest. These results could be explained by an increased sensitivity to environmental changes in the early stages of development, as the eggs in both experiments were spawned in a higher temperature before gradually lowering the temperature (Doxa et al., 2021; Fischer, 2012). The changes in larval size with high temperatures, was much less severe. Similar size decreases with increased temperature can also be seen in other species of gastropods (Horwitz et al., 2017) and can be explained by an increase in metabolic rate (Brown et al., 2004). The upper thermal limit, signified

by increased abnormal development or low survival rates (Dos Santos & Nascimento, 1985) was not found for *L. littorea* in this study, as the eggs were able to develop into hatch even at the highest temperature (23 °C). As sea temperatures in Norway rarely rise above 20 °C in winter/spring (Institute of Marine Research, 2022), the distribution of *L. littorea* should not be limited by the upper temperature range of development.

The littoral habitat of *L. Littorina* is also affected by large changes in salinity. While average sea surface salinity rarely drops below 30 ppt during early spring, abiotic effects like winds, rain, sun and river runoff can greatly impact water conditions, creating large fluctuations in salinity (Fukuda et al., 2021; Richmond & Woodin, 1996; Riser et al., 2019; Stickle & Denoux, 1976). Species living in the littoral sone need a higher tolerance for changes in environmental conditions than more deep water species (Zhang et al., 2014), including the egg and larvae stage (Anger, 1996). Due to the sensitivity to salinity in *L. littorea* embryos, increased runoffs from freshwater could impact the recruiting of the species (Génio et al., 2008). While adult animals can protect against salinity stress through behavioural responses like shell closure or emersion (Taylor & Andrews, 1988) egg and larva stages are bound to the aquatic environment. While little is known on the buoyancy of the egg capsules, they were observed sedimenting in still water. The density of the capsules and embryos might allow them to stay further down in the water column (Nissling et al., 2017), keeping them safe from the low salinity in the surface water (Fukuda et al., 2021). The capsules themselves might also help protect the eggs against UV-rays, desiccation, mechanical stress, or fluctuations in salinity (Hamdoun & Epel, 2007; Pechenik, 1983; Rawlings, 1999; Woods & DeSilets, 1997).

4.3 Effect of different microalgal diets on growth of *L. littorea* larvae

The differences between larvae fed the different diets were obvious as only the larvae reared on the two live algae *R. baltica* and *D. tertiolecta* survived until settlement, while larvae fed *Nannochloropsis* paste or Rotifer diet, and the starvation control, died after nine days. The time until settlement in this experiment was observed to be 22 days, which is shorter than previously suggested six to seven weeks (Cummins, 2002) and the 37 to 70 days found in other species of *L. littorea* at 12-14 °C (Hohenlohe, 2002). A previous study of *L. littorea* found that time until settlement only took 12-15 days at 22°C (264-330 daydegrees), which is slightly shorter than in the current study (336 daydegrees)(Branco et al., 2014). As seen in the embryonal development of *L. littorea* these differences in daydegrees can occur, making it difficult to comment on whether the differences in time are temperature-controlled, or indicate something different like suboptimal conditions or diet. During the 22 days of development in the present study the shell length of the larvae doubled in size and the larvae had a shell length of around 345 µm when they settled. Visual inspection of the larvae throughout the experiment showed clear coloration (red

or green depending on the algae feed) in the intestine of all larvae, except those who were starved, indicating that the larvae were able to feed in all four feeding regimes.

In addition to having a longer incubation period, larvae of *L. littorea* from this study had longer shell lengths when they metamorphosed, compared to the larvae incubated at 22°C (325 µm) (Branco et al., 2014). This could be caused by a similar thermal effect as seen in the egg-incubation, where the higher temperature of incubation caused a decrease in individuals' size due to an increase in metabolism (Brown et al., 2004). It should however be noted that large intraspecies variations in both shell size (300-400 µm) and time until settlement (37-70 days) have been observed in other *Littorina* species (Hohenlohe, 2002). While the reason for this variance is unknown, it could be theorized that other factors than size and time influence the settlement of the larvae.

Large variations in shell lengths were observed in the larvae fed *R. baltica*, compared to the larvae fed *D. tertiolecta*. As the larvae were spawned from the same population and kept in similar conditions, it is most likely these variations came from differences in the diet. Comparing the diet groups without the outliers in the *R. baltica* group creates a significant difference with the largest larvae fed *R. baltica*. Other than the variations, there were few differences between larvae fed *D. tertiolecta* or R*. baltica* throughout the experiment. Both groups had a high SGR in the beginning compared to second half of the experiment. Such change in growth was also observed by Branco et al. (2014), where *L. littorea* larvae hardly increased in size in the days surrounding settlement. This reduced growth could be a results of the metamorphosis, as it require a lot of energy (Labarta et al., 1999; Thiyagarajan et al., 2003), thus leaving little left for growth.

Little is known regarding the dietary requirements of *L. littorea*. Branco et al. (2014) did not investigate the dietary preference of the larvae but used a mix of different algae: *Isochrysis galbana, Nannochloropsis gaditana, Phaeodactylum triconutum, Tetraselmis suecica, Rhodomonas marina* and *Chaetoceros gracilis,* of which species of *Rhodomonas*, *Tetraselmis* and *Nannochloropsis* were used as diets in this experiment, in addition to *Dunaliella tertiolecta*. The alga types differ in size, fatty acid content, carbon content and whether they are fed as live algae or not. Highly unsaturated fatty acids (HUFA) like EPA and DHA are the often the topic of discussion when considering feed for marine organisms, as they are essential for many fish larvae (Izquierdo, 1996; Mejri et al., 2021). However, their importance in gastropod diets, especially with focus on *L. littorea*, is mostly unknown. While *R. baltica* contains both EPA and DHA, *D. tertiolecta* lacks both fatty acids. Seeing as the larvae fed *D. teriolecta* and *R. baltica* did not differ in neither shell length nor SGR, this indicates that the larvae are not dependent on the fatty acids to metamorphose. Other studies on both bivalves and gastropods show conflicting results when it comes to lipids and essential fatty acids like HUFA. While some molluscs appear to benefit from an increase in HUFA (Bautista-Teruel et al., 2011; Hendriks et al., 2003; Mai et al., 1996; Nevejan et al., 2003) but they appear to have generally low demands for dietary lipids (<6%)(Bautista-Teruel et al., 2011; Chu et al., 2021; Mai et al., 1996). Many species of gastropods can efficiently use carbohydrates as an energy source instead of

lipids, perhaps as a result of many gastropods feeding heavily on macroalgae (Bautista-Teruel et al., 2011; Whyte et al., 1990)

As the effect of lipids could be considered minimal, the cell size or carbon content could be affecting the survival success of the larvae. As the feed concentration was doubled in the algae pastes which had smaller cells, the availability of these substrates should be rather similar. However, the pattern could also be due to the larvae of *L. littorea* preferring live algae over algae pastes. A similar preference is seen in Pacific oysters (*Crassostrea gigas*), which had increased grazing and growth on when given live microalgae compared to a paste of the same species (Ponis et al., 2003).

4.4 Methodological limitations / Uncertainties and limitations of the study

The differences in daydegrees from Experiment 1B varied between 61.5 and 72 daydegrees, which makes the range approximately 10 daydegrees. While sampling during this experiment was attempted every 15 daydegrees, with the highest temperature at 22.8°C, meaning it would need to be sampled more than once every 24 hours. While this was attempted, monitoring the eggs 24 hours a day was not feasible, which led to some variation in the sampling times. This effect would be highest in the warmest temperatures, as the daydegrees passed more rapidly. When taking this into consideration, as well as only having one replica for each of the temperatures, the credibility for the decrease in daydegrees is lacking. This is also supported by the conflicting results from Experiment 2. While the given range for hatching time is probably accurate, additional research would be needed to properly investigate the decline in daydegrees. Similarly, only one replica was used for each salinity-temperature combination in Experiment 2, which makes it difficult to determine the accuracy of the model as a single datapoint could influence the results. The overall hatching success in this experiment was also significantly lower than in Experiment 1B, where >50% hatching was observed.

There was also some inconsistency in the shell size of larvae near hatch. While the newly hatched larvae used for Experiment 3 had an average shell length of 182 ± 1 µm (in 16°C), the shell length averages from Experiment 1B for 100 daydegrees old larvae range from 203 ± 1.5 µm to 224 ± 1.7 µm. This indicates that the larvae might have grown 20-40 µm after hatch, or that the egg quality was higher, producing larger spawn. Both egg batches were collected from the same population of adult snails, about one week apart. The exact reason for these results is not known but might be influenced by maternal factors like increased stress in the lab or that it was nearing the end of the spawning season (Moore, 1937; Williams, 1964).

There were some challenges during Experiment 3 and feeding the larvae. As mentioned, high bacterial growth in the flasks increased mortality for the snails and increased during

the experiment despite reductions in feed density and increased rinsing. While the exact consequences for the snail larvae are mostly unknown, it appeared to increase mortality, and making it more difficult for the larvae to feed. Also, as the snails grew it was difficult defining the exact time of settlement , as much of the development happens gradually, and settlement behaviour can be observed without the larvae fully metamorphosed (Branco et al., 2014). Lastly, sampling of dry weight was attempted, but was abandoned as larvae completely disintegrated when exposed to distilled water unlike the adults, who are able to withstand freshwater exposure for several days (Sokolova et al., 2000). This was speculated to be due to their tissue not being able to withstand the osmotic pressure at such an early stage, but such an extreme response to freshwater exposure is, to the authors knowledge, not seen in gastropods before. While shell length is a generally accepted measurement in gastropods (Branco et al., 2014; Fischer, 2012; Hohenlohe, 2002), having dry weight could help evaluate the growth in the second half of the larvae culture, as getting exact shell measurements became increasingly more difficult as the shells grew and become increasingly coiled.

4.5 FUTURE PROSPECTS

Overall, the results in this study show that the embryos of *L. littorea* are able to develop in a range of different salinities and temperatures. While the early development is generally considered a bottleneck for production, studies have found gastropod species with a larger temperature tolerance (Diederich & Pechenik, 2013), and the embryonal stage is also rather robust due to a number of protective factors loaded into the eggs (Hamdoun & Epel, 2007). Increased numbers of heat shock proteins are found in *Littorina* (*L. scutulata* and *L. plena*) with planktotrophic larvae to help the eggs survive in the unpredictable environment (Lee & Boulding, 2009). However, to produce the highest quality larvae for aquaculture production heating of the water and salinity monitoring might be beneficial.

While the larvae of *L. littorea* were able to survive until hatch on both *R. baltica* and *D. tertiolecta*, the latter microalgae might be best suited for commercial production of the snails as it produced larvae with less variation in size. While the successful rearing of larvae until settlement is a huge step forward, more research is still needed towards the settlement and early juvenile phases, which are speculated to be the real bottleneck (Branco et al., 2014; Hohenlohe, 2002; Jablonski & Lutz, 1983). While some disagreement on the area (Pechenik & Tyrell, 2015), rearing larvae through metamorphose might provide them with a fresh start towards maturation, eliminating the effect of suboptimal rearing conditions (Diederich et al., 2011; Fischer, 2012).

5 CONCLUDING REMARKS

The present study showed how different factors like temperature, salinity and larval diet can influence the growth and survival of the egg and larvae stages of *L. littorea*. Besides producing a description of the embryonal development, a significant effect of temperature was found the embryonal development, both influencing time until hatch and shell length, with a strong effect of too cold water. Salinity also influenced the eggs, as the developing embryos had decreased hatching success in lower salinity. By combining the factors, a temperature and salinity optimum was found, which can be applied into future aquaculture. The embryonal sensitivity to both cold and warm water is also useful knowledge for the conservation of the species, especially with regards to climate change and more extreme weather. The larvae showed strong preferences in algae diets, and preferred large-celled live algae, compared to smaller algae pastes. While the exact nutritional profiles for veliger larvae are not found, the study indicates that the species have a low dependence for marine lipids and might rather prefer a high carbon content.

Overall, the study proves that rearing the snail in a farming environment is feasible, and while some issues remain this opens the door for future aquaculture and increases the knowledge of the beautiful world of gastropods.

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APPENDIX 1 – SHELL LENGTHS AND SGR FOR EXPERIMENT 3

Table 4: Shell length and Specific growth rate (SGR) for *L. littorea* larvae

Diet	Day (*SGR-interval*)	Length ± SE	SGR	N
R. baltica	5	242 ± 3		49
	9 (*1-9*)	283 ± 5	0.054 ± 0.003	53
	14	301 ± 8		37
	18	321 ± 8		25
	22 (*9-22*)	350 ± 4	0.016 ± 0.001	54
	(*Total*)		0.031 ± 0.001	54
D. tertiolecta	5	236 ± 3		38
	9 (*1-9*)	274 ± 3	0.051 +. 0.001	48
	14	296 ± 4		50
	18	331 ± 5		26
	22 (*9-22*)	340 ± 3	0.017 ± 0.001	35
	(*Total*)		0.030 ± 0.003	35
Nannochloropsis sp.	5	194 ± 2		55
	9 (*1-9*)	191 ± 1	0.006 ± 0.001	50
Rotifer diet	5	198 ± 2		44
	9 (*1-9*)	195 ± 1	0.010 ± 0.001	50
Starvation control	5	187 ± 2		36
	9 (*1-9*)	190 ± 1	0.007 ± 0.002	48

APPENDIX 2 – LENGTH MEASURES AT 110 DAYDEGREES

Table 5: Length measurements of *L. littorea* larvae 110 daydegrees after spawn. The embryos were incubated in different temperatures presented in the table.

Temperature	Average ± SE	Significance code:	N
4.8			
6.9			
9.1	203 ± 1.5	c	90
11.3	219 ± 1.2	ab	90
13.7	217 ± 3.0	ab	57
16.0	223 ± 1.6	ab	94
17.3	224 ± 1.6	a	83
19.1	216 ± 1.1	b	93
20.9	203 ± 1.0	c	92
22.8	205 ± 1.2	c	90

APPENDIX 3 – ABIOTIC FACTORS

Experiment 1A: Description of embryonal development and egg morphometrics

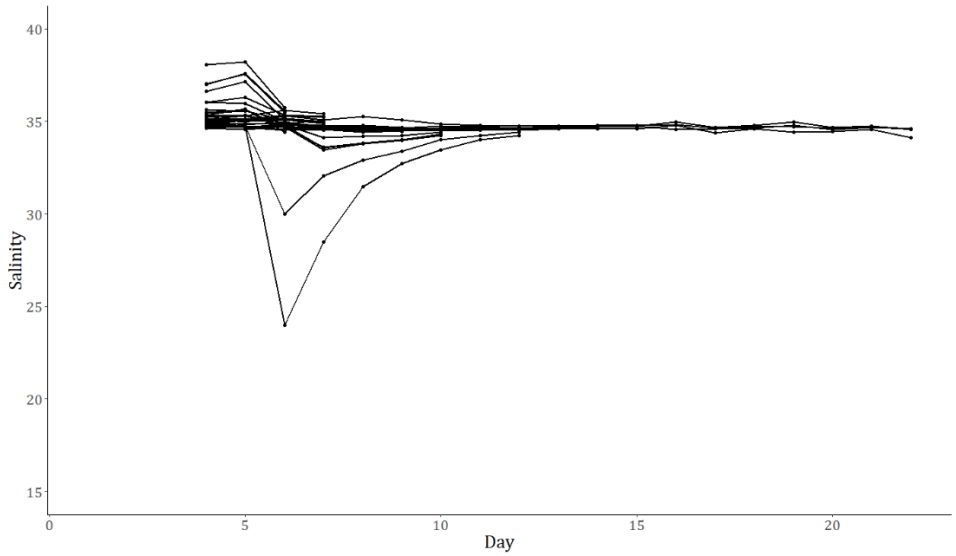

Figure 26: Salinity (ppt) measurements for all temperature treatments.

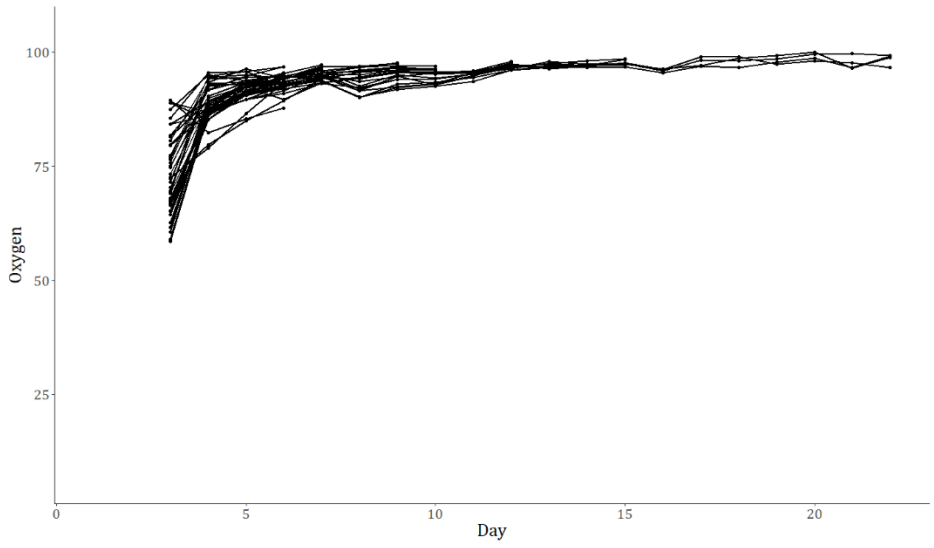

Figure 27: All measurements of oxygen in the egg development tanks. Lower values (>58.6 %) were found in the beginning of the experiment, while the average values were much higher (~97 %)

Experiment 3: Effect of different microalgal diets on growth of *L. littorea* larvae

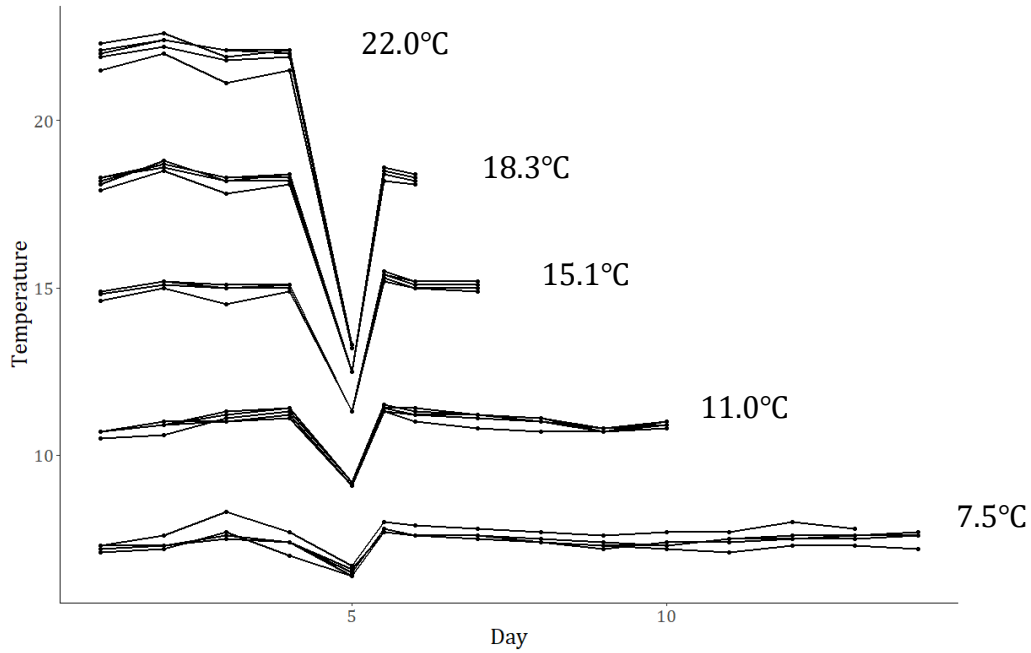

Figure 28: Temperature over time for the five temperatures used during the salinity experiment. A technical problem on day five caused the temperature to sink on day five.

APPENDIX 4 – FEEDING DENSITIES

Table 6: Feeding densities of the four microalgal treatments. All densities are in cells/mL. Days marked with an asterisk '*' signify a change in feeding density.

Day	*R. baltica*	*D. tertiolecta*	*Nannochloropsis sp.*	Rotifer diet
1	100 000	100 000	200 000	200 000
2	100 000	100 000	200 000	200 000
3	100 000	100 000	200 000	200 000
4	100 000	100 000	200 000	200 000
5	100 000	100 000	200 000	200 000
6	100 000	100 000	200 000	200 000
7 *	50 000	50 000	100 000	100 000
8	50 000	50 000	100 000	100 000
9	50 000	50 000	100 000	100 000
10	50 000	50 000	100 000	100 000
11	50 000	50 000		
12	50 000	50 000		
13	50 000	50 000		
14	50 000	50 000		
15	50 000	50 000		
16	50 000	50 000		
17	50 000	50 000		
18 *	40 000	30 000		
19	40 000	30 000		
20	40 000	30 000		
21	40 000	30 000		
22 *	35 000	25 000		
23 *	30 000	20 000		
24	30 000	20 000		
25	30 000	20 000		
26	30 000	20 000		

APPENDIX 5 – SETTLEMENT PREFERENCES EXPERIMENT 4

For the final part, substrate preferences in settling larvae were investigated. First, a pilot experiment was set up, using living snail larvae ready to settle distributed between two beakers keeping 16°C. The two beakers both contained a variety of different substrates (Table 7) were some were conditioned, meaning they had been stored with the adult snails for min. 7 days. The clean substrates had been cleaned at 60 °C before use. This was to see if the snail larvae responded from chemical cues from the adult snails. The substrates were gently removed from the water and put into a small glass container before being investigated. The substrate and the water around were thoroughly examined in a light microscope before being gently put back into the tanks. In addition to the substrates, the tanks had plenty of *U. lactuca*, which were also examined for larvae (Figure 29a). The pilot experiment lasted for 10 days and were examined every day.

The main experiment was set up using newly hatched larvae, reared in oxygenated tanks keeping 16°C. When the larvae were nearing hatch they were split between to larger tanks which contained a variety of different substrates (Table 7) set on top of *U. lactuca* (Figure 29b). The substrates were investigated twice a week, using the same method as in the pilot experiment.

Table 7: Different substrates used during the experiments

Pilot experiment	Conditioned	Rock
		Biofilter plastic (White)
	Cleaned	Rock
		Biofilter plastic
Experiment 4	Conditioned	Membrane (Black)
		PVC plastic (White)
		Scallop shell
		Rock
	Cleaned	Membrane (Black)
		PVC plastic (White)
		Scallop shell
		Rock

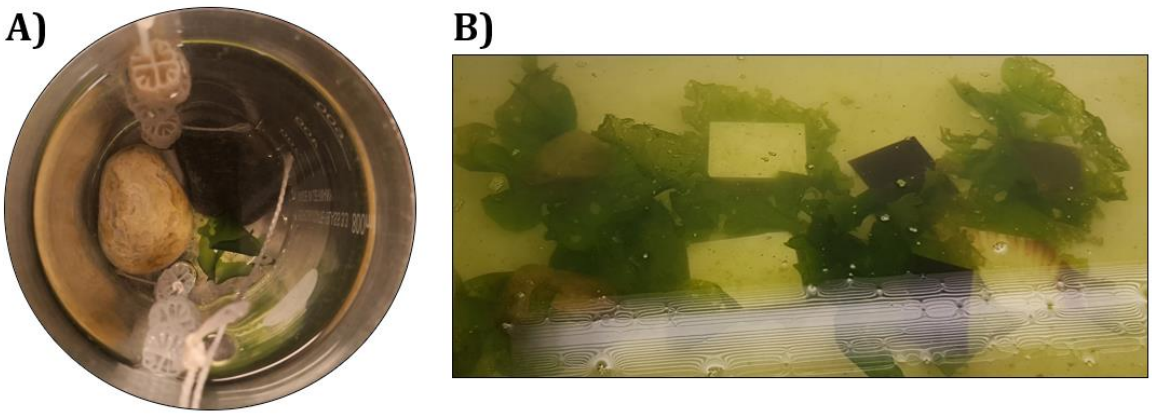

Figure 29: A) Setup of the pilot experiment. B) Setup of the final experiment, with green water after the summer

Water conditions

Water conditions (Temperature, salinity, oxygen, pH) were regularly measured in both experiments. During the pilot experiment it was measured daily, and during the full experiment it was done 4-5 days a week.

Feeding

During both experiments, the water was sampled daily, and algae concentrations were measured. The water concentration was kept at between 30 and 40 000 cells/mL, for the entire pilot-experiment, and until day 19 in the main experiment, when the feeding was stopped. Due to little aeration in the tank, most of the algae settled on the bottom, and everything was covered in a biofilm, which would provide sufficient food for the snails, which was assumed to be settling.

Problems and discontinuation of study

The experiment faced several problems, which lead to it not having significant results. The young snails were difficult to see on the dark substrate materials, and as the biofilm grew, snails attached to the substrates and were unable to move. The mobile snails were also very easy to accidentally flush of the substrate when removing it from the water. Due to the high feeding rate, and the decay of the *U. lactuca* used, the tanks became covered in algae, and most likely inhabitable for the snails. Observations became less and less, and at the end the entire tank were searched without finding a single snail. These results are therefore not included further.

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