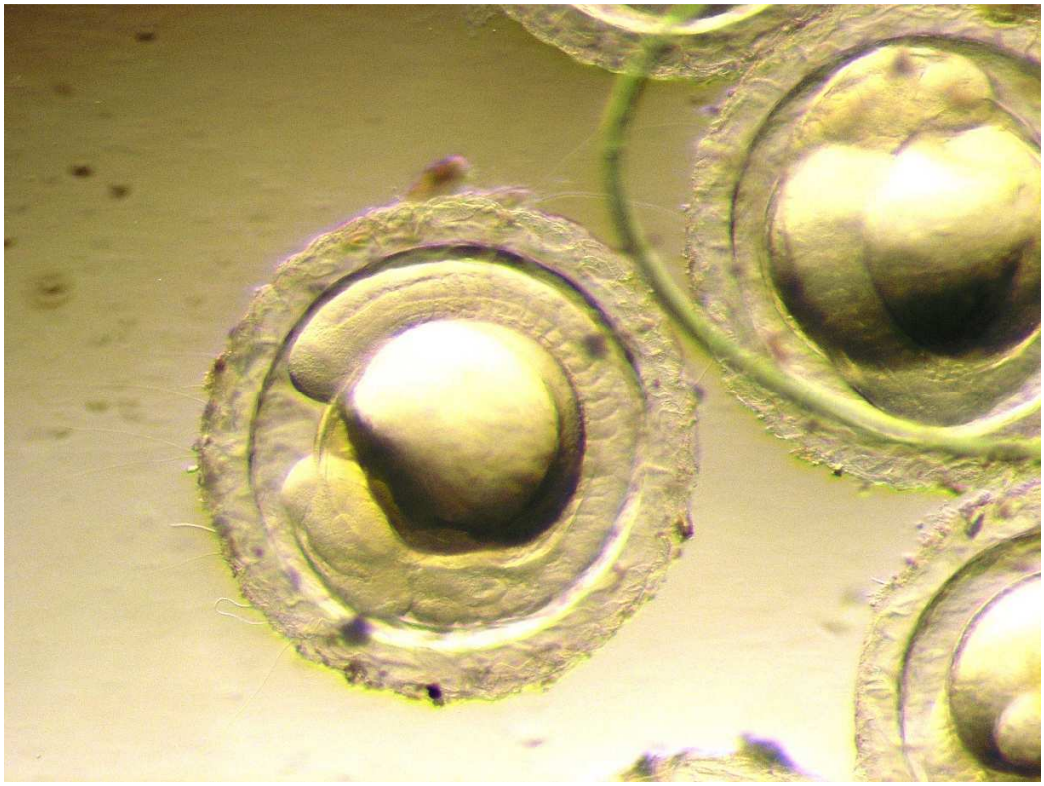
University of Bergen

Department of Biology

Master program in Aquaculture Biology

Master thesis:

Temperature dependent egg development and impact of light regimes on incubation of Ballan wrasse (*Labrus bergylta*) egg.



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Content:

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Bergen 09.09.2014 Lukasz Rembelski

1. Introduction

Atlantic salmon farming started in the 1960s in Norway and since that time has become a strong industry, with an annual 1million tonnes production in 2012. During the 50 years of development, the aquaculture industry has struggled with a number of serious biological threats like bacterial and viral outbreaks, which threatened its existence. Nowadays the salmon industry faces the problem of the ectoparasite salmon lice, which if untreated can cause significant fish losses (Wootten et al. 1982, Pike 1989), both in aquaculture, and among wild populations. The problem of salmon lice has reached the point at which traditional measures like pesticides have become ineffective. The usage of pesticides has reached its maximum and has never been so high since the beginning of the industry (Norwegian Institute of Public Health, March 2013). Fighting the salmon lice has become an international problem.

Nowadays, the only available alternatives to pesticides are cleaner fishes, like wrasse fishes, among which the most suitable for that purpose is Ballan wrasse *(Labrus bergylta).* Wrasse fishes have been used in the industry since late 1980s, but its usage is now increasing. This may cause depletion of that species along the Norwegian coastline (Espeland et al. 2010) . To avoid overfishing, the best solution is farming of wrasse, to provide a constant supply of fishes according to the industrial needs. In 2009 it became reality, when the first hatchery of Ballan wrasse was established by Marine Harvest Labrus AS in Øygarden. It was preceded by a number of research and trial rearings performed by different research institutes such as the Institute of Marine Research at Austevoll, which provided basic knowledge used for preparation of production protocols. Based on that experience, the production has been established, although there are still many areas which lack knowledge. Industrial intensive production requires knowledge of the entire life cycle of a new species, from egg fertilization through to broodstock

manipulation, which makes such a project highly risky and challenging. That is why there is a need for information, which could gradually fill in all the gaps and will encourage the aquaculture industry in production and use of Ballan wrasse as a measure in fighting salmon lice. The success of intensive production of Ballan wrasse is a win-win situation, which benefits not only industry by minimizing the loses caused by salmon lice, but also gives ecological and environmental advantages. These include prevention against degradation caused by salmon lice on wild salmon population, depletion of wild population of wrasse species, minimizing the use of pesticides and their negative impact on non-target species and others. That is why the support provided by the science institutions to Ballan wrasse production is crucial and has a reflection in many dimensions of environmental and ecological aspects. Following that way of thinking, the objective of this master theses is to provide experimentally confirmed information about the most preferable temperature to incubate Ballan wrasse egg in the temperature range from 8°C to 16°C and assess the impact of photoperiod on egg development and viability of newly hatched larvae. Because of the very limited amount of research in this area, this study could contribute valuable knowledge in that matter.

1.1 Biology of Ballan wrasse

Ballan wrasse belongs to the family of wrasse fishes (*Labridae*), and with a length from 30 – 50 cm. is the largest among the north European family (Sayer et al. 1996). It has a compressed body, big head rather small mouth with a thick fleshy lips and moderate size conical teeth (Costello et al. 1991). Coloration can vary and depends on the surrounding environment, with light green color of juvenile and darker green and brown colors with spots along the body of adult individuals (Munk et al. 2005). It has a life-span of 17 years (Sayer et al. 1996) and is a very common fish observed in Norwegian coastal waters to north the county of Trondelag and its

range also extends south to Morocco (Salvanes et al. 1993). Ballan wrasse is a protogynous, sex changing species which means that all individuals start as females and at the age of 6 years, some changes into males. They show territorial behavior and react with aggression against competitors especially during the spawning season, which occurs in early summer from April until August depending on latitude (Darwall et al 1992) . They are multiple, batch spawners, which build nests or spawn on gravel or rocks. Egg are demersal and consist of a sticky external layer, with a light-white, transparent color. The egg have an oil globule and are 0,7 - 1,1 mm in diameter (Artuz et al. 2005).

1.2 Impact of temperature on egg incubation in fish

Temperature dependence of egg development is a well described phenomenon of fish embryos and was firstly experimentally confirmed by Dannevig et al. (1895), who showed that increased temperature accelerates egg development resulting in the reduction of incubation time. The embryo stage is the most sensitive to temperature changes among all development stages of fishes (Brett et al. 1970), with its highest vulnerability in very early development during cleavage and gastrulation (Rombough et al. 1996). The tolerance to temperature change increases with the embryo age and is limited by the species temperature extremes, which is often 3°C to 6°C either side of the optimum (Rombough et al. 1996). For example Atlantic salmon embryos are more temperature resistant for upper or lower temperature challenge after the blastopore closure (Bryant et al. 1989, Servizi et al. 1992). Crossing over the borders of the optimum range has a negative impact, on hatching rate and survival and can cause increased numbers of malformations and abnormalities in the embryo. Each species has its own optimum incubation temperature, and development rates can be different among the species. Egg size is one of the factors strongly correlated to development rate, where in the same temperature smaller eggs develop faster than

big eggs (Pepin et al. 1991, Rombough et al. 1996). Temperature also affects the size at hatching and efficiency of yolk utilization, which is related to changes in metabolic costs. At lower temperatures within the optimum range maximum conversion efficiency can be observed resulting in the most efficient growth and bigger size larvae, where at high temperatures, maximum ingestion rate with maximum growth rate and small size of larvae were observed (Jobling et al. 1997, Jordaan et al.).

In the literature, there are many studies related to temperature dependent egg development of marine fish species, which test egg incubation at different temperature. Information obtained from such studies includes: development rates, daily egg mortalities, hatching rates which are used for estimating daily egg production of species , estimating stocks biomass or ecosystem modeling which are used mainly by marine ecologists and fishery scientists (Fox et al. 2003, Armstrong et al. 2001, Dickey-Collas et al. 2003). But the same methods used by ecologist in egg incubation are also used by aquaculture industry for gaining information about species optimum temperature range for use in intensive production. These data are crucial for receiving good quality larvae in successful hatchery production.

Although there are many studies about embryonic development of commercially and ecologically valuable marine species, there is very limited information about Ballan wrasse, which is narrowed to only two research studies (D'Arcy et al. 2012, Artuz et al.2005). One of the studies Artuz et al. (2005) was conducted on the population of Ballan wrasse in Marmara Sea in Turkey. Studies of embryonic development were a part of an investigation, which was followed up to the juvenile stage, with a main objective of studying spawning biology for ecological and conservation purposes. This research gives a general overview of species biology characteristics and focuses mainly on the morphological changes during development from prelarvae to juvenile stage. The egg incubation, and data collection process is poorly described without division of

embryo development into stages and without presentation of incubation results such as mortality rates and hatching rates.

In D'Arcy et al. (2012), studies were conducted on eggs provided from two different stocks of Ballan wrasse spawning naturally in Norway and fertilized artificially in Ireland. This study concentrates deeply on egg development with a main objective of standardization of development stages, which can be used as a tool for better comparison between different stocks. Embryo development was divided into 8 main stages and 14 substages, with a description of a morphological characters and key features at every sub-stage. This research also describes a temperature dependent development of egg, showing data at 50% hatching time at different incubation temperatures, which have been collected from various incubation experiments in Scotland, Ireland and Norway and summarized. It has been concluded that at various temperatures (from 10°C -17.5°C), egg development rates were different and inversely related to ambient temperature. Although experiments were conducted at different temperatures, the complete range is missing, especially on the Norwegian population, which was incubated only in 12° C. The study does not present data of mortality rates and any other indicators of larval quality like deformity rates, larval length or yolk-sack volume, which could possibly indicate the most suitable temperature for aquaculture purposes.

1.3 Impact of light on egg incubation in fish

Light can be described by intensity, spectral composition or by photoperiod. All these three properties can have influence on embryonic development, hatching rate, hatching rhythm and larval size at hatching (Downing et al. 2002). Photoperiod is a daily proportion of light to dark hours, among which constant light, constant dark and 12 light and 12 dark regimes are most

commonly observed in egg incubation research. Light is detected by the pineal gland, which stimulates secretion of melanine hormone, which is well known for control of circadian rhythm in fishes (Boeuf et al. 1999). Light can accelerate embryonic development as in the Obscure puffer *Takifugu obscurus* (Yonghai et al. 2010), or delay development as in the Atlantic halibut *Hippoglossus hippoglossus* (Helvik et al. 1992), and affects larval length at hatching as in Haddock *Melanogrammus aeglefinus,* where longer light exposure resulted in smaller larvae (Downing and Litvak 2002). Hatching rate can be influenced by photoperiod where combination of light and dark showed the best result in Zebrafish *Danio rerio* and Senegal sola *Solea senegalensis* (Villamirez et al.2013), (high hatching rate results are a consequence of low mortality, suggesting the impact of light regime on survival as well) , or not affected like in Obscure puffer (Yang et al. 2004). Thus, photoperiod can influence many aspects during embryo and larval development, and it is reasonable to identify its impact on Ballan wrasse embryos, since there is no information available on light regime preferences during the incubation process in that species.

1.3 Objectives of this study

The objective of this study is to provide experimentally confirmed information about impact of temperature and light regime on survival, development rate, hatching rate and larval length at hatching, of *Labrus bergylta* egg. The results will be used for comparison and selection of the most suitable temperature and light regime for egg incubation in artificial conditions in intensive aquaculture industry. To achieve that goal, the egg incubation experiment was designed with temperature range from 8°C to 16°C, and two light regimes with constant light (24 light) and 12 hours of light and 12 hours of dark (12 light).

2. Materials & Methods

2.1 Broodstock culture

For the purpose of this experiment eggs were provided from Marine Harvest Labrus AS in Øygarden Ballan wrasse hatchery. Total number of broodstock population in this hatchery is around 350 females and 100 males, and these fish have been selected from wild caught individuals in 2009, which were quarantined and screened against disease and parasites and stocked in two big communal tanks. Fishes in tanks are kept under controlled conditions, with water temperature 14°C, salinity 35 ppt, oxygen 87%, and photoperiod, which is designed to suit production needs for two spawning events yearly. Spawning periods are arranged in spring (around the middle of March) and autumn (around the middle of August) and last about three to four weeks. A few weeks before spawning time, the fishes are divided into groups and transferred into smaller broodstock tanks with ratio of around 40 females and 10 males. These tank are 4 meter diameter and 1,5 meter height with water volume of 15 cubic meter each, with eight tanks in total. The tanks have been equipped with specially arranged plastic bags to simulate seaweeds, and pcv pipes which both create the possibility for fish to express their natural behavior of hiding and resting in the tank. The fish spawn naturally. During the spawning period, green mats with a size of 50 x 100 cm. are placed at the bottom of the tanks as a spawning substrate. The presence of egg on the mats is checked daily, in the morning. After spawning, sticky demersal egg attach to the surface of mats, and can be taken out from the tank and assessed. If the quantity of eggs on mat are sufficient, they are transferred into the incubation unit. If the number of the eggs on the mat are too low, the mats are rinsed with seawater and placed back in tank. This routine is followed until the end of the spawning period.

2.1.1 Incubation process at Labrus AS

In the morning during daily check of broodstock tanks, green mats are taken out from the tank and checked. When the spawning occurred, mats are hanged next to each other in the green dark color, cylinder incubators. Each incubation unit has around 0,7 cubic meter volume and has a constant water flow. Incubation water temperature is 12°C with 35‰ salinity and light regime is 8 hours of light, which is provided by upper lighting, and 16 hours of dark. However, during light phase the light access inside incubator is limited by incubator depth, its dark color and mats hanging next the each other. After 8 to 9 days mats are taken out and hatched larvae are transferred to tanks.

Because of the fact that this is a production company, without research and development division, the use of degree days in data analyses process is limited. The incubation temperature had been chosen (12°C) and is kept through all incubation process during each spawning season. That is why there is no need for prediction of development time through temperature range. The data collection process during incubation is limed to a daily check of stage progression.

2.2 Egg collection for this study

The collection of eggs was planned during the autumn spawning season in 2013, and executed one week after beginning of spawning on 14th of August. After arrival to the hatchery at early morning, a routine procedure of checking the mats was followed, and one with high number of egg was chosen for further fertilization efficiency check (fertilization quality). A sample of around 100 egg was scratched from the mat surface and assessed under binocular microscope, and chosen for the experiment. Then the mat with egg was cut into smaller pieces of 30 x 50 cm. and carefully placed in a clean plastic bag filled with water from spawning tank and placed in insulation boxes for transport. Before closing the bags, basic water measurements were made: temperature 10,9°C, salinity 34,9 ppt, oxygen 87%. After that, the bags were tightly sealed,

insulation box closed and packed into the car and transported to the laboratory at University of Bergen, which took around 1 hour.

2.3 Experimental design

For the purpose of the experiment a static incubation system was chosen as an incubation method. The static incubation system is well described and is often used incubation method (Geffen et al. 2012), which consists of an aluminum block, heated at one end by HU1000 Thermocirculator (also BETTA-TECH Controls) and cooled at the other by using a CU-400 Heater/Chiller Circulator Unit (BETTA-TECH Controls, Buckinghamshire, U.K.). This provides a temperature gradient, which can be set up to suit required demand. For this experimental design, the static incubation unit was set up to provide temperature range from 8°C to 16°C and placed in a closed temperature controlled room, with ambient temperature 12°C and constant light regime (24 hours) , which were set up and automatically controlled by computer system. In the temperature range from 8°C to 16°C, five temperatures were selected for egg incubation: 8°C ± 0,5 , 10°C ± 0,5, 12°C ± 0,5, 14°C ± 0,5, 16 °C ± 0,5. Temperature was constantly monitored through the temperature range by electronic thermometer C8600 Tempscan Modular Precision Thermometer (Comark Limited, Welwyn Garden City, U.K.) and manually in others by portable thermometer. Because of the limited space in the incubation unit, two light regimes were selected: 24 light, and 12h light - 12h dark. To achieve this effect, a polyestyrene wall was installed in the middle of the incubation unit creating two sections. In first section constant light regime (24L) was provided and in second section 12 hours of light and 12 hours of dark regime (12L-12D). The light phase was provided by 1 meter long fluorescence fixtures placed one meter above the incubator providing light intensity of 26,96 µmol. The dark phase was obtained by covering the top of the incubation section by an opaque lid (top), and was moved manually every 12 hours as a

part of the sampling procedure. In each light regime (incubation section) two jars (replicate one and replicate two) for each temperature were placed giving two replicate for each temperature and light regime. The experiment was repeated twice.

2.4 Egg preparation for incubation

Two days before arrival of the egg, the incubator was set up and controlled to reach the desired temperature range. In the controlled temperature room, 12°C temperature and constant light regime 24 hour light was adjusted. After arrival to the laboratory, the insulation boxes with the egg were placed and opened in the laboratory room, where water physical parameters were measured: temperature 11,8°C , salinity 35,1 ‰, oxygen 85% in both boxes. After measuring water temperature, two tanks with 20 liters of seawater were prepared (temperature 12 °C, 35‰ salinity) and mixed with 2 ml of pyracetic acid (market name - Pyceze), where mats with eggs were transferred for 30 minutes disinfection. After that time, water in tanks was exchanged with fresh seawater (12 °C temperature, 35‰ salinity), and mats with egg were cut with scissors on small 6 x 6 cm pieces. Than 20 jars, which were previously disinfected with ethanol, washed and dried, were filled in with 500 ml of seawater. Small pieces of mats with egg were transferred into jars and placed in the incubation section and labeled with information: light regime, temperature and replicate number. Since that moment, the incubation experiment started and time was counted from the point zero, in hours [H]. To avoid thermal shock of egg, all 20 jars had initial water temperature of 12°C (35‰ salinity), and were placed in incubation section in temperature range from 10°C to 14°C. It takes around two hour for the incubator unit to warm up or cool down water for 2°C. After two hours, jars from 10°C were moved to 8°C and jars from 14°C to 16°C, what after two hours resulted in establishing desirable temperature range. Acclimatization procedure which was used, ensured that egg had a slow temperature change (2 °C every hour).

2.5 Data collection and sampling procedures

Sampling was executed according previously prepared sampling form, which was printed for every jar (replicate) (Appendix.9), where all data from experiment were noted and collected at the end of experiment. Sampling was carried out every four hours until 48 hour after incubation starts. Then the sampling interval changed to eight hours until 128 hour after incubation starts. After that time sampling interval changed to 12 hours and followed until the end of incubation process which was 320 hour after incubation began in the lowest 8°C degree temperature. Every sampling always started from 24 hour light regime and followed from the highest temperature (16°C) to the lowest (8°C). After finishing 24 light regime, sampling of 12 light/12 dark regime was performed, except samplings, which coincided with 12 hour dark phase when incubation room was covered and access was prohibited.

Firstly, egg were sampled from each jar by scraping 10 to 15 egg from the surface of mats by small spoon and placed into sampling plate chambers with a small amount of water (2 drops) which avoid them drying. The sampling plate with egg was then transferred to examination room and assessed under binocular microscope. Dead eggs were counted, and every live egg was staged and the number of egg in each stage counted. All the data were noted in each replicate sampling form and after that, egg were discarded. An egg was assessed as dead if:

- the color of the egg significantly differs from majority of egg in the sample
- development of the egg was at least two or more stages behind the rest of the samples
- embryo was severely deformed.

Secondly each jar with egg were examined on appearance of hatched larvae, which were then sucked gently by pipette to the petri dish. Then larvae were euthanized by pouring 90% concentration ethanol, and the number of larvae was counted and noted in the sampling form.

Depending on the number of hatched larvae in every jar, preferably 20 to 30 dead larvae at every sampling were selected randomly for photographic documentation. Photos were executed under binocular microscope, with specially installed camera, and afterwards named and collected on computer hard drive together with a scale photo. Finally, the larvae were discarded.
Additionally with every sampling, water temperature was measured and water was exchange every 24 hours. Water exchange was made by sucking gently water from jar by 200 ml transparent syringe, which allowed control and avoidance of discarding egg together with water. First 16°C fresh seawater was prepared by mixing warm and cold seawater from the pumping system in the lab. Then water was exchanged in the jar by sucking around 400ml of old water and gently refilling with fresh seawater. After finishing all 16°C jars, procedure was repeated for the other temperatures.

2.6 Staging criteria

Research conducted by D'Arcy et al. (2012), which were dedicated to embryonic development of two different stocks of Ballan wrasse, authors concentrate deeply on egg development with a main objective of standardization of development stages, which can be used as a tool for better comparison between different populations. As a result embryonic development of *Labrus bergyltha* was divided into eight main stages:

Ia	Zygote
Ib	Cleavage
II	Blastula
III	Gastrula
IV	Segmentation (220-360° circumference)
V	Segmentation (360-435° circumference)
VI	Segmentation (410-480° circumference)
VI+	Hatching (480-510°)

and 16 sub-stages, with a description of a morphological character and key features at every sub-stage. Basing on the above description, staging form was created, at which developmental events were divided into 13 stages starting from middle of the gastrulation process and ending on hatched larvae, giving very good resolution of development progression. Table under illustrates the division of stages with description of key features.

Stage	Morphological features
1 (III)	Mid gastrula. Germ ring is well defined and steadily envelops yolk.
2 (III)	Late gastrula. Blastoderm covers three quarters of yolk; embryonic body becomes clearly visible; the enveloping layer expands uniformly over the yolk until this point.
3 (III)	Early neurula. Circumference 180°; head distinguishable; oil droplets are migrating towards vegetal pole; yolk plug exposed; rudimentary brain. Beak-like mass of cells anterior to head. Kupffer´s vesicle (KV) appears.
4 (III)	Late neurula. Circumference 200°; rudimentary eye vesicle; beak-like mass of cells is still visible; KV enlarges somewhat; blastopore at the vegetal pole starts to close.
5 (IV)	Circumference 220°; two to four somites; eye vesicle becomes slightly more defined.
6 (IV)	Circumference 290°; oil droplets migrate closer together; melanophores appear as pigmentation begins; eyes more distinct; eight to 10 somites; KV large; otolith vesicle; beak-like mass of cells disappears from anterior of head. Flat body cavity posterio-ventral to the brain appears which contains the heart.
7 (IV)	Circumference 310°; oil droplets migrate closer together; KV starts to shrink; 16-18 somites; brain has become more distinct; body cavity expands to accommodate the heart which is tubular.
8 (IV)	Circumference 330-340°; somites number 24-26, some of which become chevron shaped; tail still entirely attached; KV reduces; brain distinctly yellow; otolith vesicle enlarges; lens and cornea evident.
9 (V)	Circumference 360-370°; pigmentation denser; 30-32 somites; KV disappears; cornea and lens distinct; tail begins separation from yolk; beginning of membranes on caudal fin; hear grows.
10 (V)	Circumference 380-410°; Oil droplets coalesce; 36-38 somites with an increasing number being chevron shaped; heartbeat 82 beats min-1; membrane on tail is more pronounced.
11 (VI)	Circumference 410-435°; 40-44 somites; tail reached anterior of eyes; pectoral fin protruding; heartbeat 87 beats min-1; pigmentation denser.
12 (VI)	Circumference 435-480°; Melanophores increase in number and location around the body; 46-48 somites; heartbeat steady at 94 beats min-1.
13 (VI+)	Circumference 480-510°; Oil droplets less conspicuous; 46-48 somites; heartbeat 93 beats min-1; otolith within vesicle are clearly visible; cornea and lens well developed , yet eyes without pigmentation. Hatching

Table 2.6.1 Embryonic development of *Labrus bergylta* , with division on main stages and sub-stages, described by D'arcy et al. (2012). Staging schema for this experiment was based on above criteria of staging features.

2.7 Data treatment

Staging data and mortality data were written down by hand in sampling schema during incubation process and at the end of experiment transferred, collected and processed in Microsoft Excel, and statistical software R. During hatching period larvae were killed, photographed, named and collected on the computer hard drive, and measured in Image J program.

2.7.1 Survival

Total survival data

Total survival data were gained by adding all egg alive from daily samples plus hatched larvae and divided by total number of egg used in the selected temperature and light regime.

$$Total\ survival = \frac{sum\ of\ egg\ alived\ in\ daily\ samples + hatched\ larvae}{Total\ number\ of\ egg}$$

Total number of egg used is a sum of all eggs sampled, hatched larvae and number of dead eggs left after the incubation finished.

Total survival data from tables were statistically analyzed in one-way ANOVA test to indicate significant difference between light regimes and temperature regimes, and two-way ANOVA test for both predictors interaction, light : temperature. (Appendix 1) For presentation purposes a box plot graph with error bars have been plotted in excel (Results - Survival, Figure 1).

Daily survival data

Statistical analyses of daily survival

Daily survival were changed into a percent number, by dividing the number of egg alive by number of egg sampled for each replicate and collected in the table. Table with daily survival data of each replicate were statistically analyzed in one-way ANOVA test to indicate significant difference between light regimes and temperature regimes, and two-way ANOVA test for both predictors interaction, light : temperature (Appendix 2). For presentation purposes a graph with mean daily survival across the temperature range has been plotted. (Results – Survival – Figure 2).

Statistical analyses of survival at stage

Mean daily survival was counted at each stage, and data collected in the table. Statistical analyses were performed with one-way ANOVA test to indicate significant difference between light regimes and temperature regimes, and two-way ANOVA test for both predictors interaction, light : temperature (Appendix 3). For presentation purposes a graph with mean survival at stage across the temperature range has been plotted. (Results – Survival – Figure 3).

Statistical analyses of slope parameter of logarithmic trend line fitted to daily survival in time.

Logarithmic trend line described by parameter ***a***- slope, was fitted to a daily survival in time for each replicate (Results – Survival – Figure 5). All parameter values for each replicate were collected in the table and statistical one-way ANOVA test was performed for light and temperature, and further Tukey contrast test for temperature. For both predictors and its interaction a two-way ANOVA test was performed (Appendix 4). For further observation and

demonstration purposes the mean values of slope, at each temperature was presented (Results – Survival – Figure 6a).

For a better understanding of influence of time on logarithmic trend line a simulation of survival was performed through four incubation periods (8°C-, 10°C, 14°C, 16°C). Total incubation time for each temperature: 8°C, 10°C ,14°C and 16°C was divided into four parts (which simulate stage intervals) and described with survival values: first stage – 1, second-0,8, third-0,6 and fourth-0,4 giving in total same mean values survival in all temperatures. A logarithmic trend line was fitted and slop parameters collected in a table and plotted in relation to temperature range, giving a curve relation, where with decreased time ***a*** slope values decrease.

2.7.2 Stage development data

Weighted age at stage (WAS)

Staging data were converted into average weighted age at stage data for all replicates. At each sampling occasion, the number of egg in stage was multiped by time at sampling. Then, results from each stage were summed up and divided by total number of egg in stage giving age at stage numbers, which were counted for each replicate and collected in a table.

$$WAS = \frac{\sum (nr.\ of\ egg\ at\ stage\ \ x\ time\ at\ sampling)}{Total\ number\ of\ egg\ at\ stage}$$

Weighed age at stage data were statistically analyzed with one-way ANOVA test to indicate significant difference between light regimes and temperature regimes with further Tukey contrast test , and two-way ANOVA test for predictors interaction, light to temperature, light to stage and temperature to stage (Results – Stage development – Figure 3).

50% hatched

Additionally age at 50% hatched (which is a time at which 50% of larvae hatched) were counted and collected in a table for further statistical analyses where one-way ANOVA test was performed to check significant difference between light regimes and temperature regimes, with further Tukey contrast test , and two-way ANOVA test for predictors interaction, light to temperature, light to stage and temperature to stage

Egg development as a logarithmic function (slope, intercept) of time and temperature

Egg development is a function of time and temperature, that is why, it is essential to check the impact of light and interaction of light to stage. That is why, age at stage data were plotted in relation to temperature and logarithmic trend line was fitted, described by parameters ***a*** and ***b***. Parameters were collected in a table and statistically analyzed in one-way ANOVA test to indicate significant difference between light regimes and stage and its interaction of light to stage (Appendix 5).

Graphic presentation was based on mean age values at 5 sub-stages (as indicators of the end of main stage, the last sub-stage of main stage, Figure 2.7.2.1) at each temperature and light regime. Logarithmic trend line was adjusted with parameters ***a*** and ***b*** and collected in the table.(Results – Figure 2).

Sub-stage	A1	A2	A3	A4	B1	B2	B3	B4	C1	C2	D1	D2	H
Main-stage	III Gastrulation				IV Seg. 220-360°				V Seg. 360-435°		VI Seg. 410-480°		VI+ 480-510°

Figure 2.7.2.1. Sub-stage and main stage progression. Color in the sub-stage row indicate the end of main stage. Mean age data from at this sub-stage were used for logarithmic trend line adjustment.

Stage duration

Stage duration time, was calculated from cumulative values of parameters ***a*** and ***b*** for each light regime (for example if we want to count time duration for stage IV, we need to minus value of slope at stage III, from slope value at stage IV) and collected in the table and presented in a results section for stage development (Results – Figure 2). Parameters ***a*** and ***b*** in relation to progressing stages were plotted. Stage duration time date were plotted in relation to time. Both graphs are presented in Results section (Figure 5 and 6) and allows for a better understanding of statistical analyses of logarithmic trend line parameters.

2.7.4 Hatching rate

Hatching rate is a percent of larvae hatched to a total number of egg (minus eggs sampled for daily survival).

$$hatching\ rate = \frac{nr.\ larvae\ hatched\ x\ 100}{total\ nr.of\ eggs - nr.of\ eggs\ sampled}$$

Hatching rate was counted for all replicates in different light regime and temperature, collected in a table and statistically analyzed with one-way ANOVA test to check significant difference between light regimes and temperature regimes, and two-way ANOVA test for predictors interaction, light to temperature (Appendix 6).

To analyze hatching in time, at each light regime and temperature, number of larvae hatched were summed at each samplings occasion (time) and transferred into percentage and plotted on time scale. Graphs were presented and described in the result section (Figure 4).

In the 12 light regime, the number of larvae that hatched were tested with one-way ANOVA to check significant difference between 12 hours of light and 12 hours of dark phase and different

temperature regimes, and two-way ANOVA test for predictors interaction, phase to temperature (Appendix 7).

2.7.4 Larval length

Photographs of larvae from each light and temperature treatment were measured with the image analysis program (Image J). Larval lengths were collected in a table, and statistically analyzed with one-way ANOVA to test for significant differences between light regimes and temperature regimes, with further Tukey contrast test. Two-way ANOVA test for predictors interaction of light to temperature was performed (Appendix 8). Results with mean values and error bars were plotted and graph presented in the result (Figure 1 , 2).

To analyze changes in larval length over time, a mean larval length at each sampling time was calculated for each temperature and plotted in relation to time.

2.8 Assessment of uncertainty the method, and collected data

The static incubation system is a well described and used method for egg incubation in the science literature (Fox et al. 2003, Geffen et al. 2012).

The experimental design was based on two incubations (one after another) during the spawning season with the same material and methods used. The first incubation finished with larvae hatching at both light regimes over the whole temperature range, with 8°C as an exception. Results from this experiment have been used for the purpose of this thesis.

The second incubation was performed on egg from the same source, but not from the same broodstock tank. The second incubation finished with no larvae hatched at either light regime and temperature treatments, with a single hatched larvae as an exception. Results from this experiment have not been taken into account in further analyses of this thesis. The reason for the

incubation failure are unknown. It is very difficult to indicate a direct cause of total mortality at second incubation, but some possible reasons could be mentioned. Eggs were obtained from a different tank (different females and males) causing variation at an individual level, which is connected with egg quality. Different spawning time could also have been a factor, since the second incubation was performed two weeks after the first incubation, and was the end of spawning season. Bacterial outbreak is another possible cause of mortality, but appropriate test were not executed. Different amount of egg in jars could also influence mortality, where at second incubation the average number of eggs used in single unit was around three times higher, and this could negatively affect oxygen level. Each of these described factors separately could cause total mortality of egg, but the possibility is also that the interaction of a few or all factors at the same time.

3. Results

For all statistics, the level of significance was $\alpha<0,05$. Common abbreviations used are: p – probability, F – F statistics from ANOVA. Numbers which describe survival are proportions.

3.1 Survival

Total survival

Looking at the distribution of total survival in both light regimes, we can distinguish a general survival trend, which starts from the lowest values at 8° and increases until reaching its highest point at 12° (optimum) and, then decreasing again. This phenomena is clearly visible in the 12 light regime, where the difference between the highest and the lowest value was 0,33, and less pronounced in 24 light regime, where the difference between the highest and the lowest value was 0,19, because of low survival values at 10° and 12° (Figure 3.1.1).

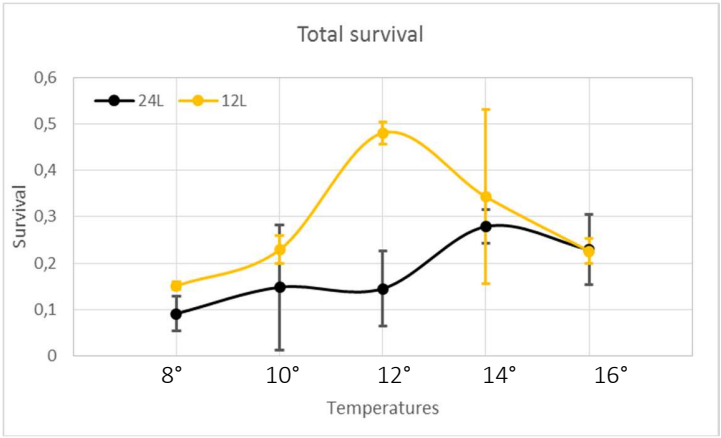


Figure 3.1.1 Mean total survival of two replicates at each light regime with error bars

In general, egg incubated in the 12 light regime had a higher survival than in the 24 light regime, with a mean survival of all five temperatures at 0,28 comparable to 0,17. Although statistically the difference was not significant (1-way ANOVA, $F_{1,18}$=3,25, p=0,08), it can still be seen that there were large differences between the light regimes. In both light regimes the worse survivals were observed at 8°C, with mean of 0,09 and 0,15. Whereas the best survival was seen

at 12°C in 12 light regime with 0,48, and 14°C in 24 light regime with 0,28. Although there seems to be large differences in survival in the different temperatures, there was no significant difference between temperatures (1-way ANOVA,$F_{4,15}$=1,49, p=0,25). There was also no significant interaction between temperature and light on total survival of eggs (2-way ANOVA,$F_{4,10}$=1,21, p=0,37).

Daily survival

Statistical analyses of daily survival based on ANOVA tests revealed significant differences between temperatures (1-way ANOVA, $F_{4,420}$=2.77, p=0.02). On average, daily survival increases from 0,32 at 8°C to its highest point of 0,46 at 14°C and shows significant difference between these two temperatures (Tukey Contrasts Test 14°C-8°C, p-value=0.01), and then decreases again to 0,33 at 16°C. There was no significant difference between the two light regimes (1-way ANOVA, $F_{1,423}$=2.9, p=0.08), but it is clearly visible that both lines vary, because of the low survival at 24 light regime at 12°C. The interaction of temperature and light was not significant (2-way ANOVA, $F_{4,415}$=1.34, p=0.25).

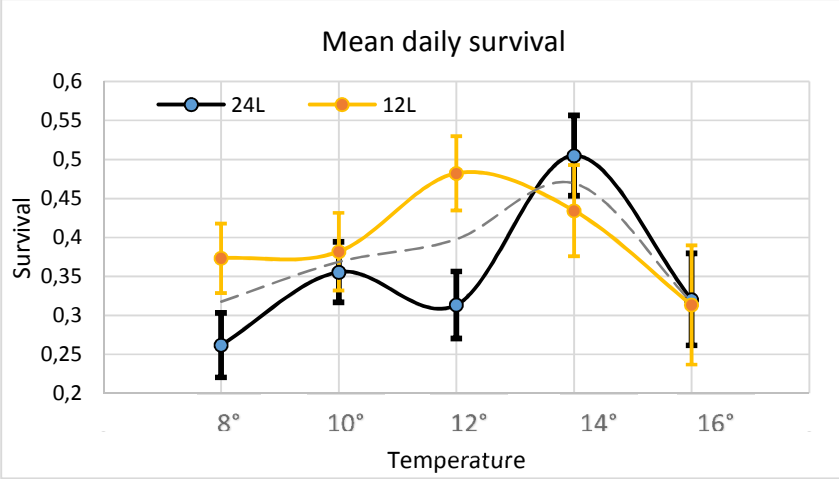


Figure 3.1.2 Mean daily survival over temperature range at 24 light and 12 light regime with standard error bars. Additionally a dashed line with mean values of both light treatment was added for demonstration of daily survival from temperature perspective.

It is useful to examine survival from the perspective of developmental stage. A statistical analyses of survival at stage, revealed no significant difference between temperature regimes (1-way ANOVA, $F_{4,55}$=0.94, p=0.4), light regimes (1-way ANOVA, $F_{1,58}$=0.26, p=0.6), or any interaction of light and temperature (2-way ANOVA, $F_{20,30}$=2.39, p=0.9). However temperature affected survival differently at particular stages, where survival in interaction of temperature and stage showed significant differences at temperature ($F_{4,30}$=12.58, p<0.05), stage ($F_{5,30}$=131.5, p<0.05) and with stage and temperature ($F_{20,30}$=2.4, p<0.05).(Figure 3.1.3)

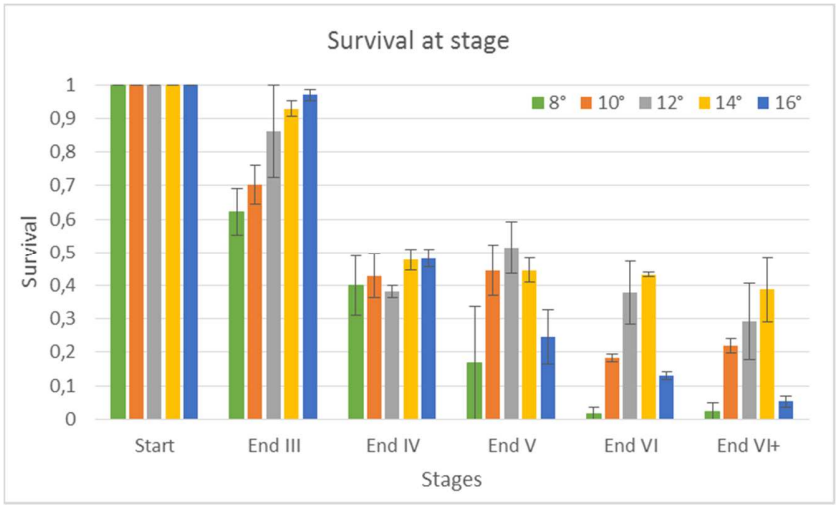


Figure 3.1.3 Mean daily survival values at main stages: III, IV, V, VI, VI+ over temperature range with standard error bars.

Because of the fact that temperature affects daily survival at a stage level and creates strong interactions, its desirable to identify main mortality events during stage progression within temperature treatment. To illustrate this the survival decrease at each successive stage and at each temperature was calculated. (Figure 3.1.4)

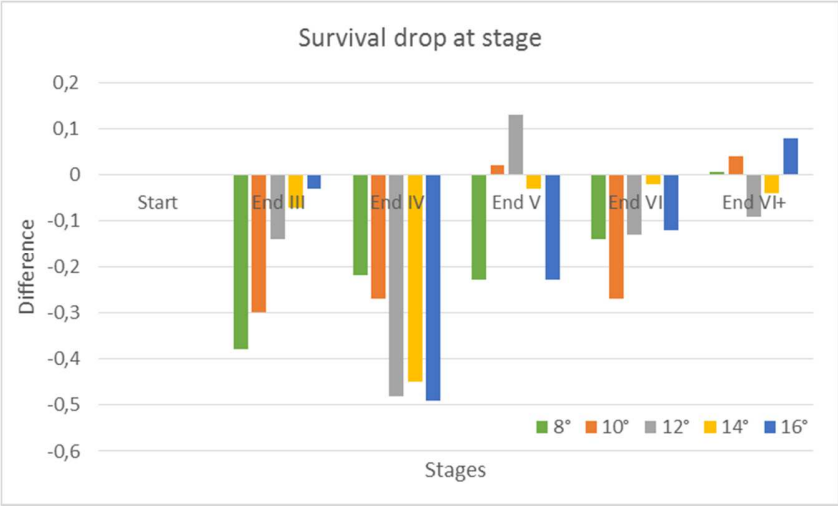


Figure 3.1.4 Decrease in survival at each stage at temperature treatments, which is a difference of mean survival at stage to a previous stage.

In figure 3.1.4, we can see that at stage III and IV sudden and severe drop of survival appeared in all temperatures, whereas at temperature 8°C and 10°C it started at stage III with a drop of around 0,3-0,4, whereas at temperature 12°C,14°C and 16°C it starts at stage IV with drop of around 0,5. This indicates gastrulation and early segmentation stage as very vulnerable stage in egg development.

Observing both figures 3.2.3 and 3.2.4 we can see that after a critical mortality event at the beginning of incubation, survival stabilized. The exceptions are two extreme temperatures: 8°C and 16°C, where survival continued to decline reaching, at the end of stage VI+, the lowest results (from 0,02-0,05) among all temperatures. Finally at the last incubation stage VI+, just before hatching, the highest survival with around 0,4 was observed at 14°C, high results at 12°C with 0,3, and mid results at 10°C with 0,22.

Statistical analyses based on ANOVA test with daily survival and daily survival at stage shows influence of light, temperature and its interactions. Another way of assessment of daily survival

date is by using logarithmic trend line fitted by the lowest square method, and described by ***a*** slope (Figure 3.1.5). Slope values shows an overall daily survival in time and intercept doesn't have a biological value (initial proportion is 1)

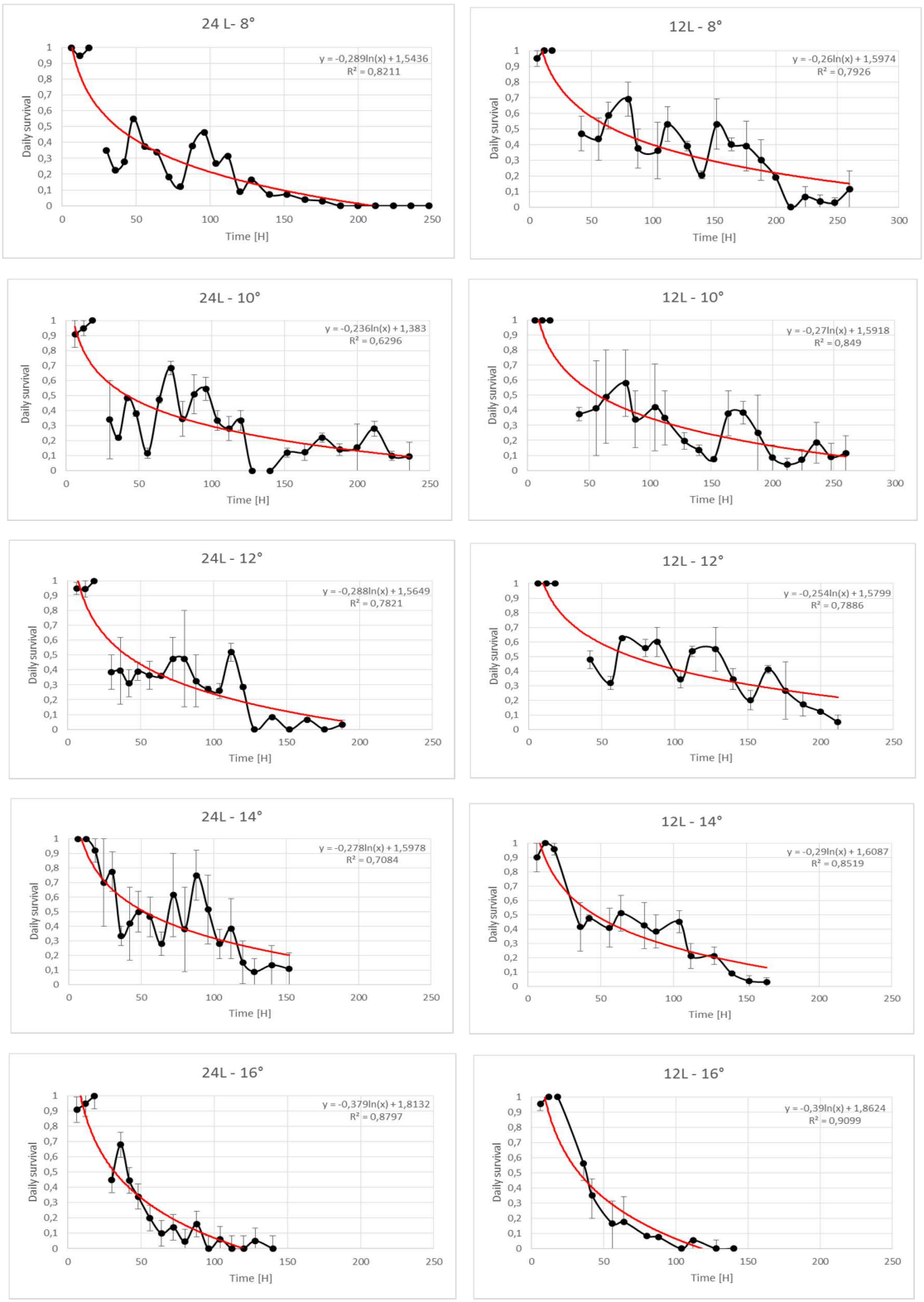


Figure 3..1.5. Daily survival over time with error bars, and with logarithmic function fitted by lowest square method described by parameters ‘a’ and ‘b’ and R2, in light and temperature treatments.

Statistical analyses of parameter ***a*** of the logarithmic function of daily survival in time showed no significant differences between light regimes (1-way ANOVA, $F_{1,18}$=0.01, p=0.92). There was a highly significant difference between temperatures (1-way ANOVA, $F_{4,15}$=15, p=0.003)

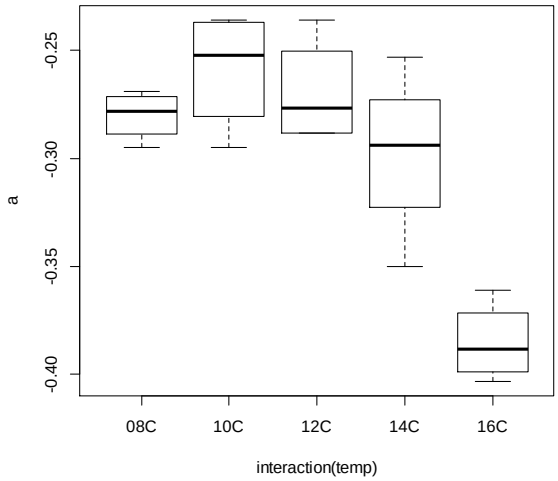


Figure 3.2.5

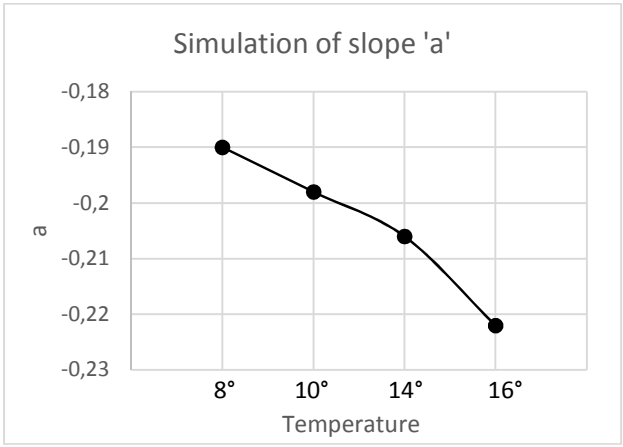


Figure 3.2.6

Figure 3.1.6 Box plots with mean values of log. trend line parameter ‘a’ with mean standard error over temperature range.

Figure 3.1.7 Simulation of slope values over different temperatures, with the same daily survival values.

Further Tukey Contrasts test revealed highly significant statistical differences between 16°C and the other temperatures (Figure 3.1.6). Low mean values of slope -0,38 at 16°C indicate the worst daily survival curve over time, and high values at 10°C with slope -0,25 the best daily survival curve. The slope of logarithmic trend line shows the overall shape of the survival curve, where negative slope values result from shorter incubation time. This has been confirmed in a simulation with the same daily survival at four different incubation durations (simulating time at 8°C,10°C,14°C,16°C). The slope of logarithmic survival trend line decreased with decreasing incubation time, resulting in a worse survival at higher incubation temperatures like 14°C and 16°C (Figure 3.2.7). Considering that fact, the appearance of higher values of slope with

increasing incubation temperature would be an indicator of better overall daily survival curve in time. This suggests that the observed mean values of slope are best at 10°C and 12°C. There was no significant difference in slope between light regimes and no interaction of photoperiod with temperature (2-way ANOVA, p= 0.3), although there were some differences between light regimes at certain temperatures (Figure 3.1.8).

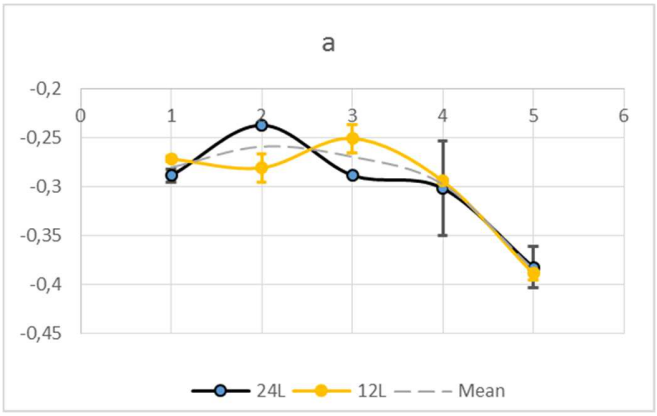


Figure 3.1.8 Values of slope ***a*** at two light regime with error bars, over temperature range connected with line. Additionally a dashed line with mean values of both light treatment was added for demonstration of daily survival from temperature perspective.

3.1.1 Total number of egg used

Labrus bergylta egg are demersal and consist of sticky layer, that allows them to attach to the spawning substrate (green mats). Because of that sticky property of the egg it was impossible to detached accurate number of egg from the mats without use of mechanical forces, which could damage the egg. Thus, egg have been incubated together with the green mats, which were cut into small equal pieces and placed in the jars. However the amount of the eggs on the cut mats varied, since they have not been covered with egg with the same density. Additionally egg are small (around 1mm) and have light white-transparent color that hindered counting and detaching of egg. Taking the above into consideration it was impossible to prepare the same amount of egg at

each incubation unit, and caused variation in egg density (Table 3.1.1.1). The variation in egg density could impact the oxygen level, between the water exchanges (every 24 hour).

			1st Incubation			
	8C	10C	12C	14C	16C	Mean
24L-R1	784	744	1190	1183	344	849
24L-R2	1175	1253	724	610	364	825
12L-R1	762	896	599	573	438	653
12L-R2	1048	734	691	542	268	656
Average						746
			2nd Incubation			
	8C	10C	12C	14C	16C	Mean
24L-R1	2299	2736	2377	1332	1561	2061
24L-R2	2639	2861	1662	1613	1471	2049
12L-R1	2236	2025	2108	1860	1384	1922
12L-R2	3446	2036	2745	2612	1508	2469
Average						2125

Table 3.1.1.1 Total number of egg used in two incubation at both light regimes (24L – 24 light regime, 12L – 12 light regime), both replicates (R1- replicate one, R2- replicate 2) and temperature regime.

The average number of egg used in the second incubation was around three times higher, than in first incubation. The numbers of eggs used in a second incubation could be too high for the water volume for the incubation unit (jar – 500 ml.) This could lead to oxygen level declines, which at the end of each water exchange interval (water shift) could possibly reach low levels, and negatively affected survival of egg. Over time it could cause temporary and repeated oxygen depletion, which could be the possible reason for total mortality in the second incubation.

3.2 Stage development

Eggs developed fastest in the highest temperature 16°C. Hatching starting 109 hours after incubation started at 16°C, and 212 hours at 8°C. Egg development in relation to temperature was curvilinear and was very suitably described by logarithmic function (mean R=0,95) at all stages.

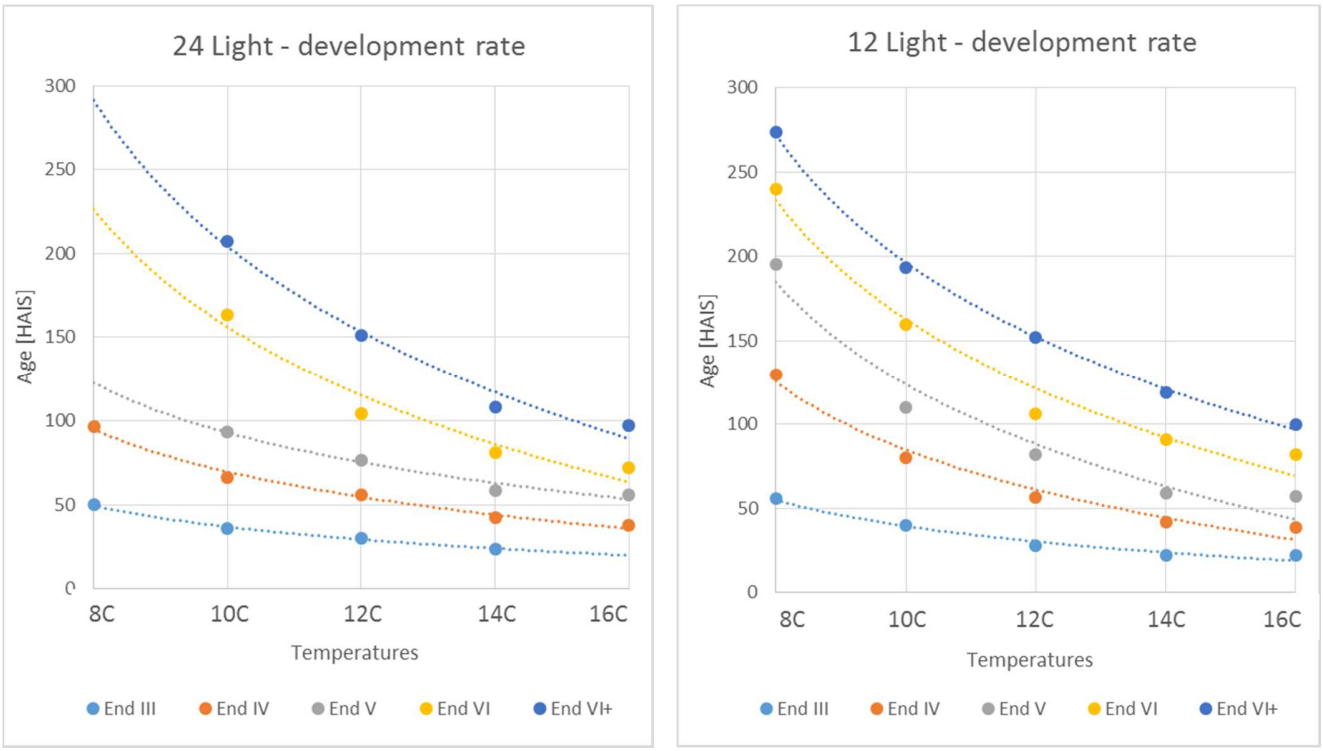


Figure 3.2.1 Age at the end of development stages: III, IV, V, VI, VI+, as a function of temperature.

Parameters of log line					Stage duration			
	24 Light		12 Light		24 Light		12 Light	
	a	b	a	b	a	b	a	b
End III	-18,49	49,69	-22,56	55,178	-18,49	49,69	-22,56	55,178
End IV	-36,82	95,07	-58,45	125,45	-18,33	45,38	-35,89	70,27
End V	-43,11	122,7	-87,82	184,87	-6,29	27,63	-29,37	59,42
End VI	-101,2	226,4	-101,8	233,42	-58,09	103,7	-13,98	48,55
End VI+	-125,4	291,1	-108,6	271,75	-24,2	64,7	-6,8	38,33

Table 3.2.1 Relation of age (end of stage) to temperature described with parameters ***a*** and ***b*** of logarithmic function. Stage duration calculated from parameters of logarithmic functions

The logarithmic parameters in Table 3.2.1, present values of slope and intercept, where ***a*** decreases with development and ***b*** increases with stage progression, showing orderly structured curves. This shows the temperature dependency of egg development, where an increase in temperature accelerates development. One-way ANOVA test of weighted mean age at stage data revealed significant differences among temperatures with $p<0,05$. Further Tukey Contrast tests revealed significant differences with $p<0,05$ in temperature 8°C and 10°C in relation to 14°C and 16°C (Figure 3.2.2).

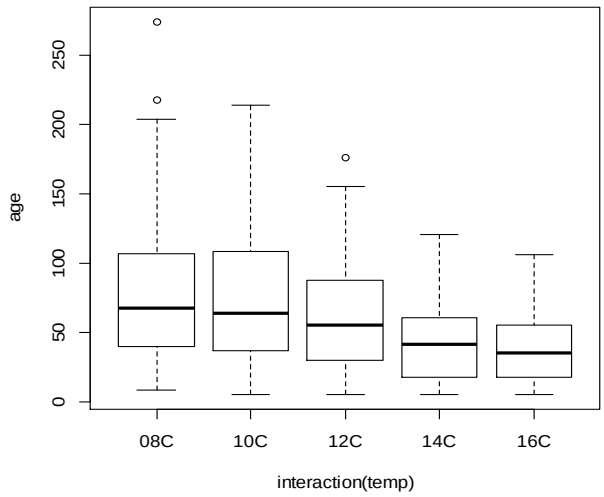


Figure 3.2.2 Box plot graph of weighted mean age at different temperature treatments.

Statistical analyses of age at 50% hatching , which reflects the cumulation over all incubation stages, also showed significant impact of temperature (1-way ANOVA test, $p<0,05$). Further Tukey Contrast test revealed significant differences between all temperatures combinations, except between 14°C to 16°C. (Figure 3.2.3).

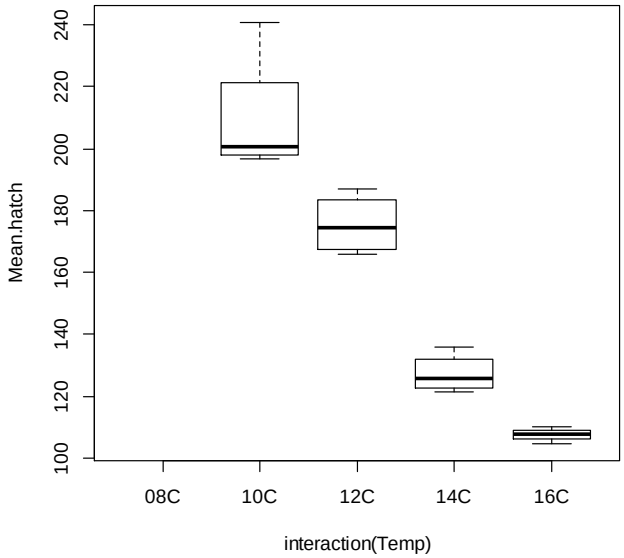


Figure 3.2.3 Relation of 50% age at stage to temperature, showing a statistical differences among the temperature treatments.

In further statistical analyses of the parameter ***a*** and ***b*** of the logarithmic function of age at the end of stage to temperature, it was shown that there was no significant difference in ***a*** and ***b*** between 24 light and 12 light incubation treatments (1-way ANOVA, ***a*** p=0,3, ***b*** p=0,5). However different light regime affected slope and intercept at different stages showing (2-way ANOVA test) significant differences at light (***a*** p<0,05, ***b*** p<0,05) and with stage and light (***a*** p<0,05, ***b*** p<0,05). This suggests, that light itself does not influence overall time of development (it does not slow down or accelerate total development period), but it does affect development at the stage level. It can be seen at Figure 3.2.4, which shows the stage duration expressed in time, which were calculated at the end of each stage for both light regimes. Short distance between slope and intercept lines in particular light regime indicate acceleration of development at stage.

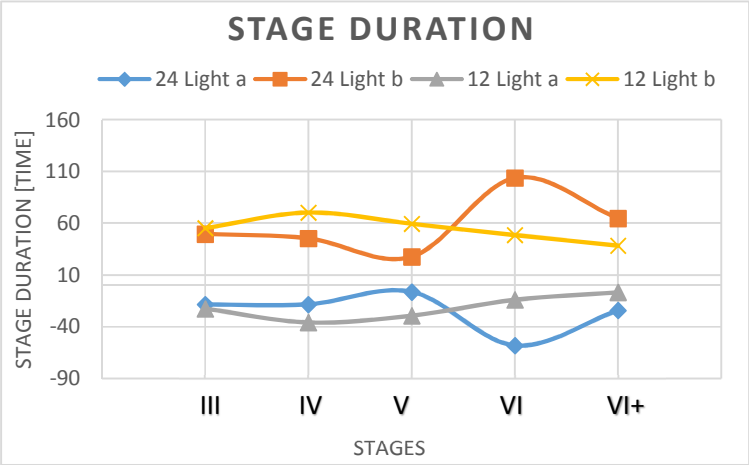


Figure 3.2.4 Stage duration (time) described with parameters 'a' and 'b' at stage at 24 light regime (line blue-slope 'a', line red -intercept 'b') and 12 light regime (line grey-slope 'a', line yellow -intercept 'b'). Data taken from table at figure 2.

If we compare slope and intercept lines of both light regimes we can clearly see that they show two different patterns, where ***a*** and ***b*** at 12 light regime were more stable and regular, with small and constant acceleration starting from stage IV until the end of stage VI+. In 24 light regime development accelerates significantly in the first half of incubation, and after stage V slows down. In the stage progression (Figure 3.2.5) we can see this phenomena with values of parameters ***a*** and ***b***.

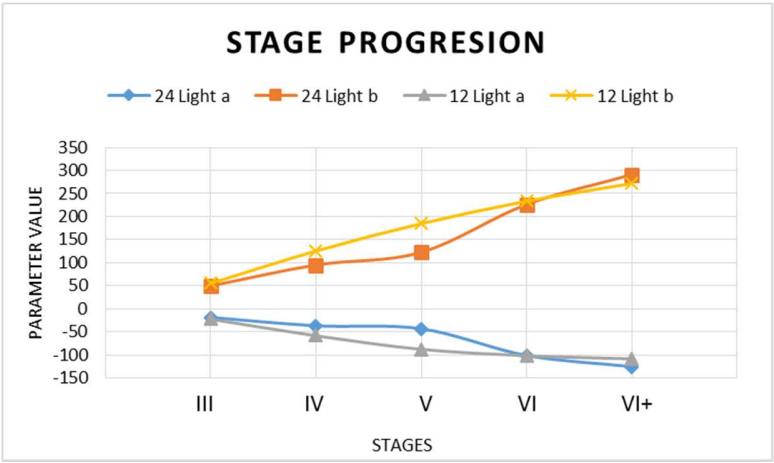
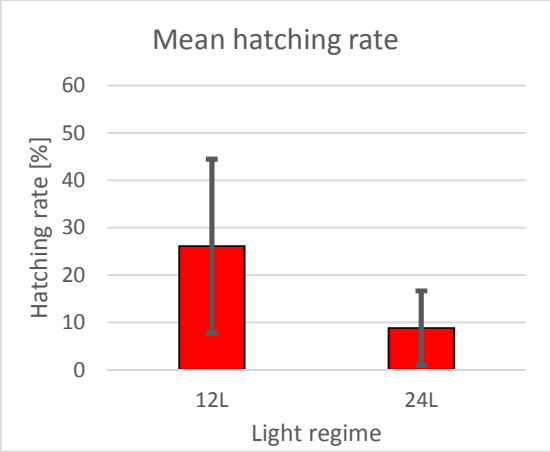


Figure 3.2.5 Stage progression (time) described by parameters ***a*** and ***b*** (data from Table 3.2.1) show values at stage at 24 light regime (line blue-slope ***a***, line red -intercept ***b***) and 12 light regime (line grey-slope ***a***, line yellow -intercept ***b***).

The reduction in intercept increase and slope decrease as a result of rapid development at stage IV and V at 24 light regime, led to smaller difference between intercept and slope (in comparison to 12 light regime), and as a consequence a reduced impact of temperature on development. At 24 light regime at stage VI and VI+ the situation is reversed, because of delayed development, leading to a reinforced impact of temperature on egg development, where the difference between intercept and slope exceeds the values of the 12 light regime.

3.3 Hatching rate

Statistical analyses of hatching rate data revealed no significant difference between light regimes (1-way ANOVA, $F_{1,18}$=4,2 , p=0.055), but the difference was noticeable, where the mean hatching rate at 12 Light regime was 26% and at 24 Light regime 8,8% (Figure 3.3.1).



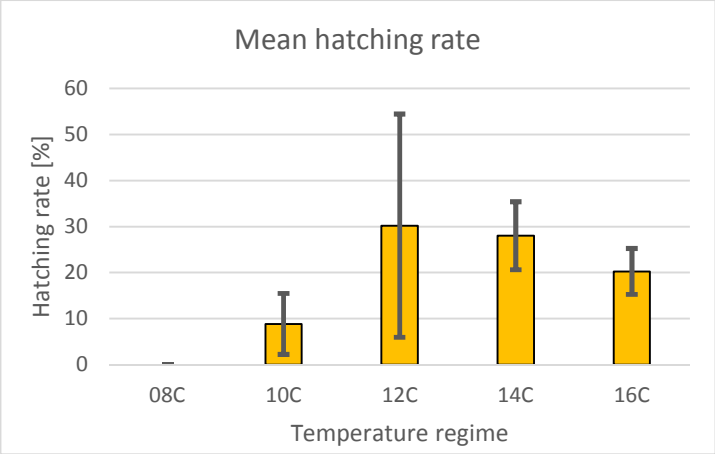


Figure 3.3.1 Mean hatching rate in percent at different light regime with standard error bars

Figure 3.3.2 Mean hatching rate in percent at different temperature regime with standard error bars

Temperature had no significant impact on hatching rate (1-way ANOVA, $F_{4,15}$=1,8 p=0.16), however the difference between temperature regimes can be distinguished. Larvae hatched from 10°C to 16°C, with the best result at 12°C with 30%, and decreasing to 20% at 16°C (Figure 3.3.2).

There was no significant temperature and light interaction (2-way ANOVA, $F_{4,10}$=1,4 , p=0,3), but it showed significant differences at light level ($F_{1,10}$=6,2 , p<0.05), suggesting that in a model with both predictors, light has bigger impact on hatching rate than temperature. However very low results at 24Light regime at 12°C caused the significant difference at light level (Figure 3.3.3).

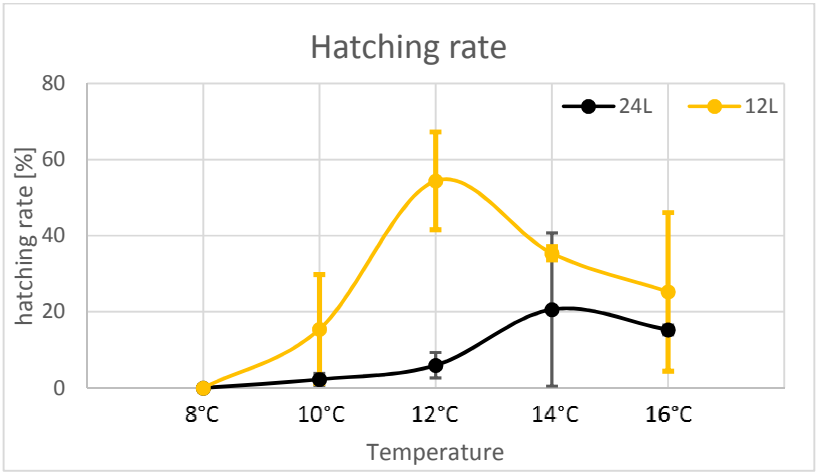


Figure 3.3.3 Hatching rate percent at different light regime and at different temperature regime with a standard error bars.

The number of larvae hatched during the hatching period showed various distribution among temperatures, but some trends could be observed. With increasing incubation time (decreasing incubation temperature), the hatching period was extended and the hatching peak was lower (Figure 3.3.4) The hatching curve changes its character from a short and intense event like at 16°C and 14°C, into an elongated curve with two shorter equal size peaks, and generally more balanced event like at 10°C.

There were significant differences between the numbers of larvae hatched in the dark phase and light phase at 12 light regime (1-way ANOVA, $F_{1,38}$=6,4 , p=0.01), indicating that the majority of larvae hatched during the dark phase. This phenomena is very clearly visible at 14°C and 12°C where proportion of larvae hatched during the dark to total number of larvae was respectively 96% and 80%, however at 10°C the numbers were almost equal 56%, and 4% at 16°C.

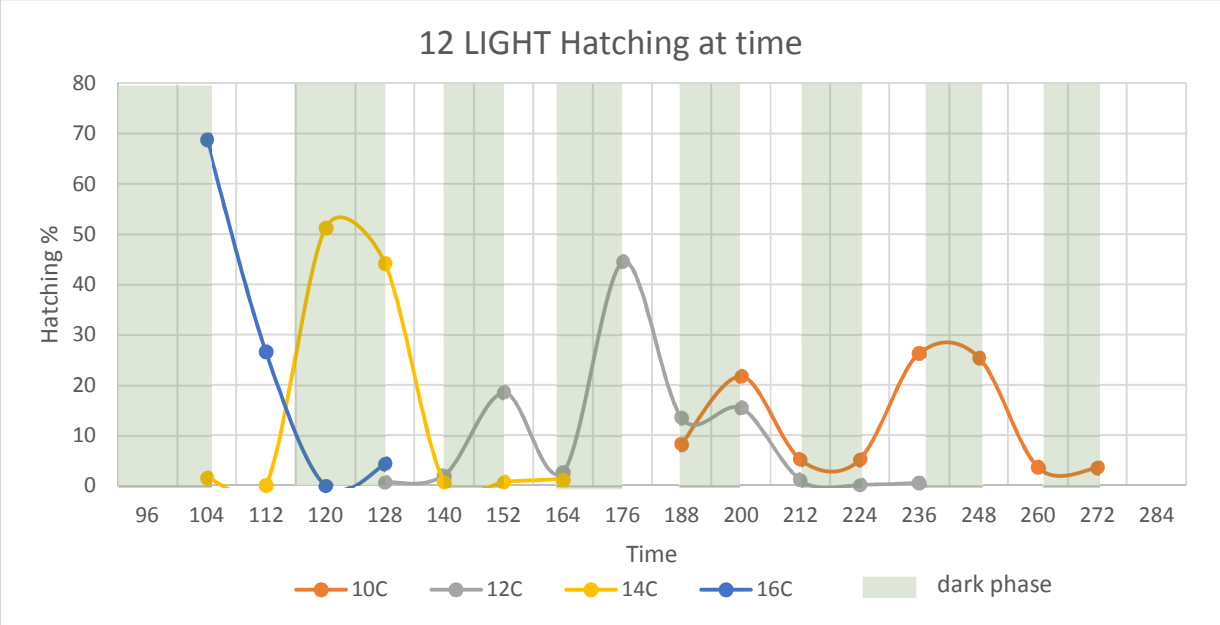
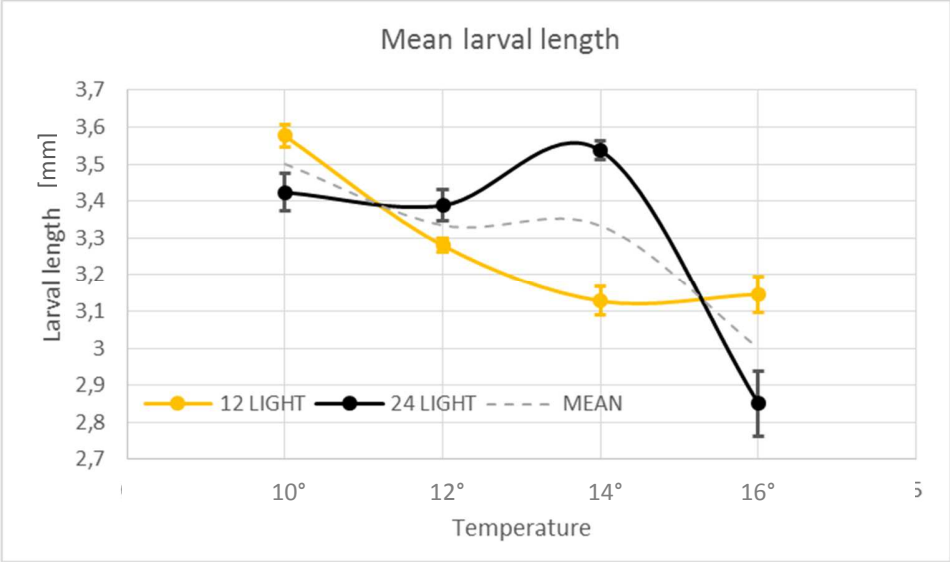


Figure 4. Larval hatching curves over time at different temperatures at 12 light regime, where grey stripes describe dark phase.

3.4 Larval size at hatching

Overall, the mean larval length was 3,35 ±0,34 (Mean ±SD). Mean larval length in the 24 light regime was 3,37 ±0,35, and in the 12 light regime was 3,33 ±0,33. There was no significant difference between the two light regimes (1-way ANOVA, $F_{1,13}$=0,01, p=0.9).



	10C	12C	14C	16C
12 LIGHT	3,57	3,28	3,13	3,15
24 LIGHT	3,42	3,39	3,54	2,85

Figure 3.4.1 Mean larval length [mm] with standard error bars at different light and temperature treatments. Additionally a dashed line with mean values of both light treatment was added for demonstration of larval length over temperature.

Temperature had a significant impact on larval length (1-way ANOVA, $4,9_{3,11}$ p=0.02), where the longest larvae (3,46 mm.) hatched at 10°C, and the shortest (3,00 mm.) at 16°C. Further Tukey Contrast test revealed significant difference between these two temperatures (16°C to 10°C p=0.01, Figure 3.4.2)

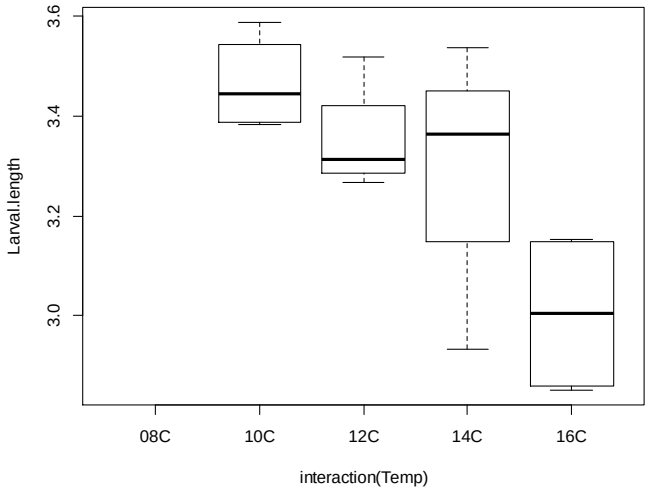


Figure 3.4.2 Larval length in mm. at different temperature treatments

Larval size at hatching varied over the hatching period and showed high variation among temperatures. To observe the trends across temperature range a logarithmic trend line was fitted to larval size data during the hatching period (Figure 3.4.2). Observing the trends, we can see that with decreasing incubation temperature larval size over time increased, where at 16°C showed downward trend, at 14°C constant trend at a same level and an upward trend at 12°C and 10°C.

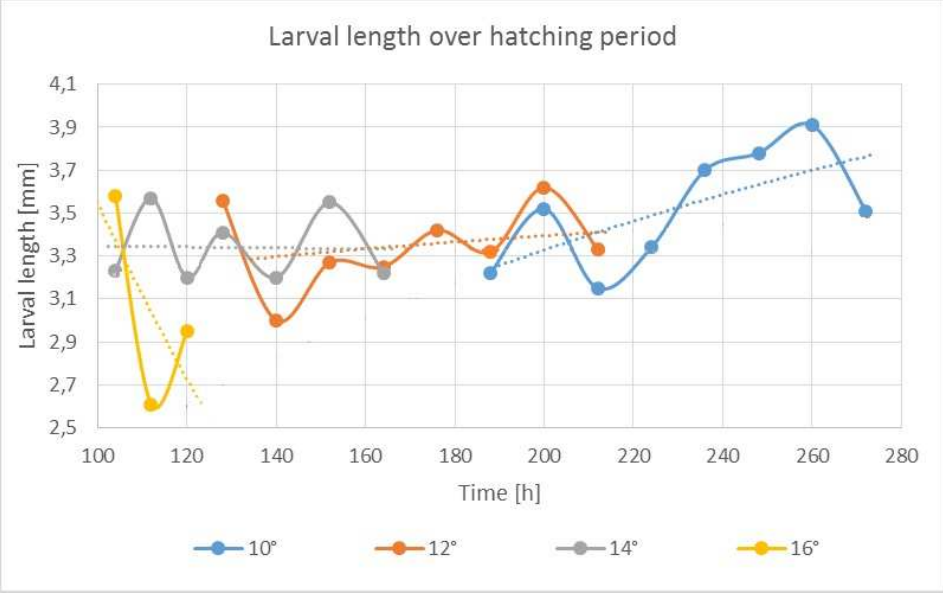


Figure 3.4.3 Larval length over time at different temperatures, with logarithmic trend line fit by the lowest square method.

4. Discussion

Temperature dependent egg incubation experiments play an important role in developing new species for the aquaculture industry. The main purpose of such experiments is to define the optimal incubation temperature and select the best temperature with the highest number of large and healthy larvae for each species. To achieve that goal many indicators are measured during incubation experiments such as: survival, hatching rate, development rate, larval length, larval weight, yolk sac volume, larval deformities and many others. These data after further analyses would indicate the most suitable incubation temperature. The purpose of that experiment is to deliver experimentally confirmed information about the impact of different temperatures and light regimes on egg survival, development rate, hatching rate and larval length during the incubation process. This information would be used for further recommendations in selection of the most suitable temperature and light regime for successful larvae production. Considering that fact, this discussion will be focused on the summary of results, and recommendation but also a comparison with other experiments on *Labrus bergylta* and on other species, for a wider context of that work.

4.1 Survival

There was no significant difference between temperatures in total survival, although temperature had significant impact on the mortality for daily survival, where the worst survival was observed at 8° and the best at 14°. There was also a very strong interaction of temperature and stage on survival at all levels (suggesting that temperature affects survival differently at the different stages). Survival at stage and slope of logarithmic function of daily survival in time was the lowest at 8°C and 16°C, and 14°C and 12°C was the best.

There was no significant difference between two light regimes but the difference is noticeable in both the statistical test on total survival and daily survival (p=0,08), where 12L regime had higher survival numbers. Very low results at 12°C at 24L regime where the biggest difference was observed between both regimes in total survival and daily survival data, have changed the average survival curve through the temperature range and as a consequence promote temperature 14°C as the best one. There was no significant difference between light regimes in daily survival at stage and daily survival over time.

Taking the above comments into consideration, thesis observations indicate that temperature 8°C and 16°C are possible upper and lower incubation temperature extremes for *Labrus bergylta*, and the temperature range from 10°C to 14°C is an optimum for egg incubation, with its peak between 12°C and 14°C. From the two tested light regimes, the most suitable in respect to eggs survival would be the 12L regime.

The impact of light regime on eggs survival has not been widely examine in the fish biology literature, and seems to not have a great influence on survival. Although the difference between the two regimes examined in this experiment could be distinguished (because of very low results at 12°C), it is difficult to state with a full confidence that light regime was the direct and only factor reducing egg survival. It is difficult to indicate the reasons of mortality in that particular example (very low results at 12°C) but it is possible that low results are the reason for the variation, because of some procedural failure or bacterial infection.

There is no published research on *Labrus bergylta*, which includes mortality rates during incubation of Norwegian populations. Mortality was reported in a Turkish population, which was >90 % for 10 °C and between 40 and 82 % at 15°C, 20°C and 25 °C (Artuz, 2005, Turkey). High survival at a higher temperatures (15°C), suggest that the Turkish population may possess higher

optimum incubation temperature than the Norwegian population ,what seems to be understandable because of higher temperature of sea water during the spawning season in Marmara Sea in Turkey.

In the bachelor thesis “Temperature dependent development rate of Ballan wrass” by Willenbrink at UIB (2013), total survival results were much higher 8°C -85%, 10°C-90%, 12°C-88%, 14°C-68%, 16°C-43%. That experiment was conducted according to the same materials and methods as trial version of this experiments (which have not been included to this work) in the spring spawning season, on eggs provided from the same source. The causes of variation in survival in the egg incubation experiments are always difficult to interpret, but some possible reasons could be suggested. Endogenous factors like egg quality and paternal crosses are considered to affect survival. Seasonal variation in egg quality can be a survival limiting factor. In the cited bachelor study high survival was observed at spring spawning, which is the natural spawning season for *Labrus bergylta*, where low survival described in this study was observed at autumn spawning, which is artificially arranged by light manipulation of the spawning season to satisfy production need. This could indicate that the autumn spawning season produces eggs with lower quality. There could be also differences in egg quality at the parental level where age of a spawning female affects egg viability and is expressed in protein and fat content level. Such phenomena was observed in gadoids and flounder fish (Kjørsvik et al.1990) where it was confirmed that protein and fat content influence survival of fertilized egg. Levels of these substances in egg vary in different female age, with the highest egg survival at middle age group. Another factor could be spawning time. *Labrus bergylta* spawning season lasts from three to four weeks, where during that period the same female can spawn several batches of egg. Mortality of egg from first to subsequent spawnings can vary. Although there is no experimentally confirmed information in

the literature about *Labrus bergylta*, it was noticed at hatchery Labrus AS basing on few years' experience and observations that survival of egg are lower at the beginning of spawning period and higher in the middle of the second half of the spawning season. These observations are consistent with studies conducted on cod egg in which mortality of batches of egg from the same female decreased significantly from first to later spawnings (Solemdal et al.,1995). That is why lower results in this study could be the reason of usage of egg from the initial spawning time. Another factor could be the experimental design, which had some small differences. Although the routine of water exchange in static incubation system was the same (every 24h), the total number of egg used in incubation jars varied, where in this experiment the average number was twice as high as in cited study. This could negatively affect oxygen level at the late hours at each water exchange shift and lead to temporary and regular oxygen level depletion, what in consequence could negatively affect egg survival in this experiment. Also the interaction of pathogens had not been tested, and cannot be exclude. Concluding each of described factors separately could influence total survival of eggs but the possible is also interaction of flew or all limiting factors at the same time, but direct indication is impossible.

Incubation upper and lower temperature limit vary among different species. In some species temperature tolerance range is short like in Atlantic cod *(Gadus morhua)* only 6°C (Forrester et al. 1964) and in others wide like in Winter flounder *(Pseudopleuronectes americanus)* 16,8°C (Williams et al. 1975). However, most of the fish egg can tolerate water temperature change of around plus or minus 5,8°C (Rombough 1996). Upper and lower limits are the borders behind which egg incubation result in 100% mortality. In this experiment across the temperature range (from 8°C to 16°C) 100% mortality was not observed suggesting that both upper and lower limits have not been reached, although its clearly seen that at the edges of temperature range survival

dropped significantly. Assuming that temperature of 12°C degree as the most suitable for incubation in respect to the highest survival, as a center of *Labrus bergylta* embryonic zone of tolerance, then the possible lower and upper limit would be respectively around 6°C and 18°C.

From the aquaculture perspective it is very desirable to identify the most vulnerable moment during embryos development. In the analyses of survival at stage data it was shown that at gastrulation and beginning of segmentation high mortality occurrence (around 50%) had been noticed. The reasons of mortality are very difficult for direct indication. However, such phenomena have been observed in other species, where in general fish embryos are the most sensitive to temperature change (Buddington et al. 1993 , Rombough 1996), but also other factors like mechanical stress (Ciechomski et al. 1967), pollutants (Westernhagen et al. 1988, Cameron et al.1992) and changing levels of oxygen (Hempel 1979), early in development during cleavage and gastrulation. These two stages are the most sensitive stages during fish life. The fact that mortality appeared through all temperature range, suggest that temperature was not a main cause of mortality in this experiment, and increases the likelihood of other factor involvement. Another critical moment in fish embryos development is the stage just before hatching, where egg are more sensitive to temperature change like in the Pacific cod *(Gadus macrocephalus)* and Atlantic cod (Forrester et al. 1964) and mechanical stress like in European plaice *(Pleuronectes platessa),* (Pommeranze et al. 1974). In this experiment mortality levels just before hatching were low, which possibly indicates that this stage is less vulnerable in *Labrus bergylta* or indicates the absence of factors that limit the survival.

4.2 Development rate

Temperature significantly affected egg development rate, where egg at 8°C developed twice as long as egg at 16°C, which confirms that development rate is slow at low temperature and accelerates with increasing temperature. The relationship between age (time [h]) and experimental temperature range (from 8°C to 16°C) at selected stages was curvilinear and was very well described by the logarithmic function. Age data of 50% hatched showed significant differences between temperatures with one exception between temperatures 14°C to 16°C.

Light did not have an impact on development rate, if we compared both light regimes, where total incubation time until 50% hatched was almost the same. However light affected development rate at the stage level where constant light regime accelerated development during the first half of incubation reducing the impact of temperature on development. The situation was reverse in the second half of incubation where development slowed down and the impact of temperature on development was reinforced. In the 12 light regime development rate was slow at the very beginning of incubation (gastrulation stage) and at subsequent stages development increased from stage to stage. From the incubation perspective it is more desirable to have a stable and constant, and easily predictable stage development like at the 12 light regime instead of an unstable development, with very variable speed (with very fast accelerations at some stages and slowdowns at the others) like at the 24 light regime. Taking above into consideration, 12 light regime would be more suitable for egg incubation in respect to development rate at stage level.

From the aquaculture perspective it is important to identify development rate at different temperatures, where according to the industrial needs the incubation process can be accelerated or slowed down. It also facilitate the production planning, since we know when new hatched larvae can be expected, and arrange all necessary facilities for further larvae culture. Higher

temperature negatively affects survival of egg and can cause increase incidents of malformation and abnormalities in the embryo. That is why development rate needs to be always considered and correlated with survival of egg, and larval health status like larval length and development state. Temperature regulation of egg incubation process has been used in aquaculture industry for a long time, where for example in Common whitefish *Coregonus lavaretus* the incubation temperature was lowered and hatch was delayed, resulting in earlier first intake of dry diet, lower mortality and more advanced development at hatching (Rösch et al.1989).

There are many laboratory experiments, which describe the influence of different temperature on development rate at many fish species, reporting relation of increasing development rate to increasing temperature, indicating ambient temperature as strong factor affecting survival and egg development (Kamler 1992, Rombough 1996). In research conducted on different populations of *Labrus bergylta* by D'arcy et al. (2012), data of time to 50% hatched at 12°C were collected and presented (Irish population -123 hours, Norwegian population – 141 hours, and Scottish population – 162 hours). Incubation time differed at the same temperature between the populations. However the comparison to this experiment is not possible because of different starting points of incubation. Light in this experiment didn't influence development rate over the whole incubation period but it did at the individual stage level. In experiments with other fishes at which photoperiod was tested it was shown that constant light regime can extend the incubation time (slowing down the development rate) like in halibut (*Hippoglossus hippoglossus),* (Helvik et al. 1992), haddock *(Melanogrammus aeglefinus),* (Downing et al. 2002) or triggerfish (Tucker 1998), which is explained by authors in photoreceptors control hatching enzyme and hatching, and is related to species-ecology of photoperiod sensitive eggs. However, there are other environmental factor which can influence to some extent incubation time (as a

result of development rate) like oxygen depletion, different salinity levels, pH values, but also parental impact of different egg size.

Development rate has been very widely described by the concept of degree days which is widely used to predict particular developmental stage of incubated fish egg, which assumed a constant and proportional increase or decrease of development rate depending on temperature, seems to not perfectly describe development rate of *Labrus bergylta*. Calculations of degree days of total incubation period at different temperatures gives high variation of values (64°D at 8°C and 86°D at 16°C). That is why accurate prediction of hatching time at selected temperature, based on temperature degree-days data of another temperature would give some error (like in this example). Development rates of *Labrus bergylta* at stages are not the same at particular temperature, that is why prediction of time at stage basing on one stage duration can cause a serious error. Also proportions of development rates at stages at one temperature can change at the other temperature, especially at the edges of temperature optimum. This observations suggests that development rate is not constant, but it can accelerate and slow down depending on the embryonic stage. There can be also some variation within the species on parental level, seasonal level and also differences at spawning time which have influence on egg quality like egg size, where bigger size egg takes longer time to develop influencing development rate. Taking the above into consideration, the use of degree days as a method for presentation and analysis of development data of *Labrus bergyla* at this experiment was abandoned.

4.3 Hatching rate

Temperature had no significant impact on hatching rate, however a general trend can be indicated, where hatching rate increase rapidly from 10°C until reaching its peak at 12°C and then gradually decrease with increasing temperature. It suggest that the optimum temperature in respect to hatching rate is from 10°C to 16°C (or even higher) with the most suitable temperature at 12°C.

Light had a significant impact on hatching rate, indicating 12 Light regime as the best one. Interaction of temperature and light had a significant impact on hatching rate at a light level, suggesting that in a model with both predictors light has a bigger influence on hatching rate than temperature.

Indication of light as a significant factor affecting hatching rate was caused by very low result at 24 Light regime, at 12°C what has influenced the final conclusion. Exclusion of 12°C degree treatment in both light regimes from statistical analyses (1-way ANOVA), indicate no significant difference between light regimes. Taking the above into consideration, it is difficult to state with a full confidence that light is significantly affecting hatching rate. It is also difficult to indicate the reasons for the very low hatching rate at 24 light regime at 12°C degree, but very low results of daily survival at this particular example have negatively affected the total survival and as a further consequence hatching rate. The reason of low survival as mentioned in ‘Survival discussion’ are difficult for direct indication, but potentially some procedural failure or bacterial infection could be one of the reasons, but also high density of egg used could affect the survival numbers, which were the highest in that particular combination of light and temperature treatment.

The impact of different light regime on hatching rate at other fishes vary but in most of the conducted research had no significant effect on hatching rate. However, among species with different daily patterns of behavior like zebrafish – diurnal, Senegal sole – nocturnal, Somalian cavefish *(Phreatichthys andruzzii)* – blind, 12 Light regime was shown to be the most suitable with the highest hatching rate through the temperature range (Villamirez et al.2013). In the same study the worst hatching rate was observed for zebra fish and Senegal sole at constant dark regime, indicating light importance in the embryogenesis of both dial type fishes. In a research conducted on haddock (Downing et al. 2002) and Obscure puffer, (Yang et al. 2004), it was shown that there was no significant differences between different light regimes in respect to hatching rate although still combination of light and dark regime had the best results.

The impact of temperature on hatching rate differs depending on species, for example in Obscure puffer the difference between temperature treatments was not significant (Yang et al.2004), but a general trend of lower hatching results at the edges of temperature optimum and the best results in the middle, have been noticed. The same trend was reported in Ruffe *(Gymnocephalus cernuus)*, where the lowest result were observed at the lowest temperature treatment and showed significant difference with the best hatching rate at middle of temperature optimum (Douglas et al 1996). In research conducted on Largemouth bass *(Micropterus salmoide*s*)* and Smallmouth bass *(Micropterus dolomieu)* , hatching rate were significantly affected by temperature (Landsman et al. 2011).

Temperature affected hatching period at *Labrus bergylta* by elongation of the hatching period with decreasing temperature. At high temperatures hatching period was short and intense where at low temperatures was more distributed in time and more balanced. From an aquaculture perspective it is more desirable to have a short and intense hatching period, which facilitates further logistic operation, since it is not necessary to wait for late larvae to hatch.

In this study, a hatching rhythm at 12 Light regime was noticed to be synchronized with the dark phase, during which most of the larvae hatched showing significant difference between the two phases. That phenomena was also observed in Sergeant major *(Abudefduf saxatilis)*, triggerfish family *Balistidae* (Tucker 1998), and was clearly demonstrated in the halibut (Helvik et al. 1992, Helvik et al. 1993). It is assumed that coordination of hatching with dark phase can be a fish strategy for limiting predation in newly hatched larvae and is related to species ecology (Downing et al. 2002).

From aquaculture perspective this hatching rhythm has little relevance since the predation threat is not an issue, but it may have a practical importance for working schedules.

4.4 Larval size at hatching

Different light regime did not have impact on larval size at hatching. However temperature had an impact on larval size, which was shown to be significant between temperatures 16°C to 10°C. A general trend was identified, where larval size (length) at hatching decreased with increasing incubation temperature (decreasing time). This trend was also reported in other fishes like Pacific cod (Alderdice et al. 1971) and toutog *(Tautoga onitis),* (Laurence et al. 1973). In most of the species, the largest larvae are produced within the optimum incubation range, like in Sablefish *Anoplopoma fimbria* (Alderdice et al.1988), halibut (Howell et al.1984) or Gilt-head seabream *Sparus aurata* (Polo et al. 1991). Although there are some contradictory examples of research showing no influence of incubation temperature on larval size at hatching like in Atlantic salmon *(Salmon salar),* (Ryzhkov et al.1976). The maximum survival in *Labrus bergylta* was noted at 12°C, which was indicated as the best incubation temperature, but this does not corresponds with the highest larval length, which was at 10°C. This can suggest that larval length at hatching is increasing with decreasing temperature until reaching the edges of temperature optimum.

The reason for small size of larvae at hatching at higher temperatures could be the result of precocious hatching, which is the effect of increased embryo mobility, and desynchronization of secretion of hatching enzymes to growth of larvae (Penaz et al.1983). Another explanation has a bioenergetics approach, where energy from yolk is used by embryo on tissue production (development) and respiration (metabolism). The proportion of energy used between these two main physiological processes is different in fish, where combination of high metabolic Q10 and low developmental Q10 will result in increase of cumulative metabolism and decrease of size at hatching with increasing incubation temperature like in *Phusa acuta* (Kamler et al.1976). Situation will reverse in fishes with higher developmental Q10 than metabolic Q10, where

cumulative metabolic energy used through development will decrease with increasing incubation temperature, and result in bigger size larvae at hatching at higher temperatures, like in Rainbow trout *(Oncorhynchus mykiss),* (Kamler et al. 1983). This phenomena can explain contradictory results of larval size at hatching in different fish species. Following that way of thinking, *Labrus bergylta* egg have higher metabolism than development proportion.

In this experiment it was also shown that larval size is bigger at the end of hatching time than at beginning, where this trend is more clear and visible at higher incubation temperatures. This phenomena was also reperted in other fishes like Pacific cod (Laurel et al. 2008), Ocean pout *(Macrozoarces americanus)* (Methven et al. 1991), and capelin *(Mallotus villous)*, (Chamber et al. 1989), Atlantic herring *(Clupea harengus),* (Geffen et al. 2002). However there are species, which have bigger larvae at the beginning of hatching period like silverside *(Menidia menidia),* (Bengtson et al. 1987). In experiment conducted on herring egg it was concluded that the differences between larval hatched at the beginning and the end of hatching period are due to continued growth, which takes place until the end of incubation process. The early hatched larvae are smaller, but as a free swimming larvae, they have a higher potential of growth, which at the end of yolk sack utilization results in the same size larvae compare to those hatching later. This suggest that there are no disadvantages of hatching earlier in respect to growth (Geffen et al. 2002).

There are also other factors influencing larval length at hatching like maternal and paternal factors and abiotic factors like salinity and dissolved oxygen level, which in case of this study could also affect the result.

Larval size of *Labrus bergylta* have been also presented in other research: 2,75-3 mm (Fives, 1976, Ireland), 2,7 ± 0,2 mm (Artuz et al. 2005, Turkey), 3,64 ± 0,1 mm (Dunaevskaya, 2010,

Norway), 3,297 ± 0,2 mm (Shchepak, 2011, Norway), 3,72 ± 0,13 mm (D´Arcy et al., 2012, Ireland/Norway), 3,64 ± 0,05 mm (Ottesen et al., 2012, Norway) and larvae from Scotland with 4,1 ± 0,1 and 4,3 ± 0,3 mm (mentioned in D´Arcy et al., 2012). Larval size in this experiment 3,35 ±0,34 correspond to the larval size of Norwegian population, but is different from other populations.

From the aquaculture perspective it is desirable to receive viable and big larvae. This experiment indicated that temperature 10°C produced the longest larvae at hatching. However the differences between larval size at other temperatures can be compensated during endogenous feeding of larvae. Taking above into consideration, the most desirable information would be the total impact of different incubation temperature on larval size after yolk sack absorption. Since the larval size at hatching can change and the final effect of temperature after yolk sack utilization is unknown, it is reasonable to indicate the best temperature based on survival as a main criteria.

5. Final remarks

Based on the results of this experiment, the most suitable temperature and light regime for Labrus bergylta eggs incubation is 12°C degrees and 12 light regime. Recommended combination of temperature and light have given the best survival and hatching rate results among those tested. The most vulnerable moment during embryos development was the end of gastrulation stage, at which time mortality increased up to 50% at stage. That is why it is reasonable to avoid any logistic procedures connected with transportation, which could causes mechanical stress during that development stage at a commercial hatchery. Total incubation time is around 160 hours (6,5 days) , with a stable and constant, and easily predictable embryo development. Larval length at hatching was ~3,3 mm, which was a middle size result, with a bigger larvae hatching at the end of hatching period. Larval length decreased at higher temperatures, and hatching rhythm of 12 Light and 12 dark photoperiod was synchronized with dark phase.

From the aquaculture point of view, it is desirable to get further information on the final effect of temperature (in a range from 10°C to 14°C), after larval hatching on survival of newly hatched larvae and their size until moment of yolk sack absorption. It is recommended to test the effect of incubation temperature on further larval development to give a wider picture and confirm the health status of hatched larvae it in further experiment.

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7. Appendix

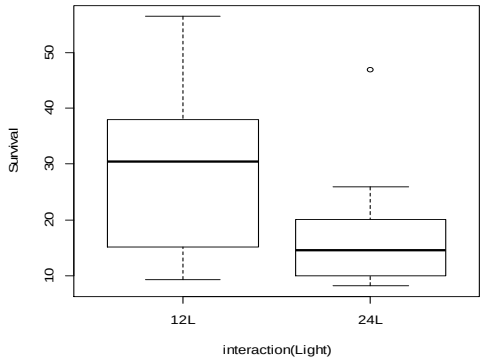
Appendix 1. Statistical analyses of total survival in relations to light, temperature and interaction of light : temperature.

LIGHT

Analysis of Variance (1-way ANOVA)

Response: Total survival

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Light	1	581.0	581.04	3.2515	0.08813 .
Residuals	18	3216.6	178.70		

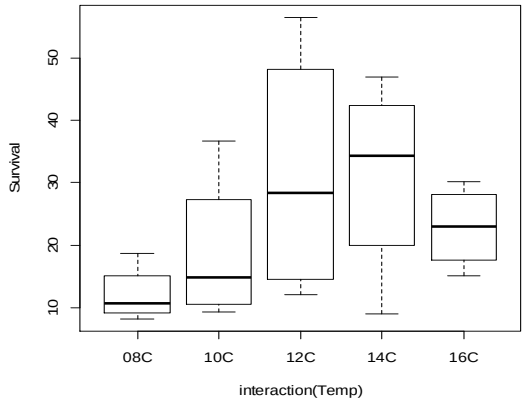


TEMPERATURE

Analysis of Variance (1-way ANOVA)

Response: Total survival

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Temp	4	1082.4	270.59	1.4948	0.2534
Residuals	15	2715.3	181.02		



TEMPERATURE : LIGHT

Analysis of Variance (2-way ANOVA)

Response: Total survival

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Temp	4	1082.37	270.59	1.8817	0.19028
Light	1	581.04	581.04	4.0407	0.07216 .
Temp:Light	4	696.26	174.06	1.2105	0.36534
Residuals	10	1437.99	143.80		

Appendix 2. Statistical analyses of daily survival data in relation to light, temperature and its interaction light : temperature.

LIGHT – predictor

Analysis of Variance One-way ANOVA

Response: Daily survival

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Light	1	0.307	0.30703	2.9106	0.08873 .
Residuals	423	44.621	0.10549		

TEMPERATURE - predictor

Analysis of Variance One –way ANOVA

Response: Daily survival

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Temp	4	1.158	0.28949	2.7778	0.02664 *
Residuals	420	43.770	0.10422		

Multiple Comparisons of Means: Tukey Contrasts

Fit: lm(formula = Daily survival ~ Temp)

Linear Hypotheses:

	Estimate	Std. Error	t value	Pr(>|t|)
10C - 08C == 0	0.04917	0.04512	1.090	0.8109
12C - 08C == 0	0.08061	0.04670	1.726	0.4175
14C - 08C == 0	0.15291	0.04972	3.076	0.0188 *
16C - 08C == 0	0.01401	0.05136	0.273	0.9988
12C - 10C == 0	0.03144	0.04744	0.663	0.9640
14C - 10C == 0	0.10374	0.05041	2.058	0.2393
16C - 10C == 0	-0.03516	0.05203	-0.676	0.9613
14C - 12C == 0	0.07231	0.05183	1.395	0.6298
16C - 12C == 0	-0.06659	0.05340	-1.247	0.7225
16C - 14C == 0	-0.13890	0.05606	-2.478	0.0971

TEMPERATURE : LIGHT - predictors

Analysis of Variance (2-way ANOVA)

Response: Daily survival

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Temp	4	1.158	0.28949	2.8016	0.02563 *
Light	1	0.334	0.33352	3.2277	0.07313 .
Temp:Light	4	0.555	0.13877	1.3430	0.25319
Residuals	415	42.882	0.10333		

Appendix 3. Statistical analyses of survival at stage in relation to stage : light, stage : temperature and light : temperature.

STAGE : LIGHT interaction

Analysis of Variance (2-way ANOVA)

Response: Survival at stage

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Light	1	0.0290	0.02899	1.3926	0.2438
Stage	5	5.3948	1.07897	51.8266	<2e-16 ***
Light:Stage	5	0.0233	0.00466	0.2237	0.9505
Residuals	48	0.9993	0.02082		

STAGE : TEMPERATURE interaction

Analysis of Variance (2-way ANOVA)

Response: Survival at stage

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Temp	4	0.4127	0.10318	12.5760	4.005e-06 ***
Stage	5	5.3948	1.07897	131.5033	< 2.2e-16 ***
Temp:Stage	20	0.3927	0.01963	2.3931	0.01493 *
Residuals	30	0.2461	0.00820		

LIGHT : TEMPARATURE interaction

Analysis of Variance Table

Response: Survival

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Light	1	0.0290	0.028992	0.2452	0.6227
Temp	4	0.4127	0.103184	0.8726	0.4870
Light:Temp	4	0.0920	0.023004	0.1945	0.9401
Residuals	50	5.9127	0.118253		

Appendix 4. Statistical analyses of parameter ***α***-slope of logarithmic function of daily survival and time in relation to light, temperature and interaction of light : temperature.

LIGHT – predictor

Analysis of Variance (1-way ANOVA)

Response: slope

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
light	1	0.000029	0.0000288	0.0101	0.9212	
Residuals	18	0.051484	0.0028602			

TEMPARATURE – predictor

Analysis of Variance (1-way ANOVA)

Response: slope

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
temp	4	0.041214	0.0103036	15.007	3.997e-05	***
Residuals	15	0.010299	0.0006866			

Multiple Comparisons of Means: Tukey Contrasts

Fit: lm(formula = Slope ~ Temp)

Linear Hypotheses:

	Estimate	Std. Error	t value	Pr(>|t|)	
10C - 08C == 0	0.02125	0.01853	1.147	0.779870	
12C - 08C == 0	0.01075	0.01853	0.580	0.976027	
14C - 08C == 0	-0.01775	0.01853	-0.958	0.869426	
16C - 08C == 0	-0.10525	0.01853	-5.680	0.000400	***
12C - 10C == 0	-0.01050	0.01853	-0.567	0.977994	
14C - 10C == 0	-0.03900	0.01853	-2.105	0.268049	
16C - 10C == 0	-0.12650	0.01853	-6.827	< 1e-04	***
14C - 12C == 0	-0.02850	0.01853	-1.538	0.555282	
16C - 12C == 0	-0.11600	0.01853	-6.261	0.000108	***
16C - 14C == 0	-0.08750	0.01853	-4.722	0.002185	**

LIGHT : TEMPERATURE interaction

Analysis of Variance (2-way ANOVA)

Response: slope

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
temp	4	0.041214	0.0103036	15.5808	0.0002683	***
light	1	0.000029	0.0000288	0.0436	0.8388814	
temp:light	4	0.003657	0.0009143	1.3826	0.3077653	
Residuals	10	0.006613	0.0006613			

Appendix 5. Statistical analyses of parameters ***a*-**slope and ***b***-intercept of logarithmic function of age and temperature regime in relation to light and stage and light.

LIGHT - predictor

Analysis of Variance (1-way ANOVA)

Response: slope

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
light	1	1135	1135.1	0.8527	0.3602
Residuals	50	66557	1331.2		

STAGE : LIGHT – predictor

Analysis of Variance (2-way ANOVA)

Response: slope

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
stage	12	57118	4759.8	34.0506	8.095e-13 ***
light	1	1135	1135.1	8.1203	0.008452 **
stage:light	12	5805	483.7	3.4606	0.003899 **
Residuals	26	3634	139.8		

LIGHT – predictor

Analysis of Variance (1-way ANOVA)

Response: intercept

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
light	1	2276	2276.1	0.3542	0.5544
Residuals	50	321275	6425.5		

STAGE : LIGHT - interaction

Analysis of Variance (2-way ANOVA)

Response: intercept

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
stage	12	306265	25522.1	106.0951	< 2.2e-16 ***
light	1	2276	2276.1	9.4618	0.004890 **
stage:light	12	8755	729.6	3.0330	0.008661 **
Residuals	26	6255	240.6		

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Appendix 6. Statistical analyses of hatching rate in relation to light, temperature and interaction of light and temperature.

LIGHT - predictor

Analysis of Variance (1-way ANOVA)

Response: Hatching rate

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Light	1	1495.2	1495.24	4.2022	0.05523	.
Residuals	18	6404.8	355.82			

TEMPERATURE – predictor

Analysis of Variance (1-way ANOVA)

Response: Hatching rate

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Temp	4	2640	660.00	1.8821	0.166
Residuals	15	5260	350.67		

LIGHT : TEMPERATURE – interaction

Analysis of Variance (2-way ANOVA)

Response: Hatching rate

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Temp	4	2640.0	660.00	2.7321	0.08994	.
Light	1	1495.2	1495.24	6.1897	0.03211	*
Temp:Light	4	1349.1	337.27	1.3962	0.30366	
Residuals	10	2415.7	241.57			

Appendix 7. Statistical analyses of number of larvae hatched at different light phase and temperature.

PHASE – predictor

Analysis of Variance (1-way ANOVA)

Response: Larvae hatched

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Phase	1	5989	5989.3	6.4622	0.01522	*
Residuals	38	35219	926.8			

PHASE : TEMPERATURE interaction

Analysis of Variance (2-way ANOVA)

Response: Larvae hatched

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Temp	3	2162.0	720.7	0.9153	0.44453
Phase	1	6325.1	6325.1	8.0335	0.00789 **
Temp:Phase	3	7526.8	2508.9	3.1866	0.03688 *
Residuals	32	25194.9	787.3		

Appendix 8. Statistical analyses of larval length at hatching in relation to light, temperature and interaction of light and temperature.

LIGHT – predictor

Analysis of Variance (1-way ANOVA)

Response: Larval length at hatching

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Light	1	0.00106	0.001061	0.017	0.8982
Residuals	13	0.80976	0.062289		

LIGHT : TEMPERATURE – interaction

Analysis of Variance (2-way ANOVA)

Response: Larval length at hatching

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Temp	3	0.46565	0.155217	7.7541	0.01255 *
Light	1	0.00113	0.001133	0.0566	0.81875
Temp:Light	3	0.20392	0.067973	3.3957	0.08286 .
Residuals	7	0.14012	0.020017		

TEMPERATURE – predictor

Analysis of Variance (1-way ANOVA)

Response: Larval length at hatching

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Temp	3	0.46565	0.155217	4.9465	0.02057 *
Residuals	11	0.34517	0.031379		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Simultaneous Tests for General Linear Hypotheses

Multiple Comparisons of Means: Tukey Contrasts

Fit: lm(formula = Larval length at hatching ~ Temp)

Linear Hypotheses:

	Estimate	Std. Error	t value	Pr(>|t|)
12C - 10C == 0	-0.11206	0.12526	-0.895	0.8076
14C - 10C == 0	-0.18770	0.13529	-1.387	0.5312
16C - 10C == 0	-0.46246	0.12526	-3.692	0.0158 *
14C - 12C == 0	-0.07564	0.13529	-0.559	0.9420
16C - 12C == 0	-0.35040	0.12526	-2.797	0.0708 .
16C - 14C == 0	-0.27476	0.13529	-2.031	0.2344

(Adjusted p values reported -- single-step method)

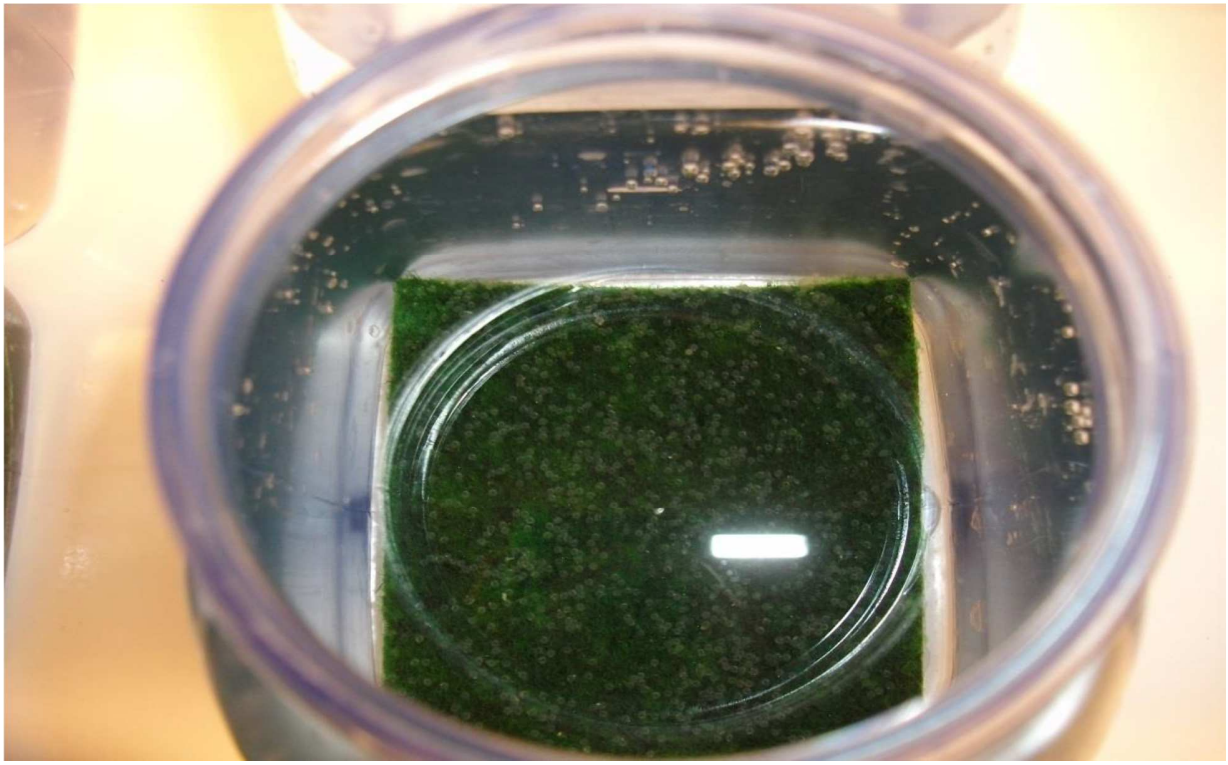
Appendix 9. Sampling form

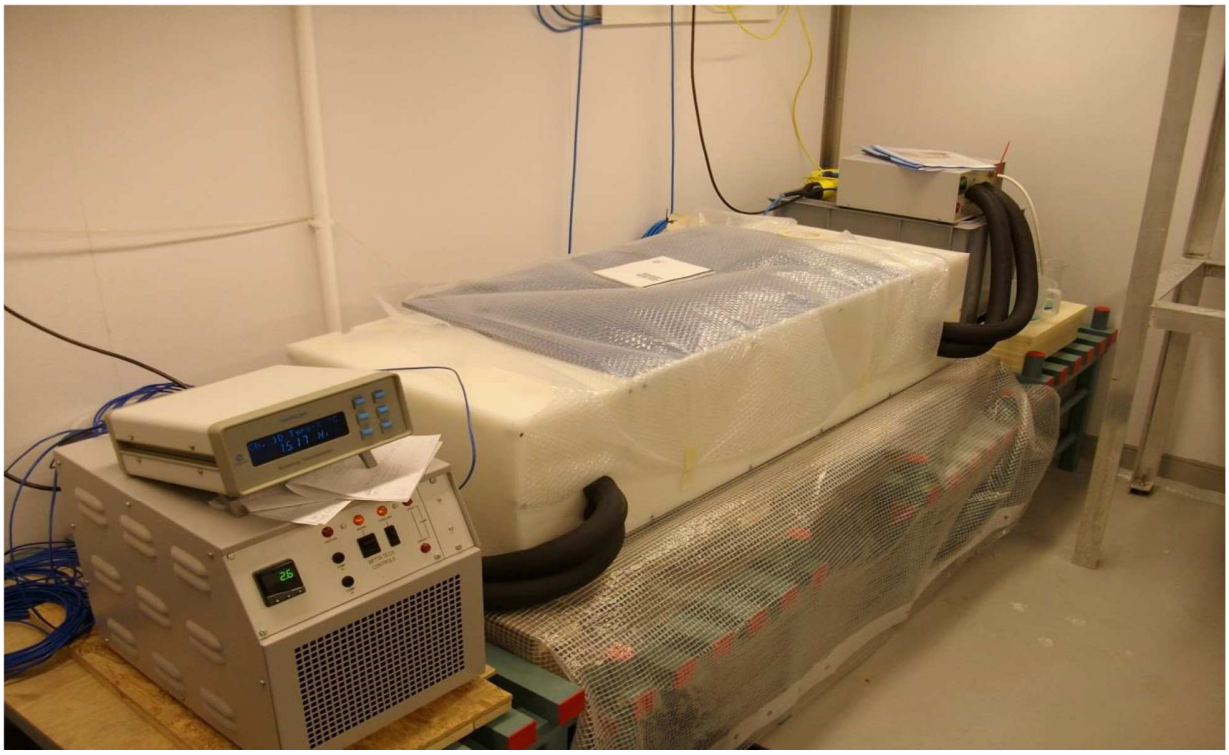
Species: Labrus bergylta											Percentage of eggs in stage								
Light regime:											Time in hours								
Temperature:																			
Sample:																			
	6	12	18	24	30	36	42	48	56	64	72	80	88	96	104	112	120		
Stage																			
Blastodisc																			
2 blastomers																			
4 blastomers																			
8 blastomers																			
16 blastomers																			
32 blastomers																			
Moderate cell size morula																			
Small cell size morula																			
Periblast zone																			
Flat morula																			
Beginning of gastrulation																			
Early gastrulation																			
End of gastrulation																			
Head visible/exposed yolk plug/beak-like mass of cells anterior to head	180°																		
Optic bulbs/blastoporus closure (200°)	200°																		
First somites (2-4)/eyes slightly more defined (220°)	220°																		
8-10 Somites/Pigmentation/Otolith vesicle (290°)	290°																		
16-18 somites/expanded cavity (310°)	310°																		
24-26 somites/eye lens formation (330-340°)	330-340°																		
30-32 somites/tail seperate from yolk/mebrane caudal fin (360-370°)	360-370°																		
Oil droplet fuse/36-38 somites/heartbeat 82 min^-1 (380-410°)	380-410°																		
40-44 somites/pectoral fin/heartbeat 87 min^-1 (410-435°)	410-435°																		
46-48 somites/heartbeat 94 min^-1/more pigments (435-480°)	435-480°																		
46-48 somites/clear otholit vesicle/eyes no pigment but wel developed	480-510°																		
Hatching or hatched/46-48 somites/heartbeat 98 min^-1 (510°)	510°																		
Dead eggs [%]																			
Number of hatched larvae																			
Number of deformed larvae																			

Appendix 10. Photos

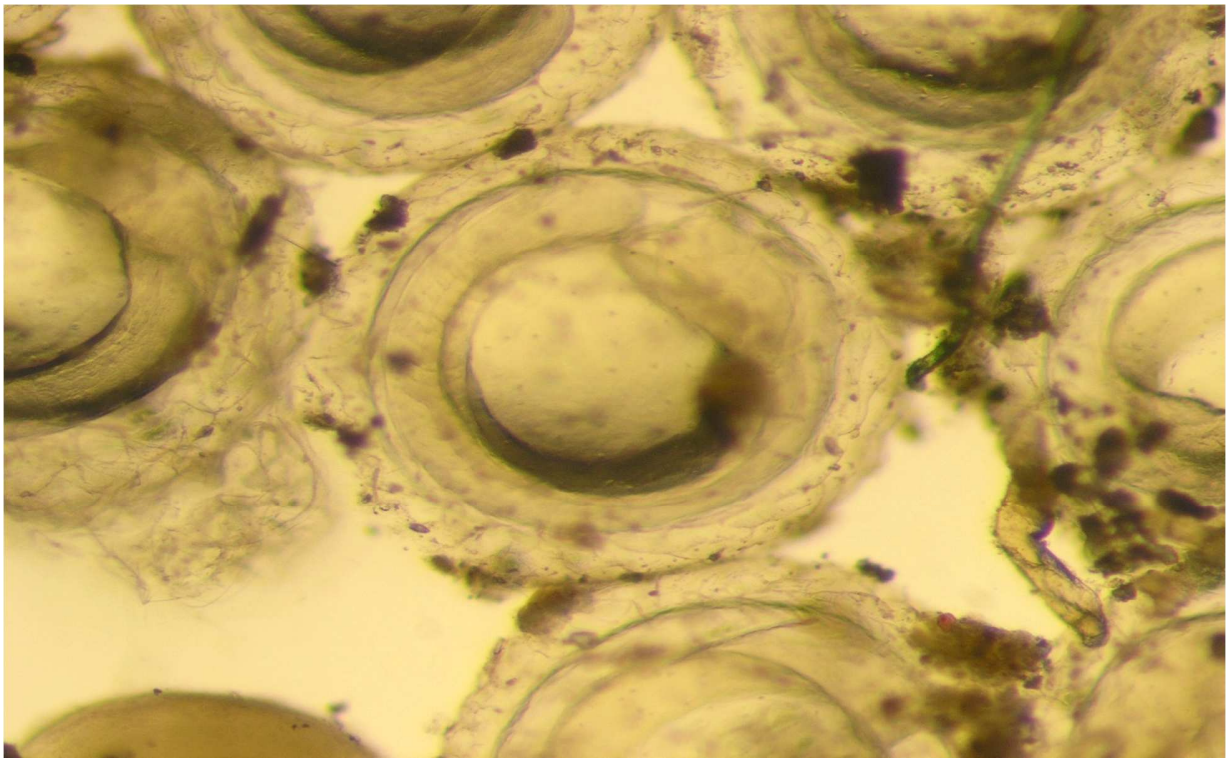
1. Spawning tank for *Labrus bergulta* with plastic imitation of plants.
2. Green mats with attached egg in the plastic bags in the insulation box.

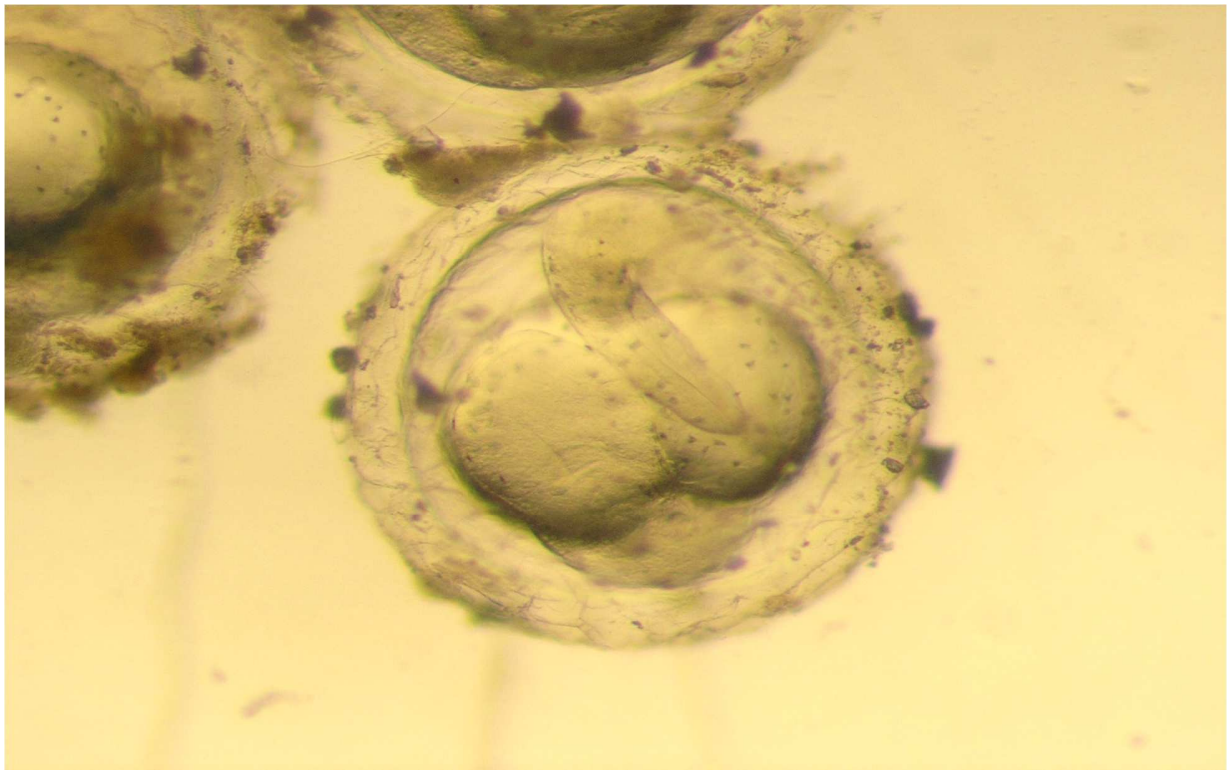
3. Plastic container with cut mats pieces 6 x 6 cm.
4. Plastic jar with green mat with attached to the surface egg (the top view)





5. Incubation unit with heating unit (on the right), cooling unit (on the left) and incubation block (in the center)
6. *Labrus bergylta* embryo , stage 9.





7. *Labru bergylta* embryo, stage 11.
8. Many dead egg (arrested around stage 5).