Effects of different temperatures and salinities on development of fertilized eggs of Ballan wrasse

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PREFACE

This master's thesis is the final part of a two-year Master of Science program at the Faculty of Biological Sciences and Aquaculture, University of Nordland, Bodø, Norway. The thesis is a scientific work of 60 credits within a field of marine aquaculture.

The work "Effects of different temperatures and salinities on development in fertilized eggs of Ballan Wrasse" is a part of International Research and Developmental (R&D) project: Organic fish production – part 1. Hatchery production of Ballan Wrasse.

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ABSTRACT

Development of Ballan wrasse is not well described and the combined effect of environmental parameters such as temperature and salinity is not known.

The main objective of the present work was to study the early development of the most promising cleaner fish *Labrus bergylta* (Ballan wrasse) which have not been described previously.

For better understanding of the tolerance of Ballan wrasse to various range of environmental conditions like temperature and salinity at early developmental stages, the effects of different temperature and salinity on morphometric characteristics of newly hatched larvae, larvae hatching success, hatching time variations were determined and malformations of early larva examined.

Larvae were hatched form eggs obtained by natural spawning from a wild caught broodstock held in captivity. The hatching success varied from 51,98% to 84,57%, the highest was found in temperature ad salinity 15°C and 33. Larvae size tended to decrease with increasing the temperature. Time of incubation for hatching varied between different temperatures: 59,3 °C days (5days of incubation, 15 °C), 67,5 °C days (4 days, 20 °C), 76,3 °C days (8 days, 10°C). The mean standard length of newly hatched larvae was 3,297±0,2 mm. Standard length at hatching among temperature treatments was highly variable, ranging from 2,6 to3,96 mm. The occurrences of abnormalities of newly hatched larvae varied from 100% in combination 20°C 26‰, 20°C 33‰ to 65,5%, with the lowest in temperature and salinity combination 15°C 33‰. Severe malformations tended to increase with increasing temperature.

The results on hatching rate, and early larval deformities, indicate that fertilized eggs of Ballan wrasse develop till hatching in a wide range of salinities, but we conclude that the optimal temperature for successful development of fertilized eggs lies between 10°C to 15°C, where highest egg hatching rate, and lowest incidence of abnormality rate was observed. The present study provides valuable information about influence of different rearing conditions during egg incubation like temperature (10, 15, 20°C) and salinity (26, 30, 33‰), and it can be useful for establishing the requirements of commercial egg incubations for further larval optimal welfare and growth.

1. INTRODUCTION

1.1. Ballan wrasse as an important fish species in commercial cold water marine aquaculture and importance of the study

In sea cage rearing of Atlantic salmon (Salmo salar L.) repeated sea lice infestations are a major problem to the industry. Sea lice (Cipepoda, Caligidae) are ectoparasitic copepods which infect salmonids of the northern hemisphere. The lice feed on the mucus, skin and blood of the host and, if they are not removed, cause open wounds exposing the fish to osmotic stress and secondary infections (Sayer, Treasurer, Costello, 1996).

Regular treatments of salmon with organophosphate pesticides are necessary once sea lice populations become established because the larval stages are not affected, and the sea lice reproduce rapidly. Besides, pesticide use can cause stress to the salmon and reduce growth (Sayer, Treasurer, Costello, 1996). A successful alternative sea-lice treatment involves cleaning species where one species of fish (the cleaner) feeds on parasites from another species (the host) (Sayer, Treasurer, Costello, 1996).

The use of north European wrasse species as cleaner-fish in the Atlantic salmon culture industry has proved a worthy alternative to pesticides (Per Gunnar Kvenseth, Norsk Sjømatsenter, Johan Andreassen, Villa Leppefisk AS, Johan Solgaard, . 2003; Per Gunnar Kvenseth, 2003a; Sayer, Treasurer, Costello, 1996; Skiftesvik, Bjelland, 2003).

. There are limited natural populations of Ballan wrasse, thus there is a growing interest in farming this species. The scientific literature about biology and ecology of this species is limited and even sparse. More over information about early development is lacking Therefore, to ensure a stable supply of Ballan wrasse to the salmon industry one has to establish methods and technology to produce this species in artificial conditions

1.2. Effect of temperature and salinity on early development

The early life-history stages of fishes are in many cases more susceptible to environmental interactions than their adult counterparts (Cingi, Keinanen, Vuorinen, 2010) Previous studies with other marine species demonstrate that temperature has a significant effect on the hatch rate (Gracia-López, Kiewek-Martínez, Maldonado-García, 2004). Metabolic rate and all other processes are influenced directly by the temperature. The temperature range of egg survival and hatching success is narrower than for other life stages (Southgate, Lucas, 2003). Both biologists/ecologists and aquaculturists are interested in the effects of salinity on marine organisms. In nature, salinity is one of the most important factors; it affects distribution of

marine organisms and the osmoregulatory capacity of an organism determines its salinity tolerance (Shi, Huang, Fu, Wang, Luo, Chen, Liu, Zhang, 2008a)

Temperature and salinity influence marine fish eggs and larval physiology, having a direct effect on hatching, growth and survival (Gracia-López, Kiewek-Martínez, Maldonado-García, 2004; Helvik, Walther, 1993; Morehead, Hart, 2003; Yang, Chen, 2005). Water temperature influences hatching rates larval sizes at hatch , time for yolk sac absorption , energy reserve take-up efficiency, larval growth and survival. Salinity affects hatching rate, larval survival, egg diameter, yolk consumption efficiency, and growth (Gracia-López, Kiewek-Martínez, Maldonado-García, 2004)

Information concerning the effects of temperature and salinity on embryonic development and survival of prelarvae of Ballan wrasse is limited. Despite the growing commercial interest only few studies have described embryonic development of wrasse and gave some information about environmental conditions for eggs rearing (Anne Berit Skiftesvik, 2003; Artuz, 2005; Dunaevskaya, 2010; Fives, 1976; Sayer, Treasurer, Costello, 1996).

Ballan wrasse has an increasing importance in commercial cold water marine aquaculture, but the production of viable juveniles could be a bottleneck. One of the problems in hatcheries reared marine coldwater species is the presence of skeletal malformations, especially those affecting the anterior part of the vertebral column (Kjorsvik, Olsen, Wold, Hoehne-Reitan, Cahu, Rainuzzo, Olsen, Oie, Olsen, 2009).

It is predicted that abnormal development of embryos is derived from the maternal factors of the eggs and the environmental factors of rearing eggs and embryos (Okamoto, Kurokawa, Gen, Murashita, Nomura, Kim, Matsubara, Ohta, Tanaka, 2009). Incubation studies at high temperatures demonstrated increased frequencies of vertebral (Brooke, 1975) and eye deformations in newly hatched lake whitefish *Coregonus clupeaformis* (Brooke, 1975; Cingi, Keinanen, Vuorinen, 2010; Naesje, Jonsson, 1988) and mountain whitefish *Prosopium williamsoni* (Keinanen, Tigerstedt, Kalax, Vuorinen, 2003; Naesje, Jonsson, 1988). A high incubation temperature may also produce smaller and shorter post-hatch fishes (Cingi, Keinanen, Vuorinen, 2010). Salinity can also induce larval deformities. Doroshev and Aronovich (1974) described that low salinity affected the oedema of the yolk sinus and pericardium cavity in the larvae of navaga *Eleginus nava*, polar cod *Boreogadus saida* and Arctic flounder *Liopsetta glacialis* (Okamoto, Kurokawa, Gen, Murashita, Nomura, Kim, Matsubara, Ohta, Tanaka, 2009; Sampaio, Freitas, Okamoto, Louzada, Rodrigues, Robaldo, 2007).

Incubation at temperature and salinity extremes may result in abnormal larvae with deformed notochord in Atlantic halibut (Ottesen, Bolla, 1998).

In general, the egg stage is vulnerable to thermal stress. Also yolk utilization efficiency in hatched larval depend on temperature. It is very important to know the temperature tolerances and optimum of a certain species to establish optimum conditions during incubation (Southgate, Lucas, 2003).

However, no studies have examined the environmental effects on eggs and post hatched effect on larvae of Ballan wrasse. Only limited information is available on Ballan wrasse tolerance to environmental conditions like temperature and salinity and only some research has been conducted using early developmental stages(Artuz, 2005).

The combined effects of temperature and salinity on survival and development of Ballan wrasse eggs have not been explored, and analysis of the effects of combinations of these environmental factors may increase understanding of the early life history of this fish.

Descriptions of cultured eggs and larvae beyond the start of hatching are not available, although there are some (incomplete) descriptions of wild-caught eggs larvae development and some information from farming experience (Artuz, 2005; Sayer, Treasurer, 1996; Skiftesvik, Bjelland, 2003). From an aquaculture perspective, it is important to know when developmental events occur (e.g. hatching, time to start feeding,) and how environment can affect these events. But this information can only be obtained from rearing experiments, that is why the following experiment was done with the great interest (Bjelland, Skiftesvik, 2006).

The main aim of this work was to examine how different temperatures and salinities influence fertilized eggs of Ballan wrasse (*Labrus bergylta*) through the period of incubation and find optimal values of these parameters during egg stage and hatching . Furthermore, find whether Ballan wrasse embryonic development is sensitive or not to different level of temperature and salinity.

To achieve the main aim following plan were laid down: to investigate the effects of temperature and salinity on the hatching rate, incubation duration, hatching time, morphometric larval characteristics at hatching and cases of deformities.

1.3. Biology of Ballan wrasse

The established name of Ballan wrasse is *Labrus bergylta*. Ballan wrasse belongs to the wrasse family (division Teleostei, order Perciformes, family Labridae) and is the largest of the north European wrasses, and may attain a total lengths of 600mm and can live maximum 25 years, as it was reported by Darwall and Costello (1992). Common lengths is 300-500 mm (Sayer, Treasurer, Costello, 1996), and life-span 17 years (Darwall, Costello, Donnelly, Lysaght, 1992). Ballan wrasse is the fish with deep set compressed body and large head. The mouth is small and protractible with thick fleshy lips. Coloration is variable and not dependent on sex or season, but may change according to surrounding background colour (Sayer, Treasurer, Costello, 1996). Juvenile Ballan (80-120 mm) may be a light green, older fish tend to brown/dark green colours with light spots all over the body (Per Gunnar Kvenseth, 2003b). (Darwall, Costello, Donnelly, Lysaght, 1992)

Ballan are recorded on eastern Atlantic coasts from Morocco to Norway. They are found in the North Sea and western parts of the Baltic, but may be rare or even absent from the Mediterranean area (Sayer, Treasurer, Costello, 1996). Juveniles are sometimes found in the intertidal; adults presence may extend below 30 m and occur on stony and rocky shores (Sayer, Treasurer, Costello, 1996).

Wrasse show territorial behaviour with aggression, which is associated with territorial defence during the breeding season. Sex reverse occur at about 6-9 years of age (16-18 cm for females and 28 cm length for males) and tends to be a year earlier in females (Darwall, Costello, Donnelly, Lysaght, 1992). This fish belongs to monandries protogynous species, which change sex after six years of age. Male guards a harem of females. On the Atlantic coasts spawning occurs in earlier summer from April and lasts to August (Artuz, 2005). However, on the west coast of Sweden records indicate spawning to occur from the end of May to the end of June (Darwall, Costello, Donnelly, Lysaght, 1992).

It spawns on gravel or rock, female build the nest and male take parental care. After one or two weeks moves to another territory when eggs hatched (Darwall, Costello, Donnelly, Lysaght, 1992). The wrasse have benthic eggs deposited in nests, and the larvae drift for some time in the water bodies (Per Gunnar, norsk sjømatsenter., Johan Solgaard., AS., 2003).

1.4. Early life history (morphometric characteristics of egg and newly-hatched larvae)

The eggs of *Labrus bergylta* demersal, sticky, spherical 0,7-1,15 mm in diameter, have creamy-white coloration with no oil globule (Artuz, 2005; Fives, 1976).

The prelarvae are hatching from the eggs with 2.7 mm (± 0.2mm) length (Artuz, 2005). The prelarvae of *L. bergylta* have a big and ovoid formed and unsegmented yolk sack. The anterior edge of the yolk sack passes beyond the vertical line from the mid of eye. Its maximum height is more or less on the level of the mesencephalon (Artuz, 2005). Body of newly hatched larva are heavily pigmented with chromatophores from posterior of the head to approximately the eight post-anal segment. Head pigment restricted to two crescent-shaped areas running longitudinally on either side of the mid-dorsal line. Anal fin membrane with a scattering of stellate chromatophores. There may be some small pigment spots along the posterior ventral margin of the body and on the primordial fin membrane in that region (Fives, 1976). The body is surrounded by dorsal, caudal and postanal swimming patterns. The preanal swimming pattern is clearly seen. Development of larva occurs planktonic (Artuz, 2005).

2. MATERIAL AND METHODS

2.1. Fish husbandry and eggs collection

The present study was done at Mørkvedbukta – Marine Research Station of University of Nordland, Bodø, Norway. Eggs of Ballan wrasse were collected from a broodstock of fish caught in Agder (Sørlandet, Norway), adapted to captivity and kept at station for two years. During the experimental year fish were kept under natural photoperiod conditions in big (5000 l) round tanks, sex composition was 10 to 20 females, and 1 – 2 males. Temperature was controlled and maintained around 7,6°C during the year and increased from 04.06.2010 and held at 9°C during the spawning season. Salinity was 33-34 ‰, oxygen level 8.3 mg l^{-}. The tanks were equipped with shelters for the fish made of plastic pipes (30 – 50 cm length), and artificial seaweeds made of black plastic bags. Because sticky benthic eggs of wrasse are difficult to collect, plastic plates were placed as a spawning substrate. The fish were feed 3 times per week with a feed composed of mixed shrimps, fish meal and fish oil. All tanks were inspected on presence of the eggs daily in morning time during spawning season. Eggs were sampled daily and checked for fertilization ratio, viability and staging. All eggs for experiment were collected from one batch and with more than 95% fertilization success. Eggs were taken at **"**morula stage"(Dulcic, Kozul, Kraljevic, Skaramuca, Glamuzina, Re, 1999; Hall, Smith, Johnston, 2004) when the blastoderm was in advanced stages of cleavage.

Part of batch of eggs was taken for checking fertilization ratio in spawning system (96%), then plates with spawned fertilized eggs were transferred in 10°C water temperature in darkness for further examining and incubation.

2.2. Experiment design

Preliminary experiment was established in June to test incubation system and to check process. The experiment started 23 June 2010 and lasted till 3 July 2010.

During this work it was checked how different temperatures and salinities (Table 1) influenced embryonic development of Ballan Wrasse eggs and if Ballan wrasse embryonic development was sensitive to temperature, salinity. Thus, eggs from one batch were incubated in 9 combination of temperatures and salinities were (Table 1).

Three closed incubation system with different types of stable temperatures were designed for the experiment. Egg were incubated in 6 multi-wells dishes (MD) with 20 ml of water and with 6 replicates (Table 1). Development and hatching were closely examined. Each combination of salinity and temperature was replicated five times to reduce the variation.

Because standardized methods for rearing wrasse eggs are not established, it was not possible to make a control group. The combinations of conditions were considered to test against each other.

The sixth MD was established for taking samples at a regular basis to check normal and abnormal development of embryos. Eggs examined were not returned to the incubation, but 2 or 3 eggs were fixed for further microscopy examination. The experiment was carried out under an artificial photoperiod (18h light:6 h darkness). Incubation experiments with batches of eggs were conducted till hatch.

Table 1. Combinations of the conditions.

MD	Combinations of temperature and salinity
1	10 C° 26‰
2	10 C° 30‰
3	10 C° 33‰
4	15 C° 26‰
5	15 C° 30‰
6	15 C° 33‰
7	20 C° 26‰
8	20 C° 30‰
9	20 C° 33‰

2.3. Experiment process

After checking fertilization ratio and developmental stage, eggs together with the plastic plates were transferred from tank in trays with seawater to cooling room (10°C). Then eggs were distributed randomly among 9 six-well dishes (about 30-40 eggs per well) by a cut plastic pipette and plastic spoon. As wrasse eggs are benthic the eggs were submerged on the bottom of the dish by themselves after few hours. The total number of eggs was estimated by counting the eggs in cooling room. Eggs were rinsed 3 times with fresh filtrated water to remove contaminations.

To obtain the experimental salinities below 33‰ required amount of sterile filtrated distilled water was added to double filtrated sea water (33‰) in which broodstock lives to the desired level. Jug with water of experimental salinities were stored in cooling room and was insulated with expanded polyester, but maintained at each of the experimental temperatures for

12 hours before adding to dishes with eggs in order to avoid thermal stress. Final salinity was measured by refractometry.

Dishes were filled with experimental seawater at one of three salinities (20 ml in each), the same was done in the experiment with European hake (Bjelland, Skiftesvik, 2006) and cod (Nissling, Larsson, Vallin, Frohlund, 1998) and were maintained at 10, 15 and 25 °C in different incubators.

Results of experiments on tolerance of cod eggs to low salinities and temperatures have been performed for Atlantic cod (Nissling, Westin, 1991) and may indicate differences in the egg salinity tolerance. That is why in our experiment the eggs from the broodstock were transported into 10 °C water and the temperature was then gradually increased from 10 to experimental of each combination (10, 15,20°C) within 2,5 days to avoid stress caused by sudden temperature changes and was maintained throughout the experiment.

By water exchange (33, 30, 26‰) salinity was gradually decreased from 34‰ (salinity of local marine water) to experimental within 2 days to avoid stress caused by sudden changes of rearing environmental condition.

Fifty percent of the water in each wells was replaced twice per day using salinity adjusted seawater of appropriate experimental temperature to eliminate the requirement for aeration, which has been found to cause physical damage to eggs in small volume of water (Hart, Purser, 1995).

For all replicates, dead eggs were removed and counted daily until first day of hatching. Dead eggs were removed from the plate with a pipette when detected during the daily inspections in cooling room (10°C).

The wells were taken out of the incubators in the morning every day, and observed under a binocular microscope for hatching and mortality. Aspects of development, such as contractions and pigmentations were noted.

The incubation was continued until hatching to estimate the total incubation time to hatching and the quality of newly hatched yolk-sac larvae.

Samples for morphometric measurements were collected by gingerly pipetting newly hatched live larvae and before fixation, anesthetized in 0,1% (m/v) solution of tricane methanesulfonate (MS-222) (Keinanen, Tigerstedt, Kalax, Vuorinen, 2003) prior to fixation for morphometric measurements. All larvae and sampled eggs were fixed in a mixture of 10% paraformaldehyde and 25% glutaraldehyde in cacodylate buffer (pH 7.2) (Appendix 1) and stored at 12°C until further analyzing of larval deformities and measuring of morphometric parameters. Fixed eggs and larvae were examined under a microscope at different magnifications.

2.4. Estimated parameters

Fixed larvae were examined under microscope. Photos were taken and then were examined for morphometric parameters of larvae and deformities. The photos of eggs were also made. All analyses of morphology were conducted using microscope Olympus SZ-12 and the software program Cell A, Olympus. Precise length measurements (0,01 mm) were obtained using an image analysis system connected to the microscope.

Diameter of fixated sampled eggs was measured.

The following morphometric measures of larvae that had been preserved in fixative for approximately 3 months were recorded:

Standard length (SL) in mm (the distance along the midline of the body from the tip of the snout to the end of the caudal fin), and myotome height at the anus (MH) of larvae, horizontal eye diameter (ED), yolk sac length (YSL), yolk sac height (YSH), yolk sac volume (YSV), egg hatching rate (%), and larval abnormality (%) were determined. Yolk sac volume (YSV) was calculated with following formula:

$$V=1/6лhl^2,$$

where h - is the short diameter, l - is the long diameter of the elliptic yolk sac (Hart, Purser, 1995; Shi, Huang, Fu, Wang, Luo, Chen, Liu, Zhang, 2008a). Means and standard deviation of these parameters were calculated.

Also development of the eyes, pigmentation gut and jaws were noted.

The number of both dead eggs and hatched larvae was counted. Percentage of hatched larvae and egg mortality were calculated relative to the number of eggs at the start of incubation, then the rates of hatching was calculated using the following formula (Bjelland, Skiftesvik, 2006):

$$\text{Hatching rate (\%)} = 100 \times \text{number of hatched larvae} / \text{number of incubated eggs}$$

Hatching period duration from first to last hatched larvae -“hatch-time” (“early”-first day, “mid”-second day or “late” –third day hatch) was calculated, which is the time interval between the period of first larvae and the period when all alive larvae have hatched from the fertilized eggs (Laurel, Hurst, Copeman, Davis, 2008). Incubation period – “time to hatch” in °C day (degree day = number of days * temperature in °C) till first portion of larvae had hatched and till last larvae hatched was calculated and used for further analysis.

Larvae that were too much deformed were not measured, but it`s deformations were recorded.

Development was considered defective if newly hatched larvae deviated from normal development and morphological differentiation (Cameron, Von Westernhagen, 1997; Vonwesternhagen, Dethlefsen, Cameron, Berg, Furstenberg, 1988).

The proportion of deformed individuals among the newly hatched larvae was calculated, the defective larvae classified to the following malformation types

- A 1. Lordosis of small degree
- A 2. Lordosis of great degree
- A 3. Kyphosis
- A 4. Scoliosis
- A 5. Downward spinal curvature in the abdominal or caudal region
- A 6. Upward spinal curvature in the abdominal or caudal region
- A 7. Axial undulation
- B 1. Lordosis of small degree accompanied by oedema
- B 2. Lordosis of great degree accompanied by oedema
- B 3. Kyphosis accompanied by oedema
- B 4. Scoliosis accompanied by oedema
- B 5. Downward spinal curvature in the abdominal or caudal region accompanied by oedema
- B 6. Upward spinal curvature in the abdominal or caudal region accompanied by oedema
- B 7. Axial undulation accompanied by oedema
- C 1. Small oedema
- C 2. Vast oedema
- D 1. Larvae with downward axial curve in abdominal and caudal regions
- D 2. “C-shaped” larvae
- D 3. “Shortened body” larvae
- E 1. Larvae with downward axial curve in abdominal and caudal regions accompanied by oedema
- E 2. “C-shaped” larvae accompanied by oedema
- E 3. “Shortened body” larvae accompanied by oedema
- F 1. Larvae with sharp deformation of spinal column, several deformations, scoliosis in the abdominal and caudal region, with asymmetrically curved tail

- F 2. Larvae with sharp deformation of spinal column, several deformations, scoliosis in the abdominal and caudal region, with asymmetrically curved tail accompanied by deformation of the yolk sac with oedema

Detailed description of types presented in chapter 3.8. “Malformations of newly hatched larvae and its characteristics”. Classification developed in this study allows to evaluate body malformations in the Ballan wrasse larvae under various environmental factors not only quantitatively, but also qualitatively.

The occurrence rate of deformities at fixated larvae was calculated using the following formula (Georgakopoulou, Katharios, Divanach, Koumoundouros, 2010; Shi, Huang, Fu, Wang, Luo, Chen, Liu, Zhang, 2008b; Tutman, Glamuzina, Skaramuca, Kozul, Glavic, Lucic, 2000):

$$\text{Deformed larvae rate (\%)} = 100 \times \text{number of deformed larvae} / \text{number of surviving larvae:}$$

The results of the thesis experiments are presented in figures and tables. Total 2246 egg were incubated and 1523 larvae were examined.

Effects of salinity and temperature on the newly hatched larvae were evaluated using the following criteria (Zhang, Shi, Zhu, Liu, Zang, 2010):

- Hatching rate (%)
- Time to hatch (in °C days)
- Hatch-time (“early”-first day, “mid”-second day or “late” –third day hatch)
- Morphometric parameters of newly hatched larvae
- The occurrence rate of deformities (%)
- Type of deformities

2.4. Statistical analysis

All statistical analyses were performed with the JMP 7 package of statistical software and Microsoft Excel software.

All statistical analyses of morphometric parameters were carried out using one or two-way analyses of variance (ANOVA).

Means among different rearing conditions were compared by analysis of variance (ANOVA). If the differences were found, then these differences were analyzed for significance ($P<0.05$) with Turkey-Kramer HSD test. Percentage date was arcsine transformed prior to statistical analysis.

3. RESULTS AND DISCUSSIONS

3.1. Egg and newly hatched larvae characteristics

L. bergylta had spherical demersal and sticky eggs of mean diameter 0,95±0,02 mm with creamy white coloration. The yolk was homogeneous and transparent, with a pale yellowish colouration. There were no visible oil droplets in the eggs (Fig. 1).

Development of the embryo was similar to that described for most marine fish larvae (Artuz, 2005; Bjelland, Skiftesvik, 2006; Dulcic, Kozul, Kraljevic, Skaramuca, Glamuzina, Re, 1999; Fives, 1976). Pigmentation appears early in development; the first sign of pigment appeared just after closure of the blastopore. Just before hatching, the embryo reached all the way round the inside of the egg, and its nose and tail touched. First contractions were noticed the day before hatching in all combinations of temperature and salinity (Fig. 2).

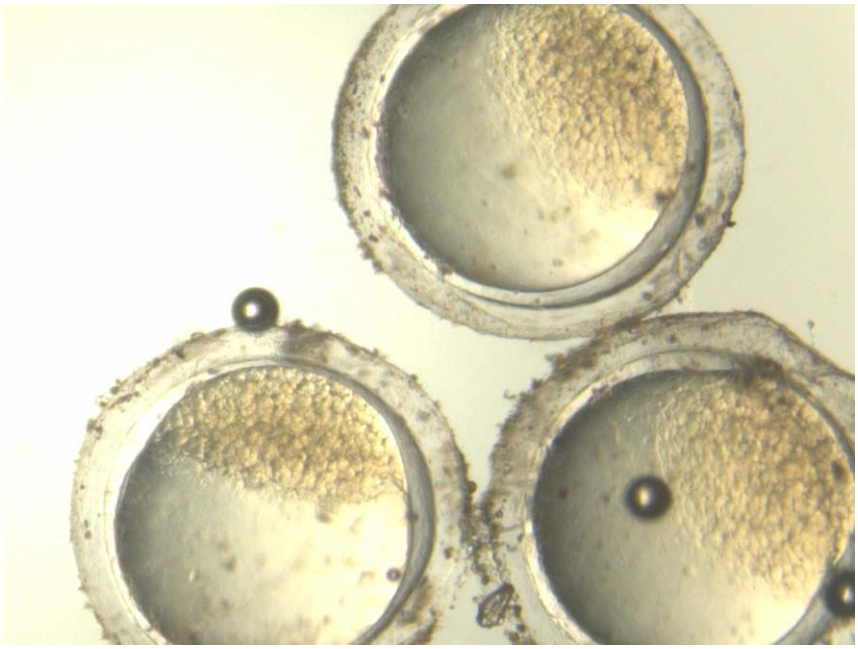

Fig. 1. Light microscopy of *L. bergylta* eggs at early stage of development.

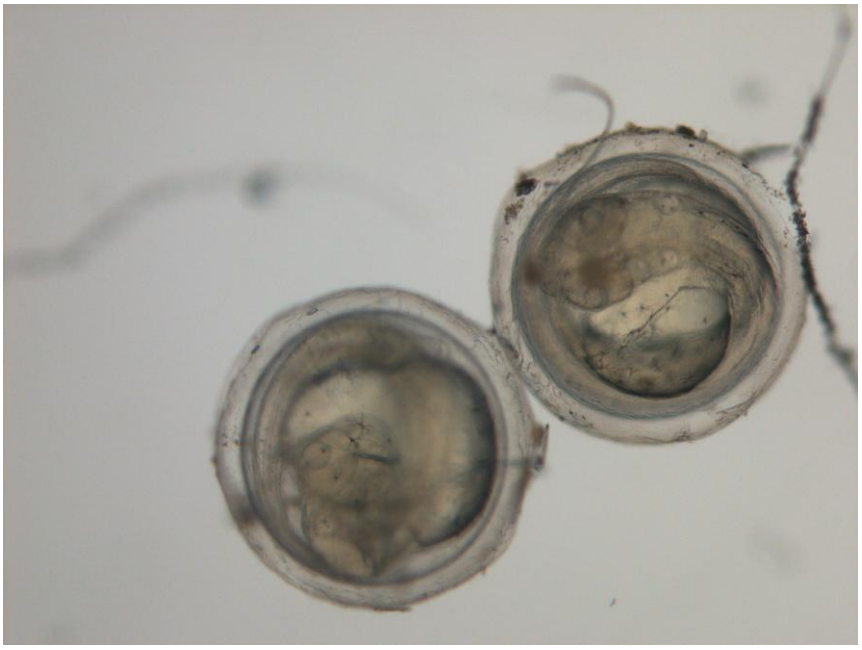

Fig. 2. Light microscopy of *L. bergylta* eggs ready to hatch.

The standard length of newly hatched larvae of *L. bergylta* were 3,297±0,2 mm. The SL of the biggest larvae was 3,961 mm, the smallest – 2,615 mm. Mean myotome height was 0,185±0,01 mm.

Larvae were transparent and had an unsegmented ovoid formed yolk sack (YSL mean 0,985±0,13; YSH mean 0,418±,045; YSV mean 0,09±0,019 mm^3).

The anterior edge of the yolk sack passes beyond the vertical line from the mid of eye. Head was pointed forwards and partly free from the yolk. Anal pore was separated, takes place after the yolk sack. The mouth was undeveloped. The eyes were not pigmented mean 0,257±0,02 mm in diameter. Otic capsules were present. The body was surrounded by dorsal, caudal and postanal swimming patterns. The pigmentation pattern consists of scattered pigment cells around the yolk, head, trunk and some areas at the posterior part of the body. Head pigment was restricted to crown region. Brown pigment was dominant (Fig. 3).

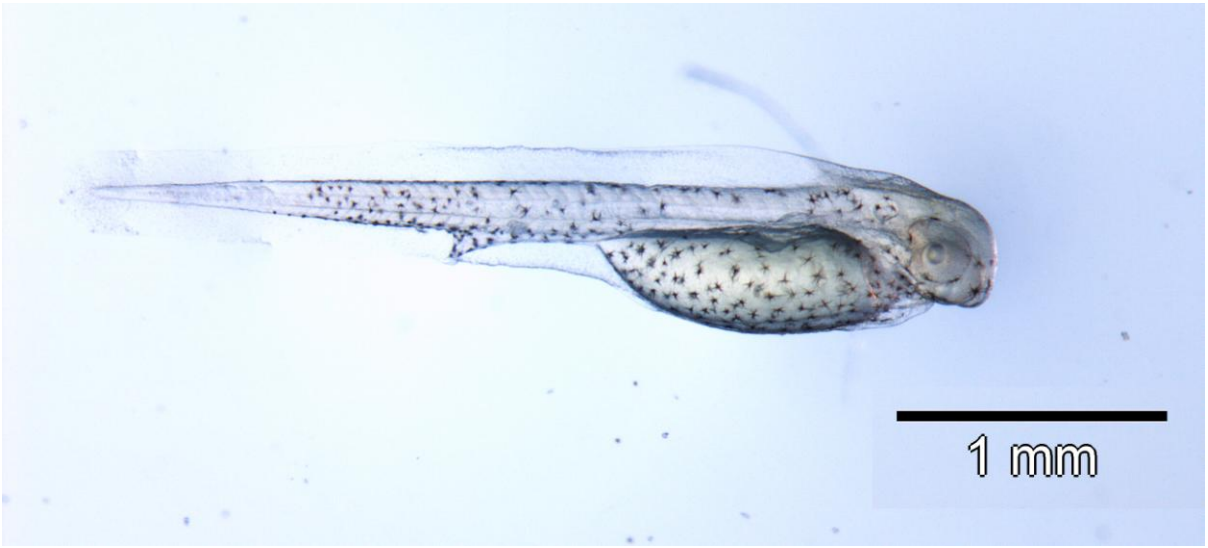

Fig. 3. Light microscopy of *L. bergylta* newly hatched larva 3,297±0,2 mm SL.

First larvae hatched approximately at 59,3 °C days (5days) after fertilization when kept in the incubators in 15°C, while the larvae kept in 20°C hatched at 67,5 °C days (4 days of incubation), and in 10°C hatched at 76,3 °C days (eight days of incubation).

3.2. Hatching rate

Even though hatching occurred in all combinations of experimental temperature and salinity, hatching rare was lower significantly lower at 20°C 30 ‰ (52,86±15,7) and 20°C 33 ‰ (51,98%±25,0) than that at 15°C 30‰ (83,3%±10,9) and 33‰ (84,57%±9,0) (Table 2). There was no significant difference among combinations - 10°C 26‰, 10°C 30‰, 15°C 26‰, 10°C 33‰, 20°C 26‰ (Fig. 4).

There was effect of temperature on hatching rate only in batches of eggs incubated in 30‰ ($F_{2,45}$ =6,07, p<0,005) and 33‰ ($F_{2,45}$ =6,9, p<0,002). There was no significant effect of salinity on temperature (p>0,05).

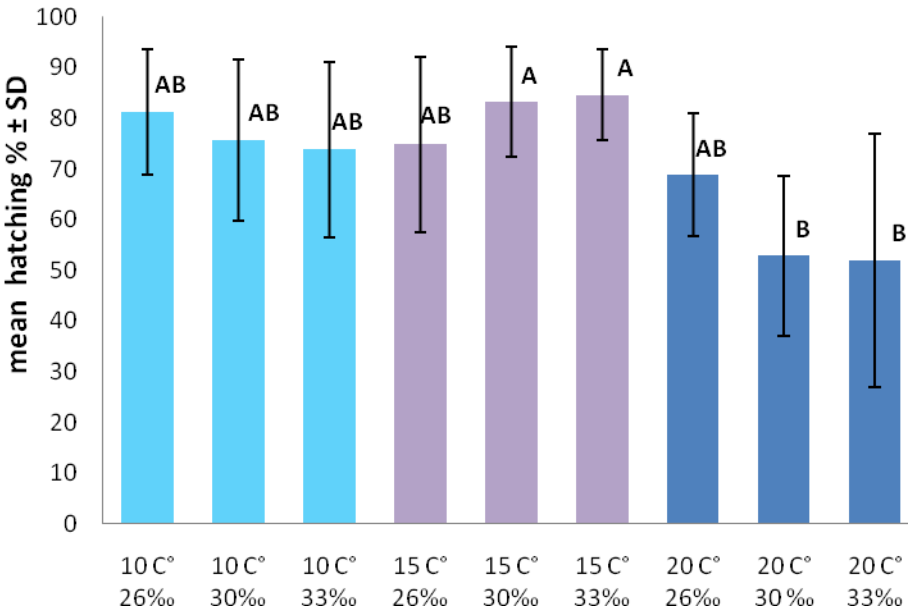

Fig. 4. Hatching rate (mean%±SD) at different temperature and salinities of Ballan wrasse eggs incubated. Different letters above each bar indicate significant differences among treatments (p <0,001).

Table 2. Hatching success of eggs of Ballan wrasse incubated at different temperatures and salinities (mean%±SD, n=6 replicates). Different letters indicate significant differences among treatments.

Temperature, °C	Salinity, ‰	Mean% ± SD	Significance
15	33	84,57±9,0	A
15	30	83,3±10,9	A
10	26	81,34±12,4	AB
10	30	75,80±15,9	AB
15	26	74,87±17,3	AB
10	33	73,83±17,3	AB
20	26	68,77±12,1	AB
20	30	52,86±15,7	B
20	33	51,98±25,0	B

3.3. Analysis of hatching rate of newly hatched Ballan wrasse larvae and hatch time

Many fish larvae hatch asynchronously from the same egg batches despite experiencing a common environment during their development (Laurel, Hurst, Copeman, Davis, 2008), and when incubated at different environmental parameters such asynchrony vary greatly.

The number of days over which the larvae hatched is termed hatch duration (Laurel, Hurst, Copeman, Davis, 2008). In the present study the hatch duration was different between different combinations of temperatures and salinities. For larvae held in 10°C it took up 75 hours from first hatched larvae to last. Larvae held in 15°C hatched for 51 hours, and in 20°C – 48 hours was needed to complete hatch (Table A4).

There was significant effect of hatch-time ("early"-first day, "mid"-second day or "late" –third day hatch) on hatching rate ($F_{3,326}$ = 5,36, p<0,001). For 10°C at first day (early hatch) main part of larvae were hatched (Table A4), but this trend did not maintain for 15°C and 20°C. For 20°C main part of larvae were hatched on second day of hatching, the same trend maintained for 15°C (Table A4).

3.4. Analysis of morphometric characteristics of newly hatched Ballan wrasse larvae

While the duration of the hatch period is generally temperature-specific, there is little likelihood that larvae emerging from eggs during the hatching period are morphometrically similar (Laurel, Hurst, Copeman, Davis, 2008). So we used morphometric characteristics to determine if there was effects of temperatures and salinities.

From the analysis of variance we can conclude that interaction of temperature and salinity during eggs incubation of Ballan wrasse has significant effect on length (SL) of newly hatched larvae (F = 4,76; p <0,001). Single effect of temperature and salinity on SL was significant F = 144,73; p <0,001; F = 5,45; p <0,004 respectively. Size at hatched among temperature was highly variable, in total ranging from 2,62 to 3,96 mm (mean 3,29±0,2). Larvae held at 10°C were longer (3,37±0,19 mm) than larvae held at 15 and 20 °C. The smallest larvae hatched at 20°C (3,14± 0,15 mm) (Fig. 5a,b) (Table A1).

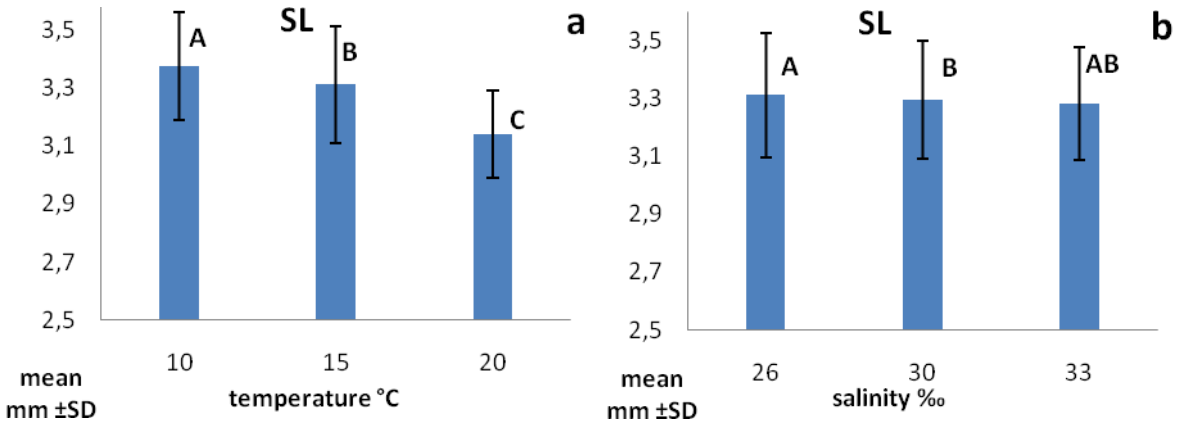

Fig. 5. Standard length mm (mean ±SD) of newly hatched larvae of Ballan wrasse incubated at different temperatures (a) and salinities (b). Different letters above each bar indicate significant differences among treatments (p <0,001).

The combination of the effects of salinity and temperature that gave the longest larvae was 10°C 26‰, but this result did not differ significantly from combination 10°C 30‰, 10°C 33‰, 15°C 30‰. The smallest SL value was observed in the combination 20°C 30‰ (Table A1).

The effect of temperature was significant in all experimental salinities (Table A2). The effect of salinity on SL was significant only in batches incubated at 20°C ($p < 0,001$) (Table A3).

Single effect of temperature and salinity on myotome height (MH) of larvae was significant $F = 88,4$; $p < 0,001$; F Ratio= 9,5; $P < 0,001$ respectively. Larvae incubated at 10°C had bigger value of MH (0,192±0,011 mm), the smallest value was found in 15°C (0,176±0,014 mm) (Fig. 6a,b).

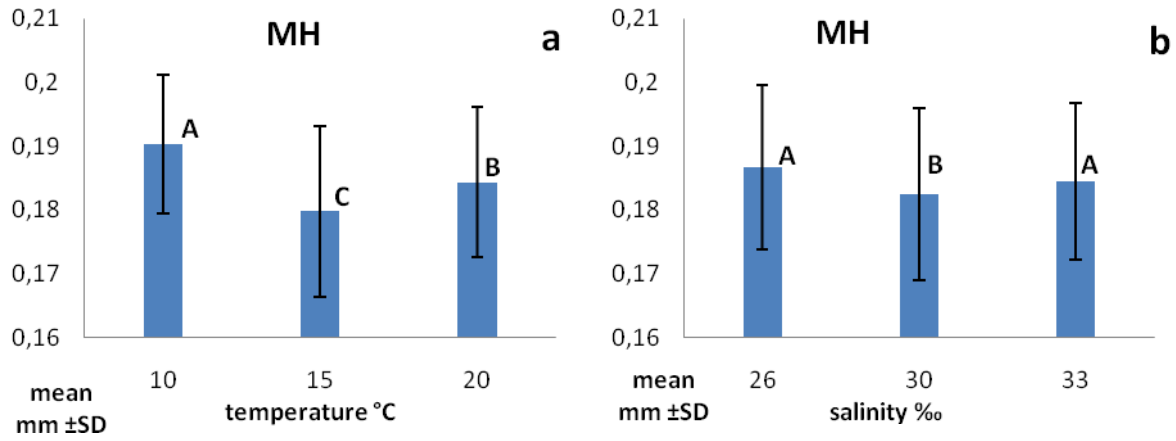

Fig. 6. Myotome height mm (mean±SD) of newly hatched larvae of Ballan wrasse incubated at different temperatures (a) and salinities (b). Different letters above each bar indicate significant differences among treatments (p <0,001).

The lowest MH of larvae was observed in combination 15°C 30‰ (0,176±0,014) and it differ significantly from other conditions (Table A1). Larvae hatched at combination 10°C 26‰ had the biggest MH value, but did not significantly diverge from 10°C 30‰, 10°C 33‰ (Table A1). The effect of temperature was significant in all experimental salinities (Table A2). The effect of salinity was significant only in batches incubated at 15°C ($p<0,001$) (Table A3).

The effect of temperature on eye diameter (ED) of larvae was significant (F Ratio= 240,9; $p<0,001$). But there was no effect of salinity on ED, and interaction of the two factors had no effect on ED. Larvae incubated at 10 °C had bigger eyes than in other conditions (0,27±0,01 mm). Whereas larvae incubated in 20°C had the smallest ED (0,24±0,01 mm) (Fig. 7). The effect of temperature was significant in all salinities (Table A2).

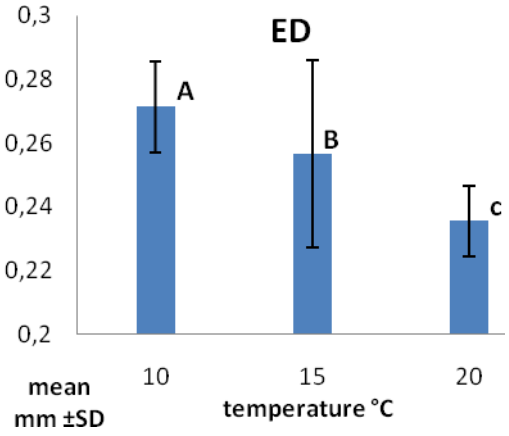

Fig. 7. Eye diameter mm (mean±SD) of newly hatched larvae of Ballan wrasse incubated at different temperatures. Different letters above each bar indicate significant differences among treatments (p <0,001).

Yolk sac length (YSL) was affected by temperature and salinity ($F = 609,6$, $p<0,001$; $F = 4$, $p<0,019$), but there were no interaction effect of this factors. The biggest value of YSL (1,07±0,08 mm) was registered in temperatures 10°C, and it was not significantly different from values in all salinities (Fig.8b). The smallest YSL value belonged to larvae hatched in temperature 20°C (0,83±0,11 mm) (Fig.8a).

There was no effect of salinity on YSL when analyzed in three experimental temperatures separately, whereas the effect of temperature was significant in all experimental salinities (Table A2). Value of YSL of larvae incubated in combination 20°C 26‰ was recognized as smallest (0,82±0,14 mm) that significantly differed from value 1,08±0,07 mm in combination 10°C 30‰ (Table A1).

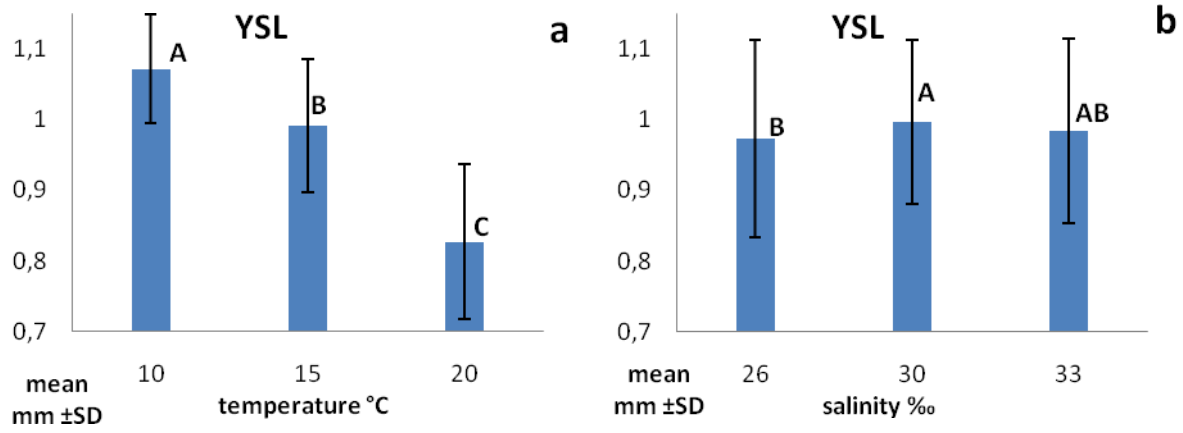

Fig. 8. Yolk sac length mm (mean±SD) of newly hatched larvae of Ballan wrasse incubated at different temperatures (a) and salinities (b). Different letters above each bar indicate significant differences among treatments (p <0,001).

Yolk sac height (YSH) was effected only by temperature (F = 26,0743, p <0,001). One way analysis showed the smallest YSH in 10°C (0,407±0,04), the biggest in temperature 20°C (0,431±0,05) (Fig. 9a). YSH values in different salinities did not differ significantly (Fig. 9b). The effect of temperature was significant in all experimental salinities (Table A2) and it was significantly different from the combination 20°C 26‰ that gave the biggest value 0,433±0,06 mm (Table A1).

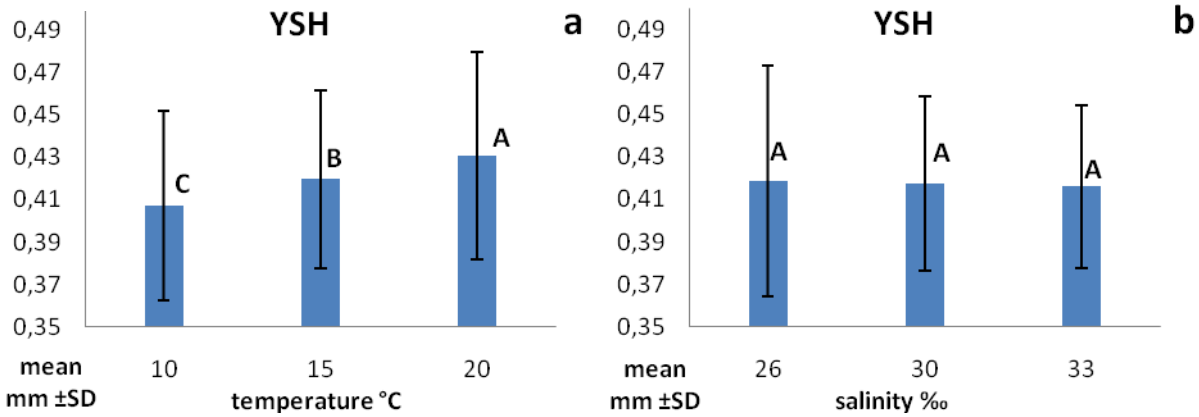

Fig. 9. Yolk sac height mm (mean±SD) of newly hatched larvae of Ballan wrasse incubated at different temperatures (a) and salinities (b). Different letters above each bar indicate significant differences among treatments (p <0,001).

Yolk sac volume (YSV) was affected only by temperature (F = 45,6804, p <0,001). One way analysis showed the smallest YSV in 20°C (0,0813±0,02 mm^3), the biggest in temperature 10°C (0,0939±0,02 mm^3) (Fig. 10a). YSV values in different salinities did not differ significantly (Fig.10b). The effect of temperature was significant in all experimental salinities (Table A2).

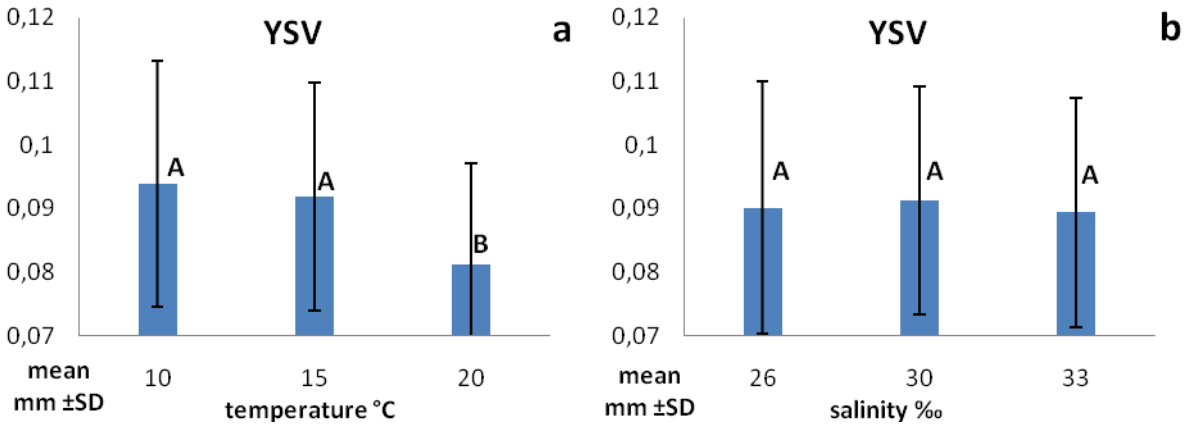

Fig. 10. Yolk sac volume mm^3 (mean±SD) of newly hatched larvae of Ballan wrasse incubated at different temperatures (a) and salinities (b). Different letters above each bar indicate significant differences among treatments (p <0,001).

3.5. Analysis of morphometric characteristics of newly hatched Ballan wrasse larvae at different time of hatching

Both temperature and hatch-time ("early"-first day, "mid-"-second day or "late" –third day hatch in this work) explained hatching size as indicated by the significant interaction term (p <0,001). Early hatching larvae were smaller than late hatching larvae, size-at-hatch (SL) generally decreased with increasing temperature (Fig. 11a), although this was most apparent in first hatching larvae (Table A5). The same trend was observed in different salinities, the size of larvae increased at late hatching (Fig. 11b). However, between the salinities there was practically no difference in size at first day and second day of hatching (Fig. 11b).

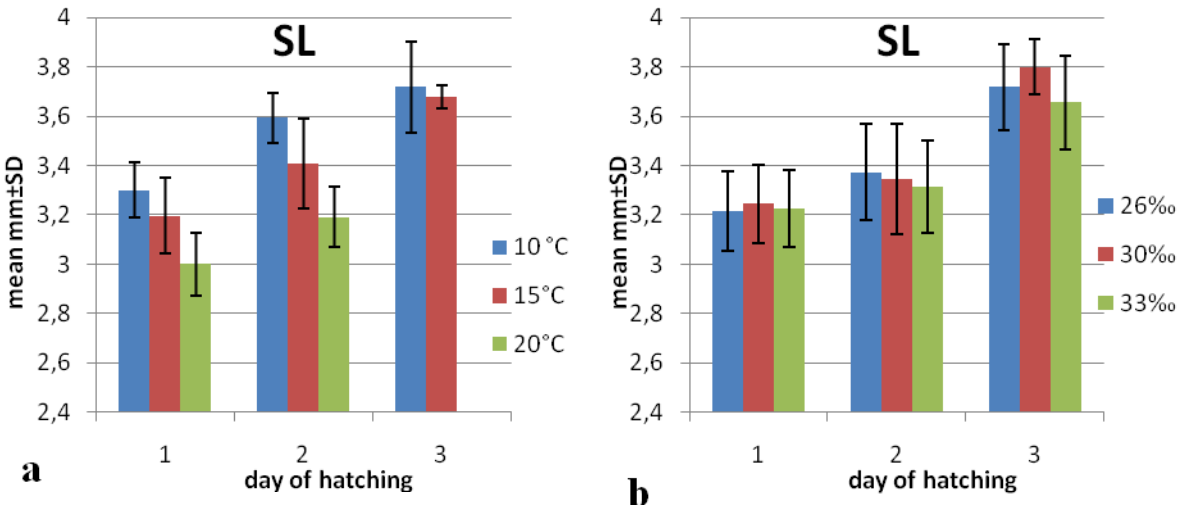

Fig. 11. Larvae size at first, second and third day of hatching incubated at different temperatures (a) (data for third day 20°C is absent because of high deformity of larvae) and different salinities (b) (mean±SD mm).

Early hatching larvae in all treatments had smaller myotome height than mid or late hatching (Table A5) (Fig. 12a,b). The eye diameter showed clear trend of increasing size with increasing incubation time in 10°C (Table A5) (Fig. 13a). For other temperature treatment there was not clear interaction of hatching duration and eye diameter (p >0,001). Eye diameter was bigger at late hatch in all salinity treatment (Table A5). There was significant difference between early, mid and late hatched larvae at 30‰ and 33‰, but there was no difference between early and late hatched larvae eye diameter in treatment 26‰ (Table A5). Larvae hatched at second day (mid time of hatch) had smaller eye diameter in all salinity treatments (Fig. 13b) (Table A5).

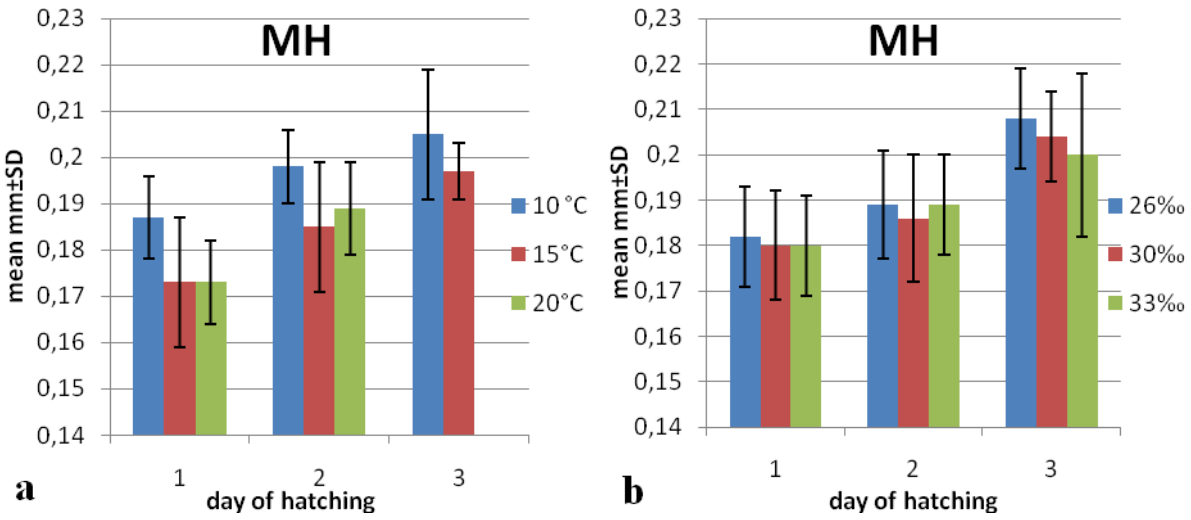

Fig. 12. Myotome height of larvae at first second and third day of hatching incubated at different temperatures (a) (data for third day 20°C is absent because of high deformity of larvae) and different salinities (b) (mean±SD mm).

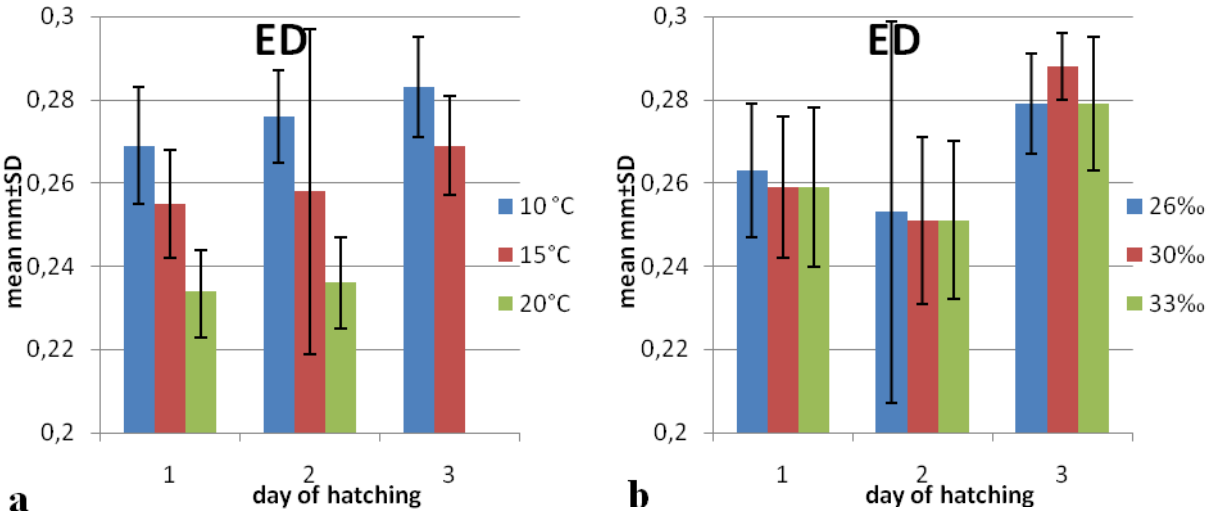

Fig. 13. Eye diameter of larvae at first second and third day of hatching incubated at different temperatures (a) (data for third day 20°C is absent because of high deformity of larvae) and different salinities (b) (mean±SD mm).

We also found an interaction between the temperature and the time of hatch ("early", "mid-" or "late") on yolk reserves. As indicated in the analysis of yolk sac length, yolk sac height and yolk sac volume, yolk reserves decreased with increasing time of hatching. Yolk reserves were significant higher in early-hatched larvae (Fig. 14a, 15a, 16,a) with the exception

of larvae at 20°C (Table A5). Interaction between the salinity and time to hatch was not clearly seen (Fig. 14b, 15b, 16b), only analysis of yolk sac volume showed the same trend of decreasing yolk reserves at increasing time to hatch (Table A5) (Fig. 16b).

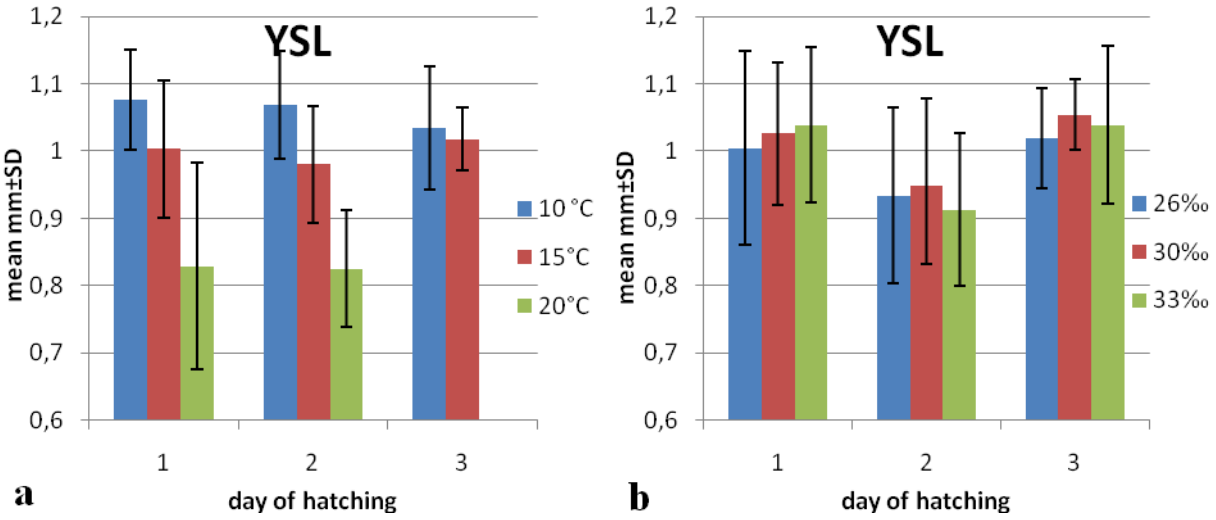

Fig. 14. Larvae yolk sac length at first second and third day of hatching incubated at different temperatures (a) (data for third day 20°C is absent because of high deformity of larvae) and different salinities (b) (mean±SD mm).

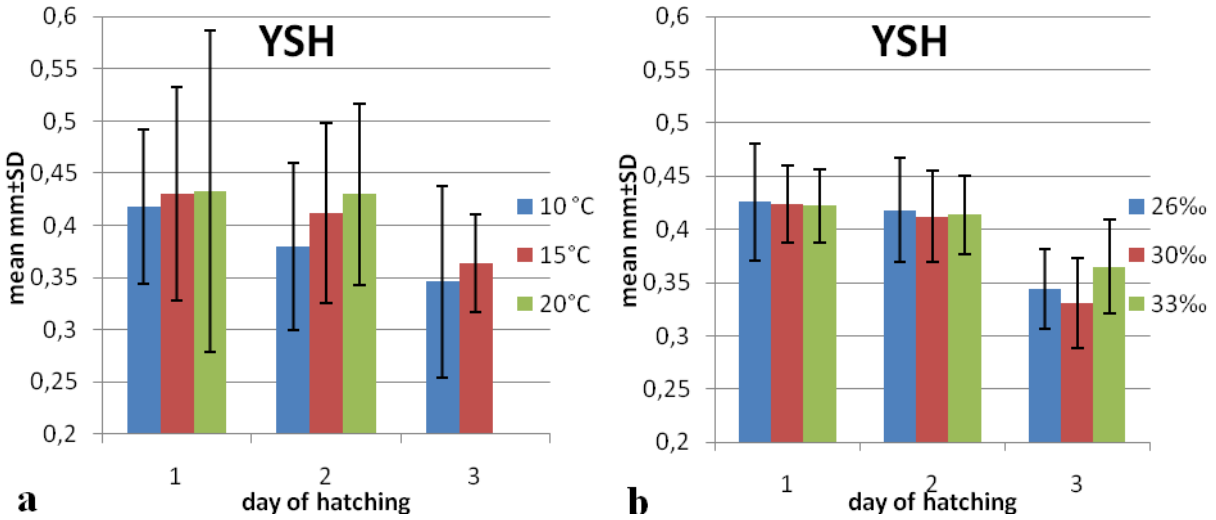

Fig. 15. Larvae yolk sac height at first second and third day of hatching incubated at different temperatures (a) (data for third day 20°C is absent because of high deformity of larvae) and different salinities (b) (mean±SD mm).

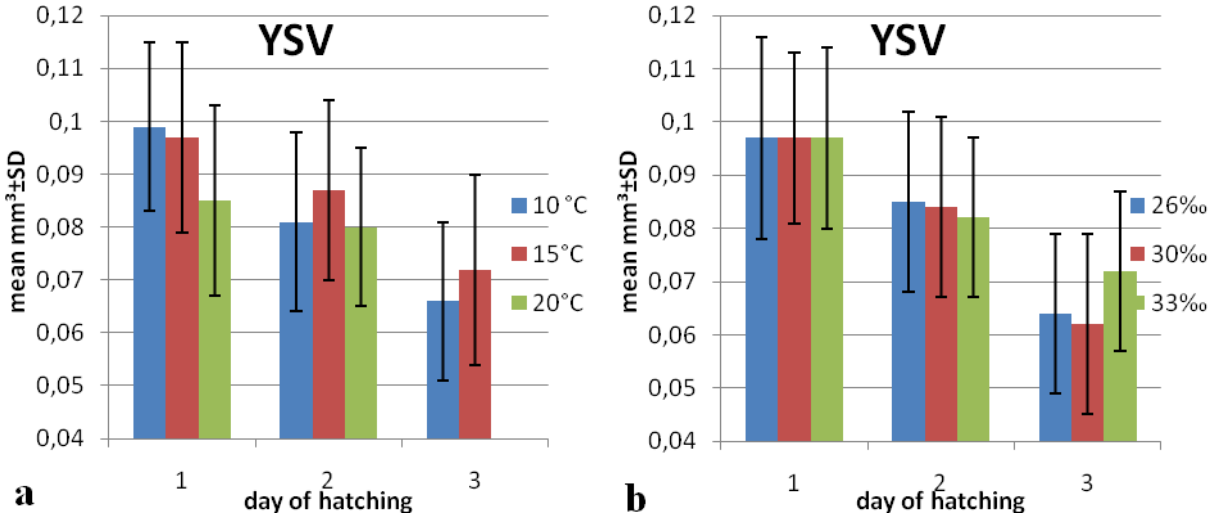

Fig. 16. Larvae yolk sac volume at first second and third day of hatching incubated at different temperatures (a) (data for third day 20°C is absent because of high deformity of larvae) and different salinities (b) (mean±SD mm^3).

3.6. Relationship of morphometric characteristics of newly hatched Ballan wrasse larvae and time to hatch (°C days)

To test the relationship between estimated parameters of newly hatched larvae of Ballan wrasse (SL, MH, YSL, YSH, YSV) and time to hatch (in °C days) in different treatments, time to hatch was plotted against parameters of larvae (mm).

The correlation between SL and °C days was positive and significant for all experimental treatments of different temperatures and salinities (10°C, 15°C, 20°C, 26‰, 30‰, 33‰) (Fig. A1).

A quite strong and significant correlation was found between the larvae length and degree day for 10°C: r = 0,686; F = 1030,9; p <0,001 (Fig. A1a). For treatment 15°C and 20°C correlations were less strong but significant: r = 0,434; F = 419,3; p <0,001 (Fig. A1b) and r = 0,377; F = 168,1; p <0,001 (Fig. A1c).

Correlation for different experimental salinities between SL and °C days was less strong than for temperatures. Only for 26‰: r = 0,37; F = 278,6; p <0,001 (Fig. A1d) correlation was the same as for 20°C and this correlation was the highest between the other salinities 30‰ and 33‰: (r = 0,261; F = 163,1; p <0,001 and r = 0,233; F = 114,9; p <0,001) (Fig. A1e,f)

The correlation between MH and °C days was positive and significant for all experimental treatments (10°C, 15°C, 20°C, 26‰, 30‰, 33‰) (Fig. A2).

The highest correlation between all treatments (temperatures and salinities) was observed for 20°C: r = 0,408; F = 192,6; p <0,001 (Fig. A2c). Comparing between temperatures it was higher than for 10°C: r = 0,342; F = 244,7; p <0,001 (Fig. A2a), which in turn was higher than in 15°C (r = 0,211; F = 146,4; p <0,001) (Fig. A2b). In case of salinities the correlations were approximately on the same level. For 26‰: r = 0,361; F = 257,4; p <0,001, for 30‰: r = 0,368; F = 269,4; p <0,001, and for 33‰: r = 0,353; F = 207,5; p <0,001 (Fig. A2d,e,f).

The correlation between ED and °C days was positive and significant only in 10°C: r = 0,134; F = 73; p <0,001 (Fig. A3 a), and in 30‰ (r = 0,042; F = 20,5; p <0,001) (Fig. A3b), 33‰ (r = 0,022; F = 8,5; p <0,004) (Fig. A3c).

The correlation between YSL and °C days was negative and significant only for 33‰ (r = -0,027; F = 10,3 ; p <0,001) (Fig. A4). In other treatment correlation was negligible and non-significant.

The correlation between YSH and °C days was negative and significant but not strong for all experimental treatments (10°C, 15°C, 26‰, 30‰, 33‰) (Fig. A5), except the correlations for 20°C, it was not significant: r = -0,004; F = 1,15; p <0,283 (Fig. A5c). For 10°C the correlations was highest among all treatments r = -0,255 ; F = 161; p <0,001 (Fig. A5a). For 15°C: r = -0,09; F = 55,8; p <0,001 (Fig. A5b). The lowest correlation showed all salinities 26‰: r = -0,098; F = 49,6; p <0,001 (Fig. A5d), for 30‰: r = -0,096; F = 49,2; p <0,001 (Fig. A5e), and for 33‰: r = -0,067 ; F = 27,5; p <0,001 (Fig. A5f).

The correlation between YSV and °C days was also negative and significant for all experimental treatments but not strong (10°C, 15°C, 20°C, 26‰, 30‰, 33‰). The strongest correlation among treatments was for 10°C: r = -0,268; F = 172,3; p <0,001 (Fig. A6a) and for 26‰: r = -0,14; F = 74,8; p <0,001 (Fig. A6d). For rest treatments the correlations were low (15°C: r = -0,125; F = 78,4; p <0,001, 20°C: r = -0,033; F = 9,7; p <0,002, 30‰: r = -0,06; F = 30,0; p <0,001, 33‰: r = -0,12; F = 52,39; p <0,001) (Fig. A5b,c,e,f).

3.7. Relationship of morphometric characteristics of newly hatched Ballan wrasse larvae, time to hatch and three different temperatures, three different salinities

To test the relationship between morphometric parameters of newly hatched Ballan wrasse larvae (SL, MH, YSL, YSH, YSV), three different temperature or three different salinity parameters (10°C, 15°C, 20°C, 26‰, 30‰, 33‰) time to hatch (in °C days), time to hatch was plotted against values of parameters of larvae (mm) (Fig. A7).

For SL analysis (ANOVA) showed that both effect of temperatures and time to hatch was significant ($F = 384,49$; $p < 0,001$ and $F = 1212,2$; $p < 0,001$) and interaction of these factors was significant ($F = 42,2$; $p < 0,001$) (Fig. A7a). Time to hatch and size was related to temperature. Effect of salinities was not significant ($F = 1,76$; $p < 0,17$), but interaction of effects of salinity and time to hatch was significant ($F = 6,51$; $p < 0,001$) (Fig. A7b).

The interaction of effects of temperature and time to hatch on MH was not significant ($F = 1,3$; $p < 0,26$). Time to hatch and MH was not related to temperature (Fig. A7c), but time to hatch and MH was related to salinities ($F = 5,6$; $p < 0,004$) (Fig. A7d).

The interaction of effects of temperature and time to hatch on ED was significant ($F = 9,08$; $p < 0,001$) (Fig. A7e), but time to hatch and ED was not related to salinity ($F = 1,2$; $p < 0,3$) (Fig. A7f).

Time to hatch and YSL was not related to temperature ($F = 1,6$; $p < 0,2$) (Fig. A7g), but time to hatch and YSL was related to salinities ($F = 8,2$; $p < 0,001$) (Fig. A7h).

The interaction of effects of temperature and time to hatch on YSH was significant ($F = 26,3$; $p < 0,001$), so the time to hatch and YSH was related to temperature and time to hatch and YSH was related to salinity also ($F = 3,2$; $p < 0,04$) (Fig. A7i,j).

Time to hatch and YSV was related to temperature ($F = 24,6$; $p < 0,001$), but not related to salinity ($F = 1,6$; $p < 0,2$) (Fig. A7k,l).

3.8. Malformations of newly hatched larvae and its characteristics

Among newly hatched larvae, even under optimum conditions, various body deformations may occur (Avery, Killen, Hollinger, 2009) (most authors observed various curvatures of the spine).

Jezierska B. (Jezierska B. , Lugowska K. , Witeska M. , P., 2000) developed a catalogue of various body deformations of common carp larvae exposed in embryonic period to cadmium, copper and lead. We took his investigation for classification of deformities in Ballan wrasse larvae rearing in different temperatures and salinities.

In the present study, a detailed individual classification of deformed Ballan wrasse larvae was done, according to the catalogue (Jezierska B. , Lugowska K. , Witeska M. , P., 2000) and other investigations (Alaya, Galzin, Quignard, Trabelsi, 2010; Andrades, Becerra, FernandezLlebrez, 1996; Fernбndez, Hontoria, Ortiz-Delgado, Kotzamanis, Estйvez, Zambonino-Infante, Gisbert, 2008; Fraser, Anderson, de Nys, 2004; Georgakopoulou, Katharios, Divanach, Koumoundouros, 2010; Kaur, Dhawan, 1993; Kingsford, Suthers, Gray, 1996; Kjorsvik, Olsen, Wold, Hoehne-Reitan, Cahu, Rainuzzo, Olsen, Oie, Olsen, 2009; Klumpp,

Vonwesternhagen, 1995; Koumoundouros, Divanach, Kentouri, 2001; Lewis, Lall, Eckhard Witten, 2004; Ługowska K., Witeska, 2004; Martinez, Moore, Schaumloffel, Dasgupta, 2003; Okamoto, Kurokawa, Gen, Murashita, Nomura, Kim, Matsubara, Ohta, Tanaka, 2009; Rombough, Garside, 1982; Shi, Huang, Fu, Wang, Luo, Chen, Liu, Zhang, 2008a; Stott, Cross, 1973; Stouthart, Spanings, Lock, Bonga, 1994; Taylor, Preston, Guy, Migaud, 2010; Tutman, Glamuzina, Skaramuca, Kozul, Glavic, Lucic, 2000). We tried to describe the deformations which were observed, also we focused on the frequency of larvae malformations.

Development was considered defective if newly hatched larvae deviated from normal development and morphological differentiation (Cameron, Von Westernhagen, 1997; Vonwesternhagen, Dethlefsen, Cameron, Berg, Furstenberg, 1988).

The results obtained from the experiment allowed to distinguish 6 major types of malformations of Ballan wrasse larvae, that made it possible to create proper classification of malformations, those major types were further divided into subtypes. The imagery of various types of defective larvae is presented in figures below (their description is given below).

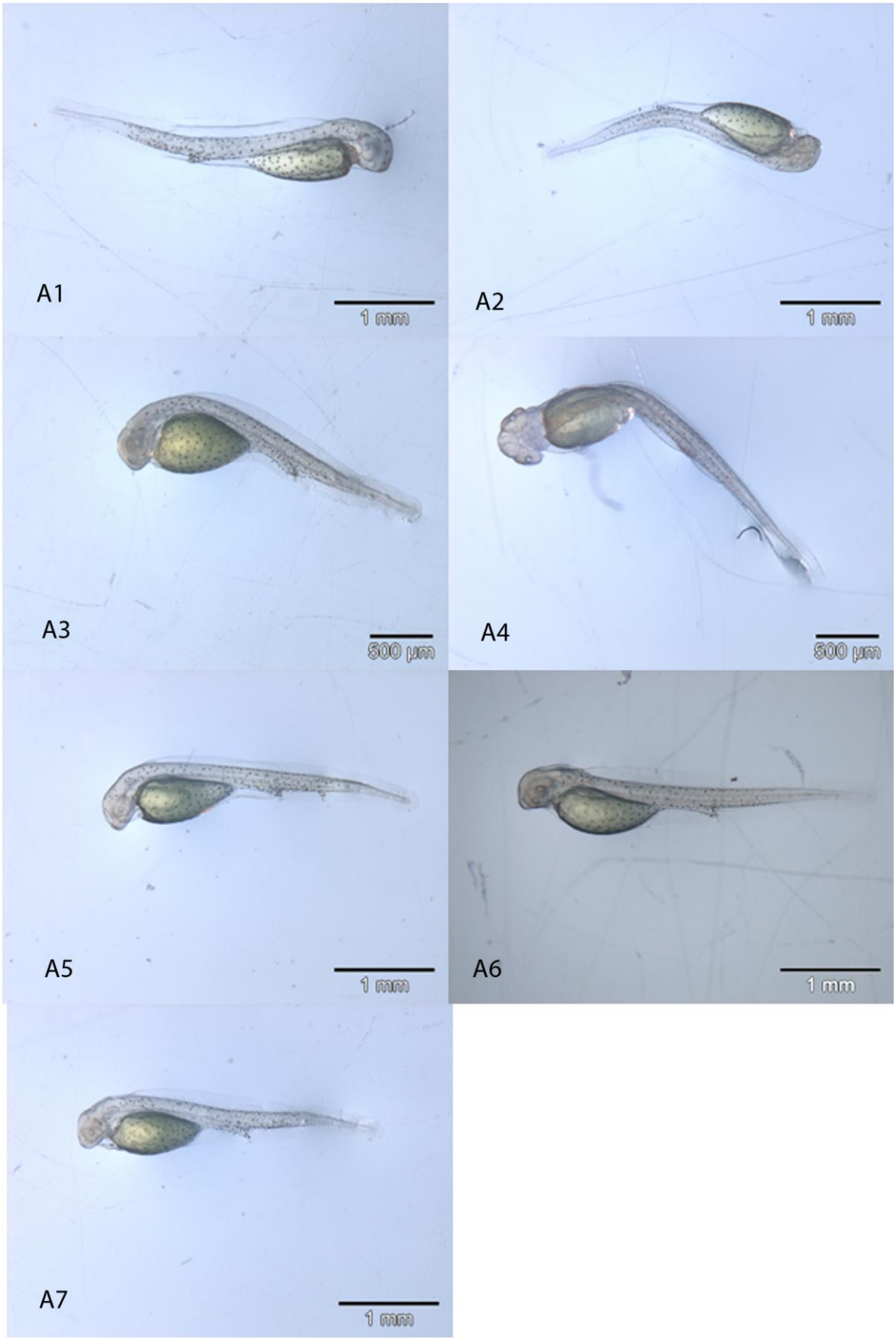

Fig. 17. "A" type of deformed Ballan wrasse larvae (see text below for descriptions).

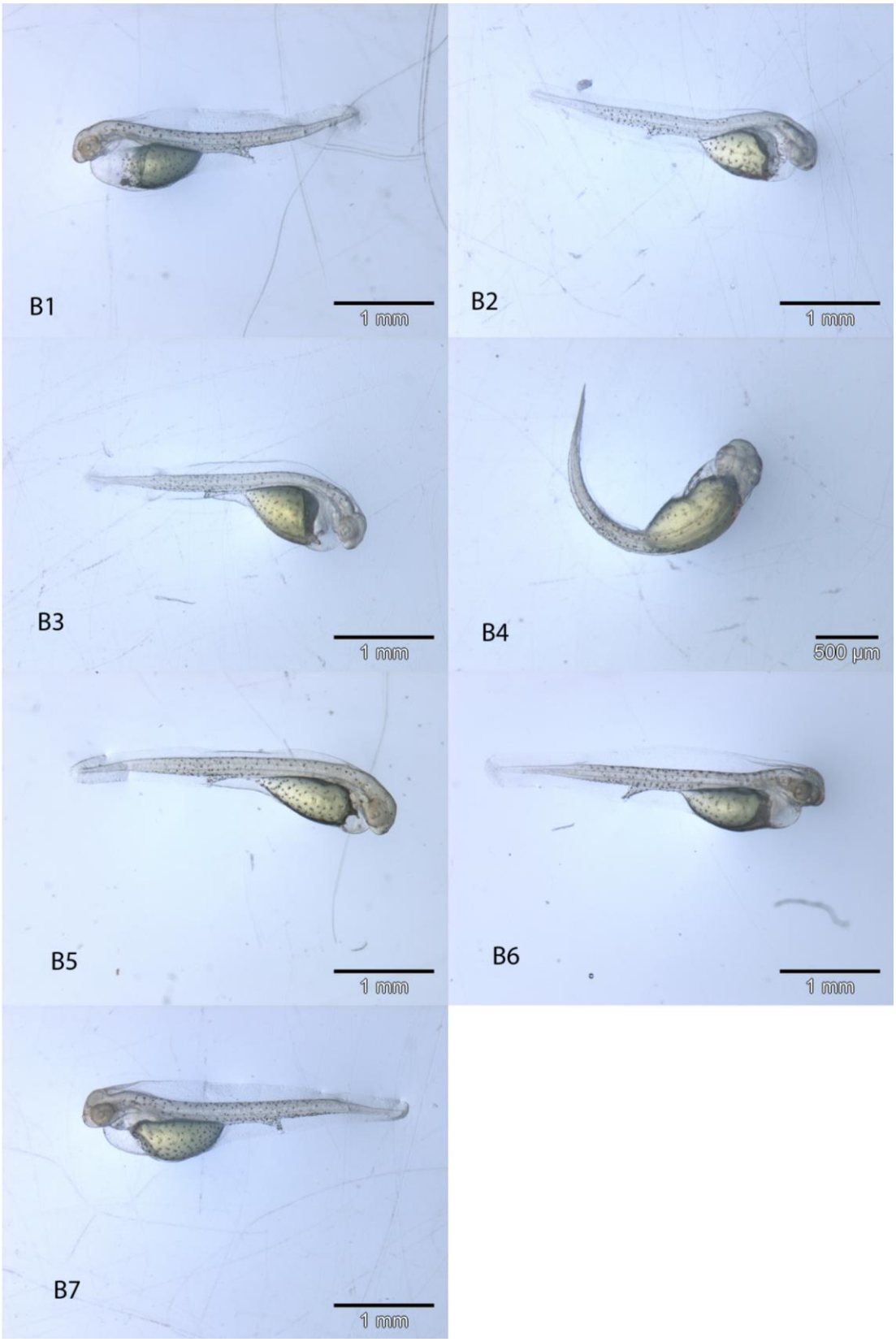

Fig. 18. "B" type of deformed Ballan wrasse larvae (see text below for descriptions).

Larvae at hatching had different types of axial deformations. The most common deformities were type A (35,2%) and type B (29,3%) and E (21,8%) from all observed cases (Fig. 19).

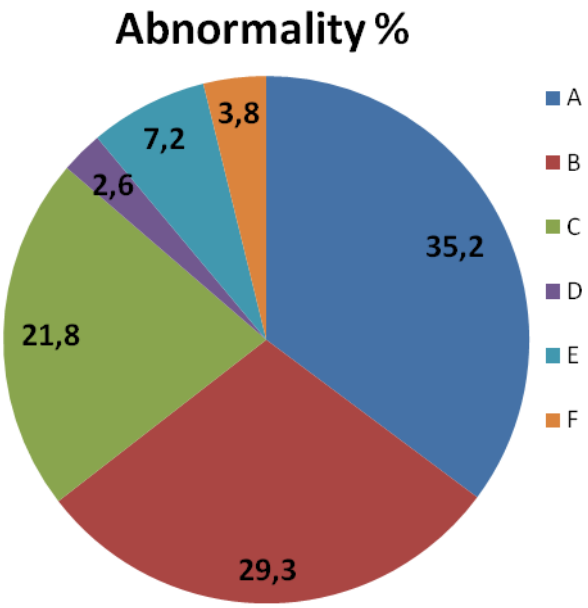

Fig. 19. Proportion of main seven types of abnormality in newly hatched Ballan wrasse larvae.

A and B types characterized as newly hatched larvae with axial (lordosis or kyphosis) or lateral (scoliosis) curvature of the vertebrae in the abdominal or caudal region (Fig. 17,18) (Fig. A7).

- A 1. Lordosis of small degree (10,6 % from total occurrence)
- A 2. Lordosis of great degree (1,9% from total occurrence)
- A 3. Kyphosis (0,8% from total occurrence)
- A 4. Scoliosis (1,6% from total occurrence)
- A 5. Downward spinal curvature in the abdominal or caudal region (9,2% from total occurrence)
- A 6. Upward spinal curvature in the abdominal or caudal region (1,3% from total occurrence).
- A 7. Axial undulation (9,8% from total occurrence)

Type B represent the same axial deformities but accompanied by slight deformation of the yolk sac with oedema (Fig. 18).

- B 1. Lordosis of small degree accompanied by oedema (4,4% from total occurrence)
- B 2. Lordosis of great degree accompanied by oedema (0,3% from total occurrence)
- B 3. Kyphosis accompanied by oedema (0,8% from total occurrence)
- B 4. Scoliosis accompanied by oedema (1,3% from total occurrence)
- B 5. Downward spinal curvature in the abdominal or caudal region accompanied by oedema (6,1% from total occurrence)
- B 6. Upward spinal curvature in the abdominal or caudal region accompanied by oedema (10,6% from total occurrence)
- B 7. Axial undulation accompanied by oedema (5,8% from total occurrence)

Lordosis is characterised by "V-shaped" dorsoventral curvature of the body axis. The degree of deformation of the vertebral column in lordotic animals varies. The degree of lordosis was evaluated by measuring the angle between the line from the first vertebra in the head region to the curvature point and the line from the curvature point to the last vertebra in the tail region (Andrades, Becerra, FernandezLlebrez, 1996). Angle less than 30°- small lordosis (A1), more than 30°- A2.

In some larvae it was several points of curvature in the vertebral column. So frequently more than one point of curvature was identified as "undulation". The caudal part and the lateral line showed an irregular trajectory.

Type C was also widespread deformity of Ballan wrasse larvae (21,9% from total occurrence). This malformations include just oedema-affected deformation of anterior part of the yolk sac without any axial deviation (Fig. 20).

- C 1. Small oedema (7,5% from total occurrence)
- C 2. Vast oedema (14,3% from total occurrence)

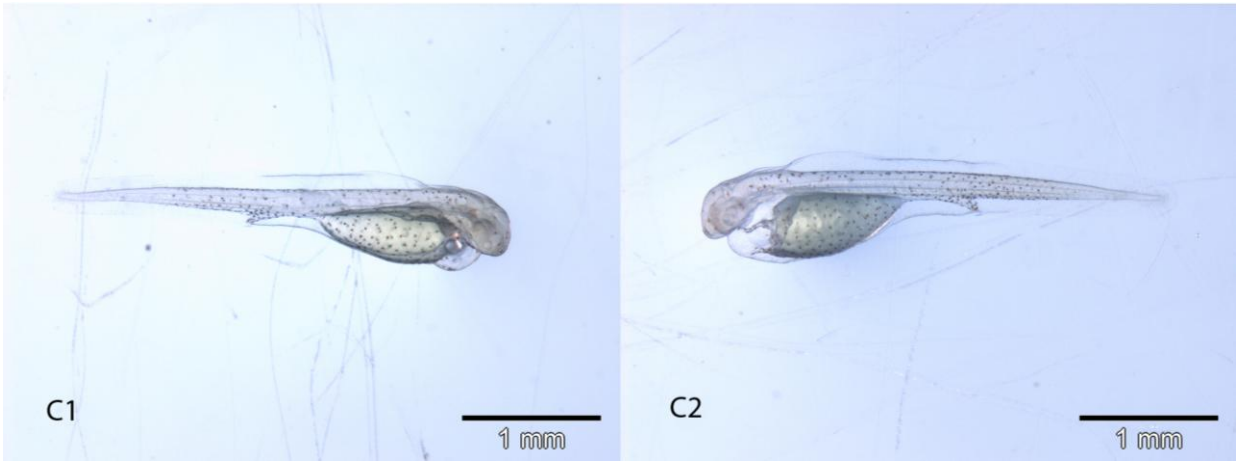

Fig. 20. “C” type of deformed Ballan wrasse larvae (see text above for descriptions).

Some larvae appeared in a partly emerged state usually with the head stick in the egg. The same was observed in Nissling experiment, but head was protruding from the egg (Nissling, Westin, 1991) (Fig. 21).

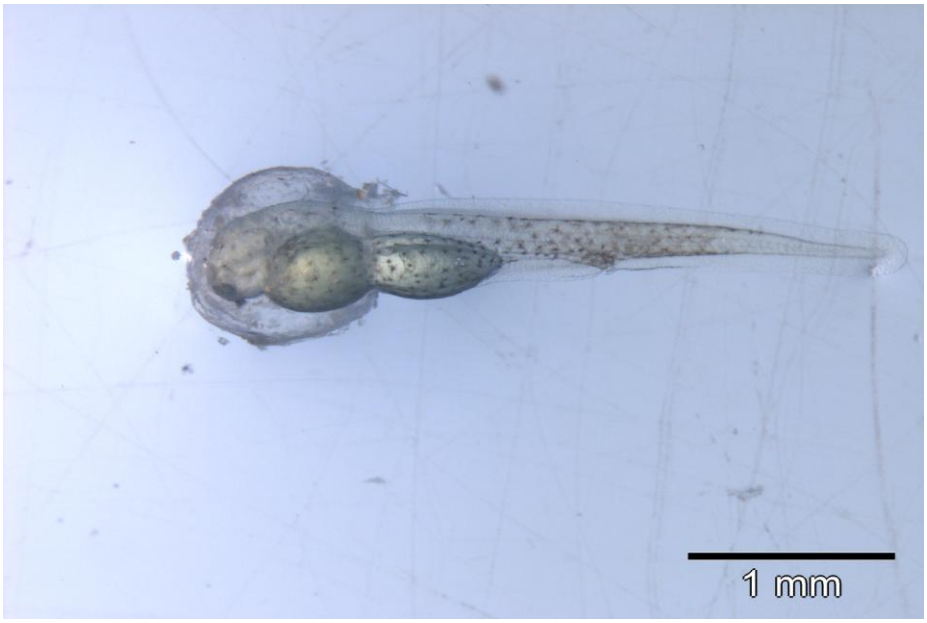

Fig. 21. Deformed Ballan wrasse larvae with the head stick in the egg (see text below for descriptions).

At early larval stages in some specimens such abnormalities of A and B type was a minor defect compare with the other, more severe axial deviations were observed – type D (2,6% from total occurrence), E (7,2%), F (3,8%), but they were not so widespread (Fig. 22, 23).

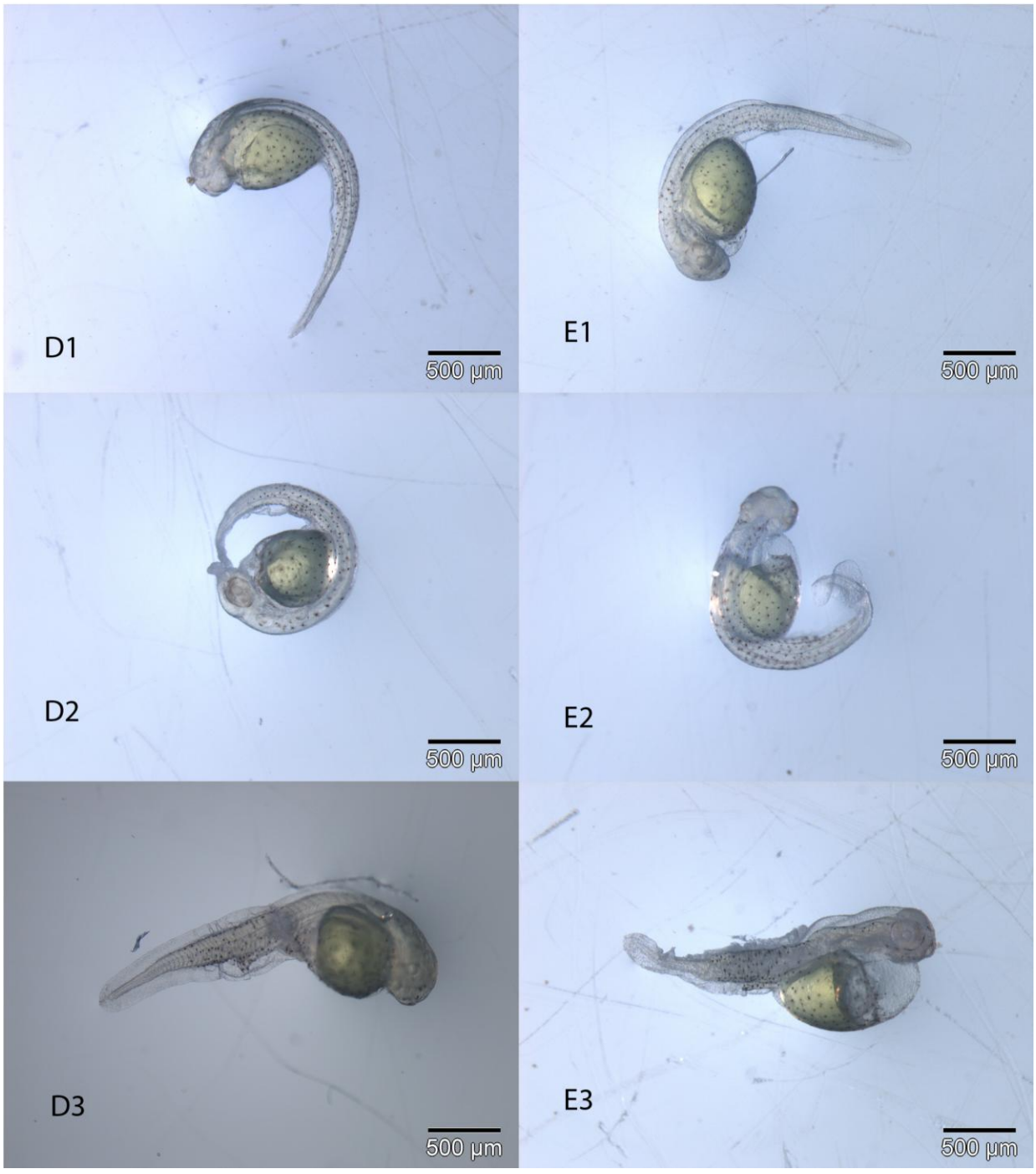

Fig. 22. "D" and "E" types of deformed Ballan wrasse larvae (see text below for descriptions).

- D 1. Larvae with downward axial curve in abdominal and caudal regions (0,7% from total occurrence)
- D 2. "C-shaped" larvae (1,6% from total occurrence)
- D 3. "Shortened body" larvae (0,3% from total occurrence)

Type E represent the same axial deformities but accompanied by slight deformation of the yolk sac with oedema

- E 1. Larvae with downward axial curve in abdominal and caudal regions accompanied by oedema (0,6% from total occurrence)
- E 2. “C-shaped” larvae accompanied by oedema (6,2% from total occurrence)
- E 3. “Shortened body” larvae accompanied by oedema (0,4% from total occurrence)

“C-shaped” larvae could be described as larvae with coiled or bent spinal column. D3 and E3 abnormalities included a small body size and deformations of the spinal column, such larvae were shorter than normal –“shortened body” (Andrades, Becerra, FernandezLlebrez, 1996),

F1 (2,5% from total occurrence) and F2 (1,3% from total occurrence) types of malformations characterised as sharp deformation of spinal column, such larvae commonly had several deformations, scoliosis in the abdominal and caudal region, the tail was often abnormally asymmetrically curved (Fig. 23). Type F2 accompanied by deformation of the yolk sac with oedema.

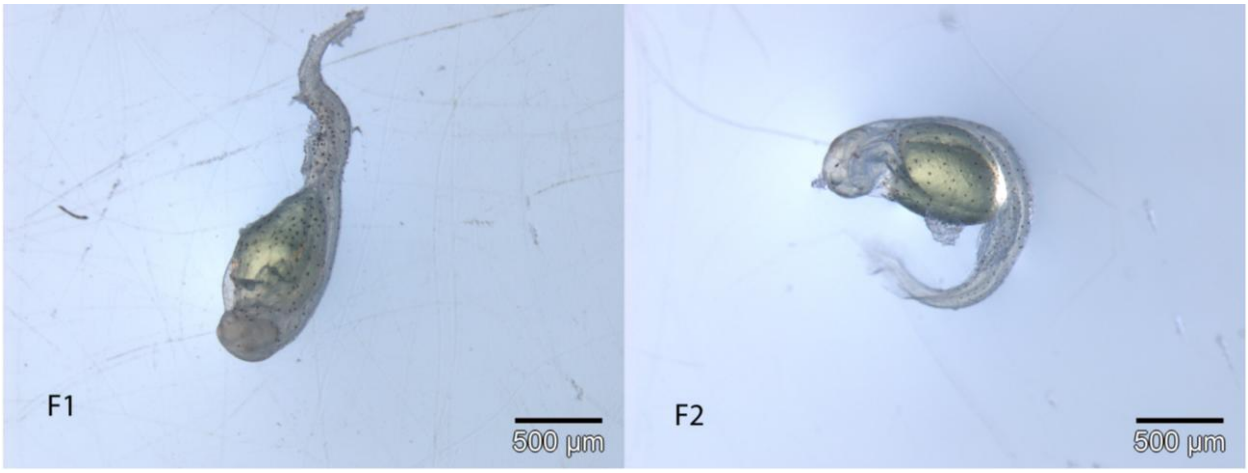

Fig. 23. “F” type of deformed Ballan wrasse larvae (see text below for descriptions).

3.9. Frequency of occurrence of malformations of newly hatched larvae

3.9.1. Total rate of abnormal larvae

The total rate of deformed larvae including as a severe deformity (type D, E, F) as minor level of deformities (type A, B) were lower at 15°C (74,6%) compare to 10°C (96%) and 20°C (99%) (Fig. A8a). In case of salinity comparison in 33‰ (87,7%) compare to 26‰ (94%) and 30‰ (88%), so the trend to increase abnormality with decrease salinity was clear (Fig. A8b).

For Chi Square test of abnormality occurrence in larvae the expected frequencies were those settings producing the highest % of normal larvae in a given treatment. Chi Square test of abnormality occurrence showed that proportion of normal larvae to abnormal was significantly higher in case of rearing temperature 15 °C than in 10°C (Likelihood Ratio=170,9, p <0,001) and 20°C (Likelihood Ratio=225,8, p <0,001), and in case of rearing salinity 33‰ than in 26‰ (Likelihood Ratio=50,7, p <0,001) and 30‰ (Likelihood Ratio=5,9, p <0,015). At second day of hatching proportion of normal larvae to abnormal was significantly higher than at first (Likelihood Ratio=46,7, p <0,001) and last (Likelihood Ratio=9,9, p <0,001).

Comparing deformation rate between different combinations of temperatures and salinities the highest rate of deformed larvae (100%) was in combinations 20°C, 26‰ and 20°C, 33‰. Combination 20°C 30‰ gave 99% deformed larvae (Fig. 24). The highest rate of normal larvae was in combination 15°C 33‰ were 34,6% of larvae were without any malformations (Fig. 24). Combination of 15°C 30‰ (27,9%) and 15°C 26‰ (13,3%) also gave high rate of normal larvae in comparison with other combinations. Rate of normal larvae for combinations of 10°C and 26‰, 30‰ was less than 5%. At 10°C and 33‰ rate of normal larvae was 5,5%.

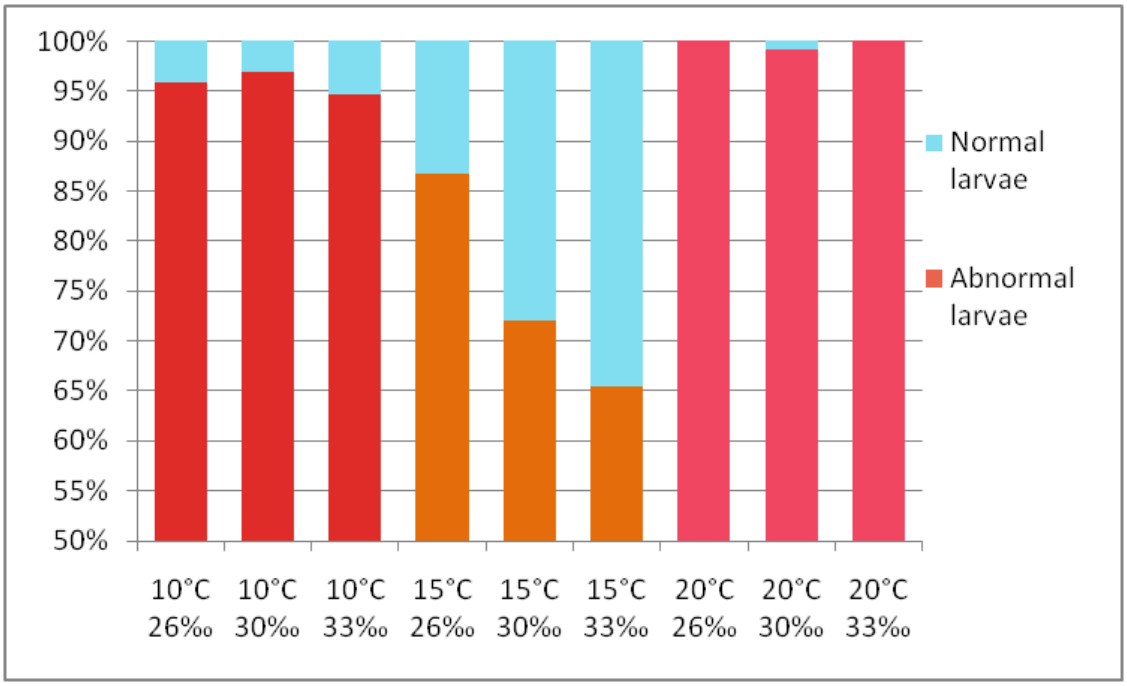

Fig. 24. Proportion of normal to abnormal newly hatched larvae of Ballan wrasse incubated at different combinations of temperatures and salinities.

3.9.2. Rate of deformities in abnormal larvae

Among newly hatched larvae six types of body malformations were distinguished The data on the frequency of deformed larvae in series A-F are summarised in Table A6.

The detailed examination of the larvae showed vertebral deformations to be most common from all defects recorded (Table A6). Larvae malformations were also accompanied with yolk sac and pericardial oedema (Table A7 type B, C, E, F2).

The occurrence rate of big pericardial/yolk sac oedema were lower at 10°C compared to 15°C and 20°C, in 30‰ compare to 26‰ and 33‰ (Table 3, marked as C2). The relative frequencies of larvae just with axial deformity demonstrated an upward trend from higher temperature to lower (Table A6, marked as A deformities), while the same axial deformities but with oedema (marked as B) and more severe deformities (marked as D and E) showed upward trend from lower to higher temperature (Fig. 25a) from 1,53 to 3,39% (D type) and from 0,38 to 19,37% (E type) (Table A6). For salinities there was not clear trend of increase in deformations, the proportions of deformities in all salinities were practically at the same level (Fig. 25b)

Larvae with a sharp bend in the notochord type D2, E2 were observed in all combinations of temperature and salinity, but the occurrence of these malformations showed clear trend to increase from lower temperature to higher (Table 3).

3.9.3. Rate of deformities in abnormal larvae at different hatch time

As it could be seen from abnormalities distribution between early, mid-, late hatch time, the most part of normal larvae 16% were hatched at second day of hatching (mid hatch time) (Fig. A9).

The rate of axial malformation like A type tended to decrease from 50% to 6% at more late hatching. The same malformations but accompanied with yolk sac and pericardial deem (type B) did not followed the same trend (Fig. A9).

With more late hatching ratio of newly hatched larvae with oedema increased from 12% to 34% (Fig. A9). The same trend was correct for more severe abnormalities as D type, E type , and the most complex type of abnormality as F type (Fig. A9).

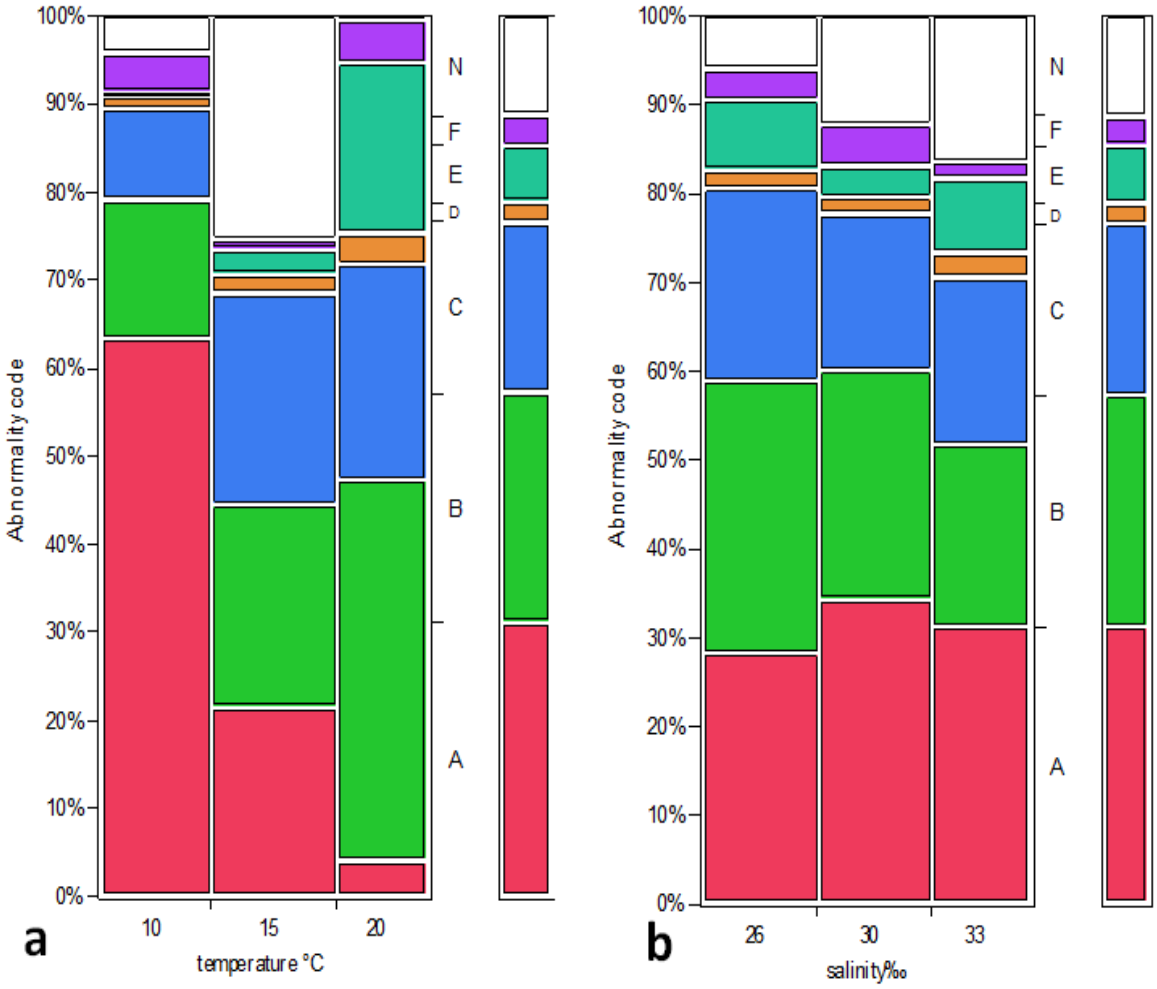

Fig. 25. Frequency of occurrence of deformities in newly hatched Ballan wrasse larvae (N - normal) in different temperatures (a) and different salinities (b).

Table 3. Distribution of different types of abnormality singled out in newly hatched Ballan wrasse larvae incubated at three different temperatures and three different salinities (mean % from total) (N-normal).

Type of abnormality detailed	Temperature ,°C			Salinity, ‰		
	10	15	20	26	30	33
A1	25,24	1,87	0	11,36	8,49	8,05
A2	4,4	0,51	0	1,1	2,08	2,01
A3	0,96	0,68	0,48	0,37	0,57	1,34
A4	1,91	1,36	0,73	1,83	1,13	1,12
A5	7,46	13,46	1,69	6,96	9,81	7,83
A6	1,91	1,19	0,24	1,1	0,94	1,57
A7	21,61	2,56	0,97	5,49	11,32	9,4
B1	4,02	2,56	5,81	5,13	3,4	3,13
B2	0	0,17	0,73	0,73	0	0
B3	0,38	0,51	1,45	0,92	0,94	0,22
B4	0,57	0,68	2,66	1,28	0,94	1,34
B5	1,53	7,16	7,75	6,59	6,23	2,91
B6	3,25	7,5	19,85	10,44	8,3	9,4
B7	5,93	4,43	5,08	5,68	6,04	3,36
C1	4,59	11,75	1,94	4,95	7,92	7,16
C2	5,74	12,1	22,52	16,67	9,62	11,63
D1	0,19	0,68	0,97	0,18	0,75	0,89
D2	0,57	1,53	2,42	1,83	0,75	1,79
D3	0,76	0	0	0,18	0,38	0,22
E1	0	0,51	1,21	0,18	1,13	0,22
E2	0,19	1,7	17,68	7,33	2,45	6,94
E3	0,19	0,51	0,48	0,18	0	1,12
F1	4,59	0,85	1,21	2,2	3,58	0,67
F2	0	0,34	3,87	1,28	0,94	1,34
N	4,02	25,38	0,24	6,04	12,26	16,33

4. DISCUSSION

In this chapter, the results from our experiment with Ballan wrasse were compared with and provided documentary evidence of information about other marine coldwater species collected during the development of intensive culture systems. Several authors have described larvae and juveniles of Ballan wrasse caught in the wild (Artuz, 2005; Fives, 1976), and spawned from broodstock fish adapted to captivity (Dunaevskaya, 2010). So larvae in our experiment were compared with artificially produced and wild-caught larvae of Ballan wrasse.

L. bergylta had spherical demersal and sticky eggs of mean diameter 0,95±0,02 mm with no oil globules, which differed from mean diameter in Artuz (2005) - 1,1 mm with one oil globule, but is comparable with Munk (2005) and Fives (1976) results (0,7-1,15 mm).

The mean length of newly hatched larvae was 3,297±0,2 mm. Size-at-hatch among temperature treatments was highly variable, ranging from 3,96 to 2,61 mm.

This findings are inconsistent with previous findings for artificially fertilized wrasse eggs 3,64±0,05 mm (Dunaevskaya, 2010), as well as for larvae hatched from eggs collected in ichthyoplankton surveys 2,7 to 3 mm (Artuz, 2005; Fives, 1976). This difference in larval size could be explained by origination of the eggs, environmental conditions of broodstock living and larvae rearing parameters such as temperature, salinity, water flow, oxygen level and light regime (Dunaevskaya, 2010). If compare with other labrid species the mean length of newly hatched larvae of wrasse was larger than of *Ctenolabrus rupestris* (1,95-2,19 mm), *Symphodus cinereus* (3,0 mm), *Symphodus melops* (2,5-3,0 mm) (Dulcic, Kozul, Kraljevic, Skaramuca, Glamuzina, Re, 1999; Kimura, Kiriyama, 1993), but smaller than of brown wrasse *Labrus merula* (3,76 mm) (Dulcic, Kozul, Kraljevic, Skaramuca, Glamuzina, Re, 1999).

Information concerning the effects of temperature and salinity on embryonic development and survival of prelarvae of Ballan wrasse is limited, but it has been suggested that temperatures 10°C are desired to normal development during embryonic development before hatching for eggs from broodstock from Marine Research Station of University of Nordland, Bodø, Norway (Dunaevskaya, 2010). In Artuz work (2005) the average temperature of development of Ballan wrasse eggs was 17,5°C. Artuz also pointed out that eggs tolerate temperature range from 10°C to 25°C, but with different mortality rates.

The results of this work showed that tolerable salinity and temperature range of Ballan wrasse eggs was within the experimental salinities and temperature (10°C, 15°C, 20°C, 26‰, 30‰, 33‰), because larvae hatched at all treatments.

Even though hatching occurred in all combinations of experimental temperature and salinity, hatching rates at 20°C 30 ‰ (52,86%±15,7) and 20°C 33 ‰ (51,98%±25,0) were significantly lower. The results showed that there was no significant difference among combinations 10 C° 26‰, 10C° 30‰, 10C° 33‰, 15C° 26‰, 20C° 26‰.

It has been recorded for herring and sole eggs (Devauchelle, Alexandre, Lecorre, Letty, 1987) that hatch rates are highest in combinations of high salinity and high temperature, and low salinity and low temperature, the opposite is true for eggs of Baltic Sea turbot (Kuhlmann, Quantz, 1980). In our case the highest hatching rate occurred at 15°C, and the optimal salinity for larvae was between 30‰ (83,3%±10,9) and 33‰ (84,57%±9,0). According to the statistical analysis, there was effect of temperature on hatching rate only in batches of eggs incubated in 30‰ and 33‰ ($p < 0,005$). There was no significant effect of salinity on temperature ($p > 0,05$).

The morphology of newly hatched larvae described here agrees well with the descriptions of Dunaevskaya (2010), Artuz (2005), Munk (2005) and Fives (1976).

Embryonic development was completed approximately at 59,3°C days (5days) after fertilization when kept in the incubators in 15°C, while the larvae kept in 20°C needed an additional 8,2°C days (hatched at 67,5°C days, 4 days of incubation), and additional 17°C days in 10°C (hatched at 76,3°C days, eight days of incubation). These last values is comparable with that reported by Artuz, who observed hatching for 8,3 days in 10°C (Artuz, 2005) and finding from hatchery from Mørkvedbukta – Marine Research Station of University of Nordland, Bodø, Norway 8 days of incubation at 10°C (Dunaevskaya, 2010 personal conversation).

As in cases of many other fish asynchronously in hatching occur even if eggs were from the same batches (Laurel, Hurst, Copeman, Davis, 2008), and when they are incubated at different environmental parameters such asynchrony vary greatly. So in present work a high proportion of the larvae hatched asynchronously (within 3 days). In present study the hatch duration was different between different temperatures and salinities. For larvae held in 10°C it took up 75 hours from first hatched larvae to last. Larvae held in 15°C hatched for 51 hours, and in 20°C – 48 hours was needed for complete hatching. There was significant effect of hatch duration on hatching rate. For 10°C at first day (early hatch) main part of larvae were hatched, but this trend did not maintain for 15°C and 20°C, where main part of larvae were hatched on second day of hatching.

In an intensive rearing context, this is a very interesting question, because from the day of hatch it is easy to estimate the developmental state of all larvae before start feeding (Bjelland, Skiftesvik, 2006).

The reason why larvae hatch asynchronously is unknown and requires further investigation, we can predict that mechanical stress can result in synchronous hatching among eggs ready to hatch in the course of daily examination during experiment.

While the length of the hatch period is generally temperature-specific, larvae emerging from eggs during the hatching period are morphometrically similar (Laurel, Hurst, Copeman, Davis, 2008).

Temperature is reported to affect development and metabolic rates in marine fish and is undoubtedly an important environmental component of early and late hatching and subsequent vital rates. For example, laboratory studies have shown that increases in egg incubation temperature produces smaller larvae or larger larvae (Avery, Killen, Hollinger, 2009; Cingi, Keinanen, Vuorinen, 2010; Gracia-López, Kiewek-Martínez, Maldonado-García, 2004; Hart, Purser, 1995; Laurel, Hurst, Copeman, Davis, 2008; Morehead, Hart, 2003). At the same time, low temperatures can extend the hatch period such that early hatching fish larvae may be smaller than their late hatching siblings(Laurel, Hurst, Copeman, Davis, 2008). A similar increase in development rate with temperature has been recorded with turbot, sole and flounder (Hart, Purser, 1995).

As it was shown the high incubation temperature may produce smaller and shorter post-hatch fish (Blaxter, 1992; Buckley, Smigielski, Halavik, Laurence, 1990; Naesje, Jonsson, 1988).

This statement go with results from present study where newly hatched larvae tended to be larger at cooler temperatures. Larvae at 10°C were longer (3,37±0,19 mm) than at 15°C and 20°C. The smallest larvae hatched were observed at 20°C (3,14±0,15 mm). Combination of the treatment gave the longest larvae at 10°C 26‰, but this result did not differ significantly from combination 10°C 30‰, 10°C 33‰, 15°C 30‰. The smallest larvae length was in combination 20°C 30‰. As it was shown in statistical analysis single effect of temperature and salinity on larvae length was significant.

Larvae incubated in conditions of 10°C 26‰ had the biggest value of myotome height (0,192±0,011 mm), but this results did not significantly diverge from 10°C 30‰, 10°C 33‰. Single effect of temperature on eye diameter of larvae was significant, but statistical analysis did not revealed effect of salinity, and interaction of two factors had no effect on eye diameter. Larvae incubated at 10°C had bigger eyes than in other conditions, whereas the smallest ED had larvae incubated in 20°C.

Early hatching larvae were smaller than late hatching larvae, size-at-hatch generally decreased with increasing temperature, although this was most apparent in early hatch larvae. The same was observed in experiments with Pacific cod larvae (Laurel, Hurst, Copeman, Davis, 2008). As Laurel J. Reported, the same trend was observed in late-hatching cod larvae. Late

hatched larvae were generally larger and had smaller yolk sack than early hatched larvae in cod and capelin *Mallotus villous* (Chambers, Leggett, Brown, 1989), wolffish *Anarhichas lupus* (Ringo, Olsen, Boe, 1987) , ocean pout *Macrozoarces americanus* (Methven, Brown, 1991), walleye pollock *Theragra chalcogramma* (Porter, Bailey, 2007). But in contrast, Atlantic silverside *Menidia menidia* larvae hatching later in the hatch cycle at a given temperature were smaller than earlier hatching larvae (Bengtson, Barkman, Berry, 1987).

In present study under each temperature treatment, late-hatched larvae had less endogenous reserves (YSL, YSH, YSV) than early-hatching larvae. As it is predicted by other scientists, the reduced yolk reserves in late hatching larvae can lead to reduced survival for these individuals after hatch, suggesting that late-hatched larvae must start feeding on exogenous food sources sooner than early-hatched larvae (Laurel, Hurst, Copeman, Davis, 2008).

Cingi S. (2010) in experiments with whitefish wrote that the higher the incubation temperature, the faster the development and thus the smaller the number of vertebrae. He also supposed yolk-sac larvae could have had sublethal deformities that would affect swimming behaviour. Deformed and smaller newly hatched fishes are likely to have reduced chances of survival (Cingi, Keinanen, Vuorinen, 2010). Thus comprehensive studies examining post-hatch effects of temperature on larval growth and survival of Ballan wrasse larvae are necessary.

The degree to which hatch synchrony is temperature dependent and the morphometrics of early and late hatching have been examined in several species, but it gives disembodied data of separated components of such interactions (Laurel, Hurst, Copeman, Davis, 2008). And as for Ballan wrasse there is no any data of such effects. So this area is needed to be investigated.

In the present study incubation temperature influenced larval hatch, higher temperature synchronized hatch, whereas cooler environments extended the hatch period.

Temperature influences the size and can effect survival of pre-feeding larvae by driving embryological development and metabolic demands from the egg stage through to hatch (Hart, Purser, 1995). High incubation temperature reduced the development time of embryo, leading to earlier hatching. As temperature increased embryonic development was faster, therefore the time elapsed from the first to the last larva to hatch was shorter, in agreement with Gracia-López and Kiewek-Martínez (2004).

Late-hatched larvae had generally wide myotome and had bigger eyes than early hatched larvae. The ecological significance of such variance of morphometric characteristics (e.g. yolk size, length, eye diameter) is suggested to be a “bet-hedging strategy” by parents to ensure some of their offspring are able to survive in a variable food environment, either by the larvae being capable of feeding immediately at hatch or having sufficient endogenous reserves to survive until food becomes available (Laurel, Hurst, Copeman, Davis, 2008)

Body malformation were observed to happen in the newly hatched larvae even if the embryonic development took place in unpolluted and well-oxygenated water, at an optimal temperature (Jezierska B. , Lugowska K. , Witeska M. , P., 2000).

A detailed visual evidence of deformations in newly hatched larvae of Ballan wrasse is lacking. So, in the present study we tried to classify various types of body malformations of the newly hatched larvae of Ballan wrasse reared at different temperature and salinity during the embryonic development. We described the deformations which observed and indicated the body parts or organs affected, we also focused on the frequency of larvae malformations. Larvae at hatch had different types of axial deformations and that could be related to notochord alterations during embryogenesis (Andrades, Becerra, FernandezLlebrez, 1996).

Ballan wrasse larvae from different treatments were affected by different body malformations, and the proportions of abnormal individuals were different between combinations of different temperatures and salinities as well. The lowest frequency of defects was recorded in treatment 15°C and 33‰, the highest rate of deformed larvae (100%) was in combinations: 20°C, 26‰and 20°C, 33‰. We also concluded that “mid” time of hatch (second day of hatching) give the most viable larvae.

The most common deformities of newly hatched Ballan wrasse larvae were axial (lordosis or kyphosis) or lateral (scoliosis) curvature of the vertebrae in the abdominal or caudal region. At early larval stages in some specimens more severe axial deviations were observed accompanied by deformation of the yolk sac with oedema, but they were not so widespread. About 4% of hatched larvae had such sharp deformation of spinal column like scoliosis in the abdominal and caudal region with abnormally asymmetrically curved tail. The occurrence of larvae with a sharp bend in the notochord showed clear trend to increase from lower temperature to higher.

The frequency occurrence of larvae with big pericardial/yolk sac oedema increased with increasing the temperature. The most common malformations in this study involving various vertebral abnormalities accompanied by oedema were more frequent in the larvae kept at 20°C.

The development of oedema appears to be a response to suboptimal rearing conditions, with unfavourable temperatures as the most important factors (Ottesen, Bolla, 1998). Ultimately these pathological manifestations may reduce the chances of larval survival up to first feeding as in many other species, for example Atlantic halibut (Oliveira, Domingues, Grisolia, Soares, 2009; Ottesen, Bolla, 1998).

Malformations may be caused by the disturbances occurring at early developmental stages or may evolve during laborious hatching (Cameron, Von Westernhagen, 1997; Jezierska B. , Lugowska K. , Witeska M. , P., 2000; Koumoundouros, Maingot, Divanach, Kentouri,

2002). Embryos with mild deformities can survive until hatching, and slightly deformed yolk-sac larvae may survive, but appear to be less viable. The spinal curvature might be a response to environmental stress, including exposure to extremes of salinity (Okamoto, Kurokawa, Gen, Murashita, Nomura, Kim, Matsubara, Ohta, Tanaka, 2009).

Lordotic specimens may further show unbalanced growth. But lordosis in larvae does not totally interfere with swimming and feeding behaviour and is not deleterious (Andrades, Becerra, FernandezLlebrez, 1996; Ługowska K., Witeska, 2004). The primary causes of lordosis emanated from defects in axial structures during embryonic development. Therefore, congenital lordosis could be due to (Andrades, Becerra, FernandezLlebrez, 1996):

- genetic causes;
- composition of the yolk;
- environmental causes such as light, temperature, mechanical shock, pollutants, infections, etc., that could affect broodstock fish or embryos.

In culture exploitations, spinal malformations are frequently seen in newly hatched larvae (Andrades, Becerra, FernandezLlebrez, 1996). The high level of spinal malformations appearing in hatchery fish is an important problem for the development of this industry. This is often associated with growth depression, leading to high mortality rates. The etiology of these syndromes is not well understood. Environmental factors such as mechanical or thermal shocks, presence of pollutants in the water, radiation, salinity, oxygen depletion and light intensity have also been reported to cause aberrations in development (Andrades, Becerra, FernandezLlebrez, 1996).

We don’t know the exact optimal range of temperature and salinity, but the best combination of temperature and salinity between tested was 15°C and 33‰. The most optimal temperature range is probably lies between 10 - 15°C for wrasse eggs. It is close to, but a little wider, than the broodstock spawning temperature environment (10 - 12°C). It is not surprising that optimal temperature for embryos is close to but a little higher the range of the natural spawning temperature, because broodstock fish have been adapted to captivity for two years, this finding has also been demonstrated for some other fishes (the eggs of turbot, tawny puffer eggs) (Zhang, Shi, Zhu, Liu, Zang, 2010). The temperature at which broodstock lived and were kept before or during spawning might affect the temperature tolerance of their spawned and fertilized eggs (Gracia-López, Kiewek-Martínez, Maldonado-García, 2004).

In the present study, the broodstock were maintained in seawater of 33% salinity and this may have affected the optimal salinity range required for the eggs. Similar results have been shown for greenback flounder *Rhombosolea tapirina* (Hart, Purser, 1995) After fertilisation,

wrasse eggs were tolerant of a wide range of salinities (26 - 33%) with an optimal temperature range of 10 - 15°C.

The development of Ballan wrasse for aquaculture is still at an early stage. This study provides some small new basic information about egg incubation and hatching. All areas require more research, and broodstock that now can be kept in captivity increases the chance of success in further investigation. Due to the high influence of temperature and salinity on eggs and larvae, the results of the present study will have implications for the culture of Ballan wrasse. For this species, there are no data for the effects of temperature and salinity on hatch rate and morphometrics of newly hatched larvae.

5. CONCLUSION

In this study, we investigated the effects of salinity and temperature on larvae of Ballan wrasse. The effects of salinity on embryos and larvae of Ballan wrasse are likely to be different under various temperatures. But further studies are needed to clarify the combined effect of salinity and temperature on embryos and larvae of Ballan wrasse.

The mean length of newly hatched larvae was 3,297±0,2 mm. First larvae hatched approximately at 59,3 °C days (5days) after fertilization when kept in the incubators in 15°C, while the larvae kept in 20°C hatched at 67,5 °C days (4 days of incubation), and in 10°C hatched at 76,3 °C days (eight days of incubation).

Despite the larvae hatched at all treatments and thus tolerate the experimental salinities and temperatures (10°C, 15°C, 20°C, 26‰, 30‰, 33‰), the highest hatching success was related to 15° 33‰ environmental conditions. The length of the hatch period was temperature-specific. Higher temperature synchronized hatch, whereas cooler environments extended the hatch period.

Larvae emerging from eggs incubated at different temperatures and salinities were not morphometrically similar. Size-at-hatch generally decreased with increasing temperature. Larvae held at 10°C were longer, had bigger value of MH, had bigger eyes. Early hatching larvae were smaller than late hatching larvae, but late-hatched larvae had less endogenous reserves (YSL, YSH, YSV), had generally wide myotome and had bigger eyes than early hatched larvae.

The larvae from different treatments were affected by different body malformations, and the proportions of abnormal individuals were different between the series as well. The most common deformities of newly hatched Ballan wrasse larvae were axial (lordosis or kyphosis) or lateral (scoliosis) curvature of the vertebrae in the abdominal or caudal region. The occurrences of abnormalities of newly hatched larvae were less at 15°C 33‰. Severe malformations tended to increase with increasing temperature.

The results on hatching rate, and early larval deformity, indicate that fertilized eggs of Ballan wrasse develop till hatching in a wide range of salinities, but we conclude that the optimal temperature for successful development of fertilized eggs lies between 10°C to 15°C 33‰, where highest egg hatching rate, and lowest incidence of abnormality rate was observed.

The result of this study could be useful in improving the production of this species through incubation and larval culture. But the optimal temperature and salinity range for embryo development and larval culture of the species need to be further determined.

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7. APPENDICES

Appendix 1

EM-fixative

Ref.: Heidi Ludvigsen, Faculty of Biosciences and Aquaculture, University of Nordland.

SOLUTIONS:

A. Buffer

Caccodylic acid sodium salt trihydrate (Sigma-Aldrich CO250) 20.8 g

Sucrose, C12H22O11 140 g

1% Calcium Chloride, CaCl2 20 ml

Distilled water 2 l

Mix and adjust the pH to 7.2 with 1 N HCl

B. Stem solution

Paraformaldehyde 25 g

Distilled water 250 ml

Buffer 1 l

Dissolve paraformaldehyde in distilled water by heating (not more than 80°C), using an exhaust hood. Add concentrated NaOH (few droplets) until pH becomes 7.1-7.3. Cool and mix with buffer. Store the solution at 4°C.

FIXATION:

Mix stem solution and 25% glutaraldehyde in proportion 12.5:1 (stem solution (12.5 ml), glutaraldehyde (1 ml).

Appendix 2

Table A1. Morphometric parameters in mm. of newly hatched larvae of Ballan wrasse incubated at different temperatures and salinities (mean±SD). Different letters indicate significant differences among treatments.

Temperature, °C	Salinity, ‰	Parameter	Mean±SD	Significance
10	26	**Standard length mm**	3,39±0,2	A
10	33		3,37±0,18	AB
10	30		3,36±0,17	AB
15	30		3,33±0,19	ABC
15	26		3,31±0,24	BC
15	33		3,29±0,19	C
20	26		3,19±0,13	D
20	33		3,14±0,15	DE
20	30		3,07±0,15	E
10	26	**Myotome height mm**	0,192±0,011	A
10	30		0,19±0,009	AB
10	33		0,189±0,012	ABC
20	33		0,187±0,012	BCD
20	26		0,184±0,01	CDE
15	26		0,183±0,014	DE
20	30		0,183±0,012	DE
15	33		0,18±0,12	E
15	30		0,176±0,014	F
10	26	**Eye diameter mm**	0,27±0,12	A
10	33		0,27±0,02	A
10	30		0,27±0,01	A
15	26		0,26±0,04	B
15	33		0,26±0,01	B
15	30		0,25±0,01	B
20	26		0,24±0,01	C
20	33		0,24±0,01	C
20	30		0,23±0,01	C
10	30	**Yolk sac length mm**	1,08±0,07	A
10	33		1,07±0,09	A
10	26		1,06±0,08	A
15	30		0,99±0,08	B
15	33		0,99±0,1	B
15	26		0,98±0,09	B

20	33		0,84±0,08	C
20	30		0,83±0,07	C
20	26		0,82±0,14	C
20	26	**Yolk sac height mm**	0,433 ±0,06	A
20	30		0,431 ±,03	AB
20	33		0,427 ±0,03	ABC
15	26		0,423 ±0,05	ABC
15	33		0,419 ±0,03	ABCD
15	30		0,417 ±0,04	BCDE
10	30		0,412 ±0,04	CDE
10	26		0,404 ±0,05	E
10	33		0,404 ±0,03	DE
10	30	**Yolk sac volume mm^3**	0,0965 ±0,02	A
15	26		0,093 ±0,02	A
10	26		0,0924 ±0,02	A
10	33		0,0922 ±0,02	A
15	33		0,0916 ±0,02	A
15	30		0,0911 ±0,02	A
20	26		0,0822 ±0,02	B
20	30		0,0808 ±0,01	B
20	33		0,0805 ±0,02	B

Appendix 3

Table A2. Effect of temperature on morphometric parameters of Ballan wrasse newly hatched larvae in different salinities.

Salinity, ‰	F ratio	Prob > F
	Standard Length	
26	$F_{2,1292}$=38,6	p<0,001
30	$F_{2,1292}$=78,9	p<0,001
33	$F_{2,1292}$=35,7	p<0,001
	Myotome height	
26	$F_{2,1294}$=26,4	p<0,001
30	$F_{2,1294}$=60	p<0,001
33	$F_{2,1294}$=18	p<0,001
	Eye diameter	
26	$F_{2,1293}$=101,7	p<0,001
30	$F_{2,1293}$=82,6	p<0,001
33	$F_{2,1293}$=64,1	p<0,001
	Yolk sac length	
26	$F_{2,1296}$=251,4	p<0,001
30	$F_{2,1296}$=210,2	p<0,001
33	$F_{2,1296}$=163,8	p<0,001
	Yolk sac height	
26	$F_{2,1295}$=16,6	p<0,001
30	$F_{2,1295}$=5,1	p<0,006
33	$F_{2,1295}$=7,7	p<0,001
	Yolk sac volume	
26	$F_{2,1295}$=14,2	p<0,001
30	$F_{2,1295}$=22,2	p<0,001
33	$F_{2,1295}$=12,9	p<0,001

Appendix4

Table A3. Effect of salinity on morphometric parameters of Ballan wrasse newly hatched Ballan wrasse larvae in different temperatures.

Temperature, °C	F ratio	Prob > F
	Standard Length	
10	$F_{2,1292}$=0,7	p<0,48
15	$F_{2,1292}$=2,2	p<0,11
20	$F_{2,1292}$=9,8	p<0,001
	Myotome height	
10	$F_{2,1294}$=2,8	p<0,06
15	F $F_{2,1294}$=16,9	p<0,001
20	$F_{2,1294}$=2,3	p<0,1
	Eye diameter	
10	$F_{2,1293}$=1,2	p<0,311
15	$F_{2,1293}$=2,8	p<0,06
20	$F_{2,1293}$=0,3	p<0,776
	Yolk sac length	
10	$F_{2,1296}$=0,8	p<0,43
15	$F_{2,1296}$=1,4	p<0,25
20	$F_{2,1296}$=1,2	p<0,3
	Yolk sac height	
10	$F_{2,1295}$=1,9	p<0,148
15	$F_{2,1295}$=1,3	p<0,285
20	$F_{2,1295}$=0,4	p<0,636
	Yolk sac volume	
10	$F_{2,1295}$=3	p<0,049
15	$F_{2,1295}$=0,5	p<0,581
20	$F_{2,1295}$=0,2	p<0,759

Appendix 5

Table A4. Hatching success % of eggs of Ballan wrasse incubated at three different temperature from first hatch larvae to last by hours (mean hatched % from total±SD, n=6 replicates). Different letters indicate significant differences among treatments.

Hours after first larvae hatch	Significance	Mean% ± SD
	Temperature 10°C	
0	A	25,72±13,51
3	A	29,02±9,95
12	A	29,08±8,03
27	B	5,54±2,30
33	B	12,73±7,15
51	B	6,65±3,53
57	B	6,16±3,15
75	B	3,10±0,33
	Temperature 15°C	
0	CD	8,13 ±5,42
3	BC	14,26 ±8,14
9	BC	13,14 ±7,72
12	BC	14,77 ±7,73
24	CD	7,98 ±3,64
27	A	26,61 ±9,55
33	B	17,69 ±13,04
51	D	3,69 ±3,08
	Temperature 20°C	
0	ABCD	16,70 ±17,41
6	CD	8,46 ±6,60
9	ABCD	17,80 ±9,85
12	ABCD	9,21 ±6,13
15	ABC	27,27 ±11,46
21	ABC	23,98 ±15,05
24	A	27,86 ±13,31
30	AB	25,63 ±12,61
33	ABCD	12,39 ±10,25
36	ABCD	5,93 ±2,93
39	ABCD	4,87 ±1,44
45	D	3,09 ±0,38
48	BCD	7,75 ±3,21

Appendix 6

Table A5. Morphometric parameters of newly hatched larvae of Ballan wrasse depending on hatch time (first, second, third) incubated at different temperature and salinities (mean±SD mm). Different letters indicate significant differences among days in treatments.

Temperature, °C or Salinity, ‰	Hatch day	Significance	Mean ± SD
Standard Length			
10 °C	3	A	3,718±0,185
10°C	2	B	3,593±0,102
10°C	1	C	3,300±0,113
15°C	3	A	3,679±0,047
15°C	2	B	3,408±0,183
15°C	1	C	3,196±0,154
20°C	2	A	3,191±0,122
20°C	1	B	3,001±0,127
26‰	3	A	3,719±0,173
26‰	2	B	3,374±0,195
26‰	1	C	3,214±0,162
30‰	3	A	3,800±0,113
30‰	2	B	3,347±0,225
30‰	1	C	3,246±0,159
33‰	3	A	3,656±0,189
33‰	2	B	3,315±0,188
33‰	1	C	3,226±0,158
Myotome height			
10 °C	3	A	0,205±0,014
10°C	2	B	0,198±0,008
10°C	1	C	0,187±0,009
15°C	3	A	0,197±0,006
15°C	2	B	0,185±0,014
15°C	1	C	0,173±0,009
20°C	2	A	0,189±0,010
20°C	1	B	0,173±0,009
26‰	3	A	0,208±0,011
26‰	2	B	0,189±0,012
26‰	1	C	0,182±0,011
30‰	3	A	0,204±0,010
30‰	2	B	0,186±0,014
30‰	1	C	0,180±0,012
33‰	3	A	0,200±0,018

33‰	2	B	0,189±0,011
33‰	1	C	0,180±0,011
Eye diameter			
10 °C	3	A	0,283±0,012
10°C	2	B	0,276±0,011
10°C	1	C	0,269±0,014
15°C	3	A	0,263±0,012
15°C	2	A	0,258±0,039
15°C	1	A	0,255±0,013
20°C	2	A	0,236±0,011
20°C	1	A	0,234±0,010
26‰	3	A	0,279±0,012
26‰	1	A	0,263±0,016
26‰	2	B	0,253±0,046
30‰	3	A	0,288±0,008
30‰	1	B	0,259±0,017
30‰	2	C	0,251±0,020
33‰	3	A	0,279±0,016
33‰	1	B	0,259±0,019
33‰	2	C	0,251±0,019
Yolk sac length			
10 °C	1	A	1,076±0,074
10°C	2	AB	1,068±0,080
10°C	3	BB	1,034±0,092
15°C	3	AB	1,018±0,047
15°C	1	A	1,003±0,102
15°C	2	B	0,980±0,0862
20°C	1	A	0,829±0,154
20°C	2	A	0,825±0,087
26‰	3	A	1,019±0,075
26‰	1	A	1,004±0,144
26‰	2	B	0,934±0,131
30‰	3	A	1,054±0,052
30‰	1	A	1,026±0,106
30‰	2	B	0,948±0,116
33‰	1	A	1,039±0,115
33‰	3	A	1,039±0,117
33‰	2	B	0,913±0,114
Yolk sac height			
10 °C	1	A	0,418±0,038
10°C	2	B	0,380±0,038
10°C	3	C	0,346±0,042
15°C	1	A	0,430±0,041
15°C	2	B	0,412±0,040
15°C	3	C	0,364±0,039

20**°C**	1	A	0,433±0,060
20**°C**	2	A	0,430±0,044
26**‰**	1	A	0,426±0,055
26**‰**	2	A	0,418±0,049
26**‰**	3	B	0,344±0,037
30**‰**	1	A	0,424±0,036
30**‰**	2	B	0,412±0,043
30**‰**	3	C	0,331±0,042
33**‰**	1	A	0,422±0,035
33**‰**	2	A	0,414±0,037
33**‰**	3	B	0,365±0,044
	Yolk sac volume		
10 °C	1	A	0,099±0,016
10**°C**	2	B	0,081±0,017
10**°C**	3	C	0,066±0,015
15**°C**	1	A	0,097±0,018
15**°C**	2	B	0,087±0,017
15**°C**	3	B	0,072±0,018
20**°C**	1	A	0,085±0,018
20**°C**	2	B	0,080±0,015
26**‰**	1	A	0,097±0,019
26**‰**	2	B	0,085±0,017
26**‰**	3	C	0,064±0,015
30**‰**	1	A	0,097±0,016
30**‰**	2	B	0,084±0,017
30**‰**	3	C	0,062±0,017
33**‰**	1	A	0,097±0,017
33**‰**	2	B	0,082±0,015
33**‰**	3	C	0,072±0,015

Appendix 7

Table A6. Presence of main seven types of abnormality singled out in newly hatched Ballan wrasse larvae incubated at three different temperatures and in three different salinities (mean % from total) (N-normal).

Type of abnormality	Temperature, °C			Salinity, ‰		
	10	**15**	**20**	**26**	**30**	**33**
A	63,48	21,64	4,12	28,21	34,34	31,32
B	15,68	23	43,34	30,77	25,85	20,36
C	10,33	23,85	24,46	21,61	17,55	18,79
D	1,53	2,21	3,39	2,2	1,89	2,91
E	0,38	2,73	19,37	7,69	3,58	8,28
F	4,59	1,19	5,08	3,48	4,53	2,01
N	4,02	25,38	0,24	6,04	12,26	16,33

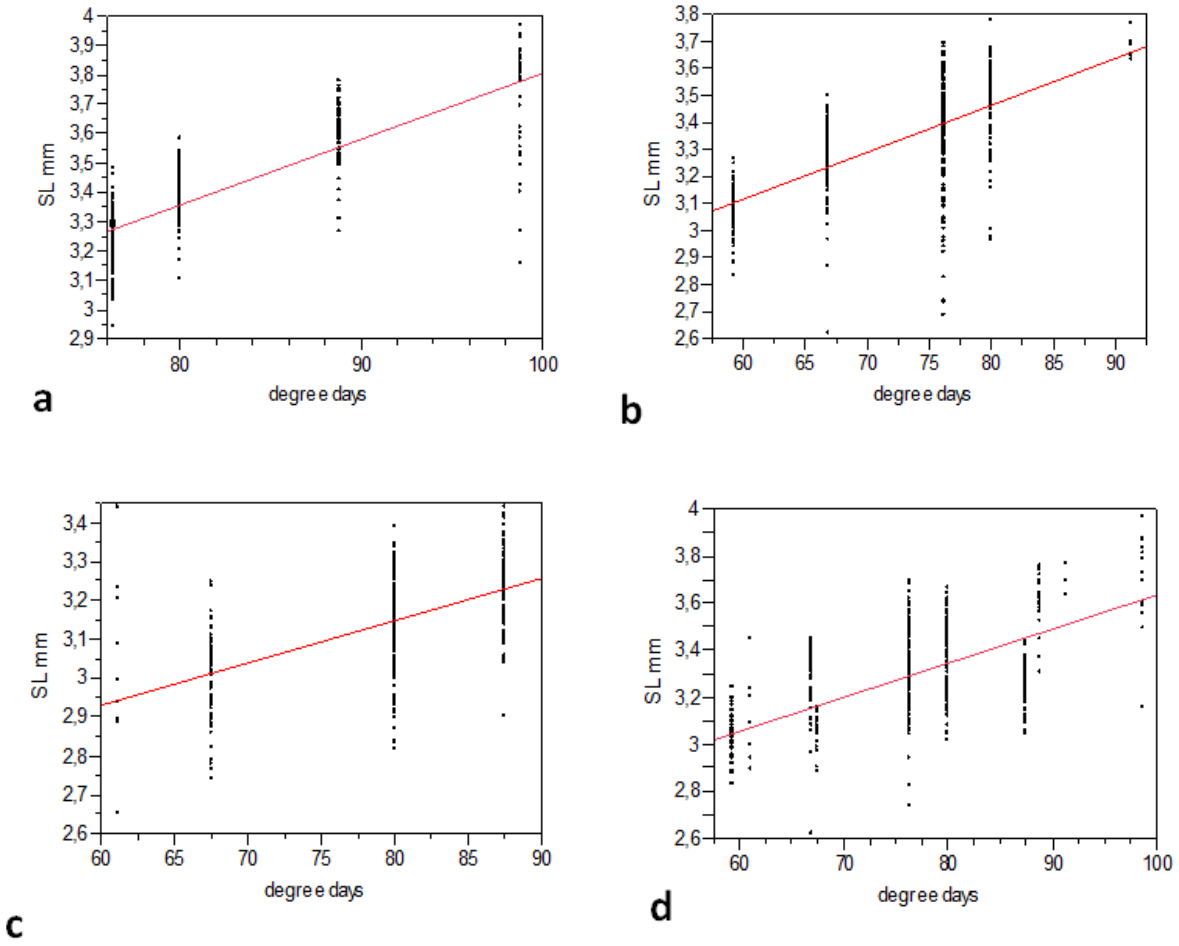
Appendix 8

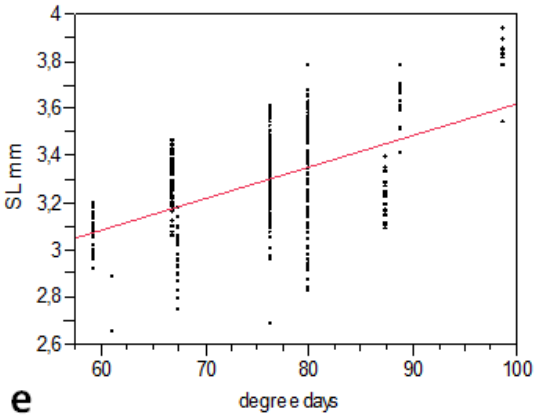
Table A7. Frequency of occurrence of different types of abnormality singled out in newly hatched Ballan wrasse larvae (mean % from total).

Type of abnormality	Occurrence %
A1	10,6
A2	1,9
A3	0,8
A4	1,6
A5	9,2
A6	1,3
A7	9,8
B1	4,4
B2	0,3
B3	0,8
B4	1,3
B5	6,1
B6	10,6
B7	5,8
C1	7,5
C2	14,3
D1	0,7
D2	1,6
D3	0,3
E1	0,6
E2	6,2
E3	0,4
F1	2,5
F2	1,3
total	100

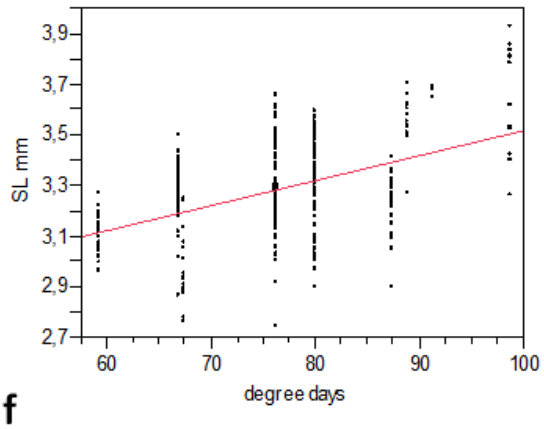
Appendix 9

Fig. A1. Correlation between SL of newly hatched Ballan wrasse larvae and time of incubation at three different temperature and three different salinity (degree day, n=6 replicates) a- 10°C (SL mm = 1,546 + 0,023*degree days), b- 15°C (SL mm = 2,086 + 0,017*degree days), c- 20°C (SL mm = 2,273 + 0,011*degree days), d-26 ‰ (SL mm = 2,193 + 0,014*degree days), e-30‰ (SL mm = 2,286 + 0,013*degree days), f-33‰ (SL mm = 2,524 + 0,009*degree days).




SL mm
4
3,8
3,6
3,4
3,2
3
2,8
2,6
60
70
80
90
100
degree days

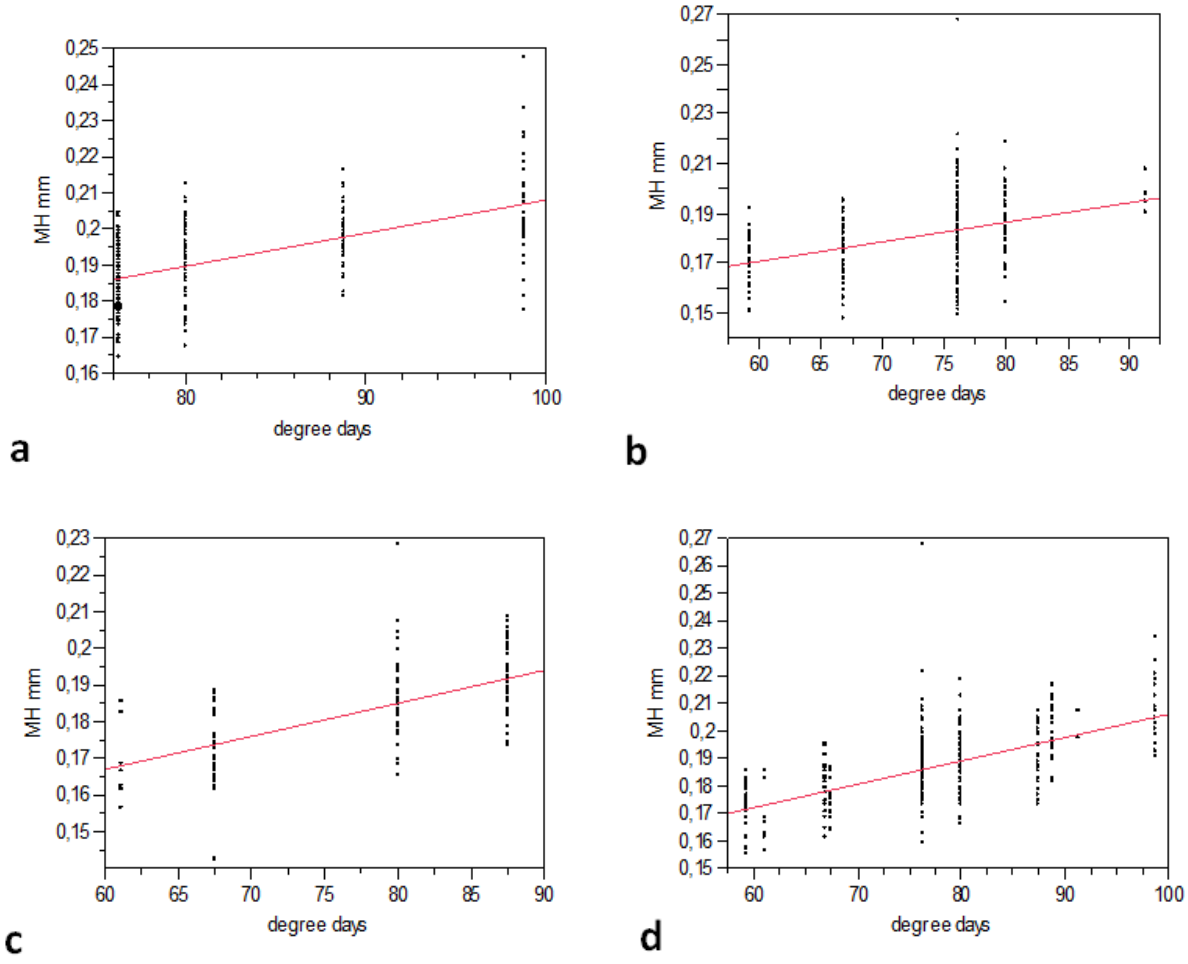
e

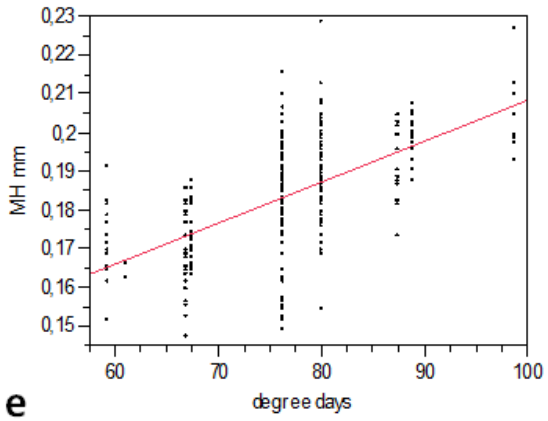

SL mm
3,9
3,7
3,5
3,3
3,1
2,9
2,7
60
70
80
90
100
degree days

f

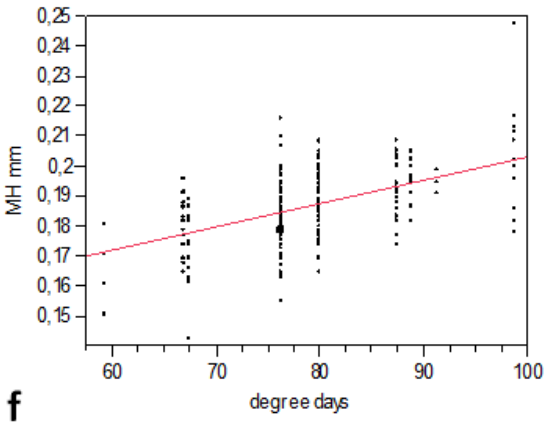
Appendix 10

Fig. A2. Correlation between MH of newly hatched Ballan wrasse larvae and time of incubation at three different temperatures and three different salinities (degree day, n=6 replicates) a- 10°C (MH mm = 0,115 + 0,001*degree days), b- 15°C (MH mm = 0,123 + 0,001*degree days), c- 20°C (MH mm = 0,113 + 0,001*degree days), d-26 ‰ (MH mm = 0,121 + 0,001*degree days), e-30‰ (MH mm = 0,103 + 0,001*degree days), f-33‰ (SL MH mm = 0,125 + 0,001*degree days).




MH mm
0,23
0,22
0,21
0,2
0,19
0,18
0,17
0,16
0,15
60
70
80
90
100
degree days

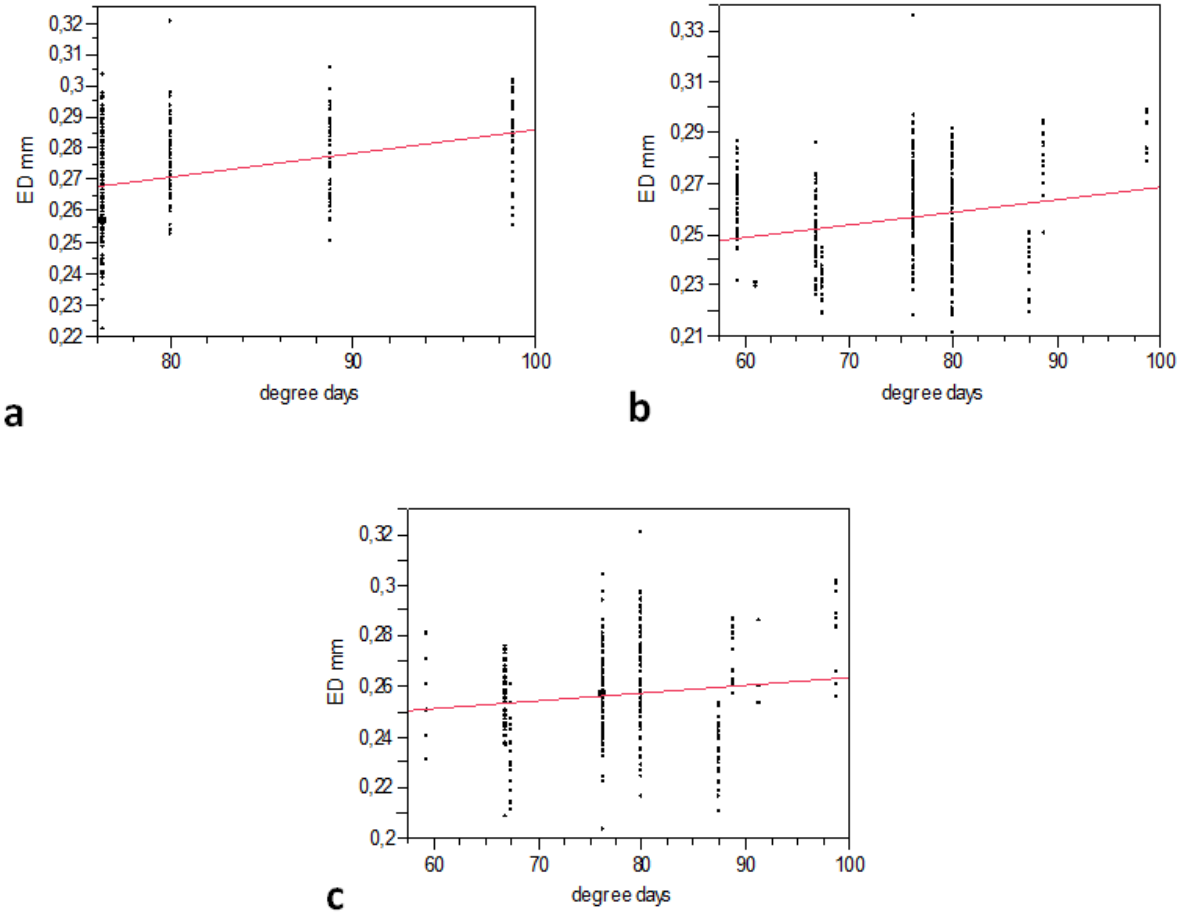
e


MH mm
0,25
0,24
0,23
0,22
0,21
0,2
0,19
0,18
0,17
0,16
0,15
60
70
80
90
100
degree days

f

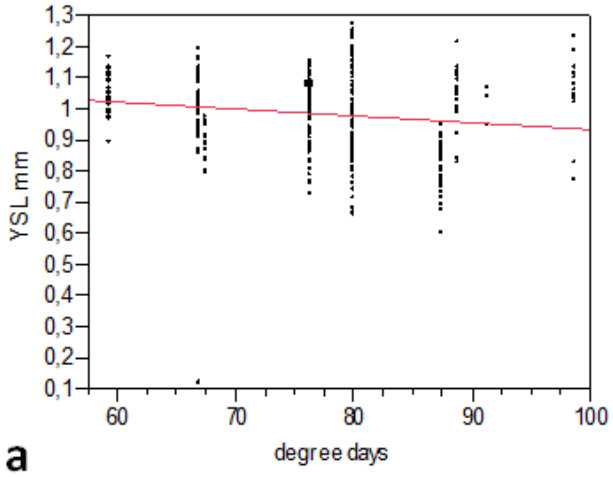
Appendix 11

Fig. A3. Correlation between ED of newly hatched Ballan wrasse larvae and time of incubation at three different temperatures and three different salinities (degree day, n=6 replicates) a- 10°C (ED mm = 0,21 + 0,001*degree days), b- 30‰°C (ED mm = 0,219 + 0,001*degree days), c- 33‰ (ED mm = 0,232 + 0,001*degree days).



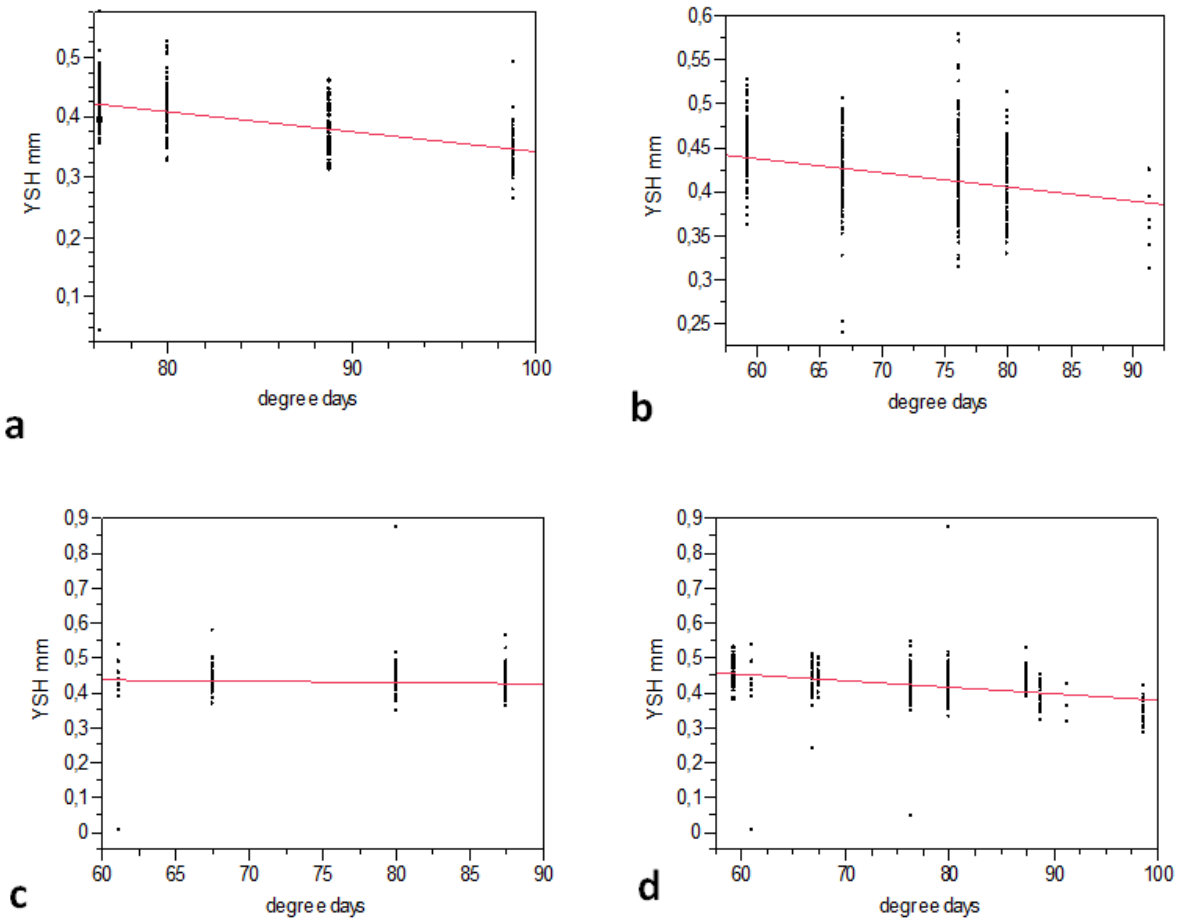
Appendix 12

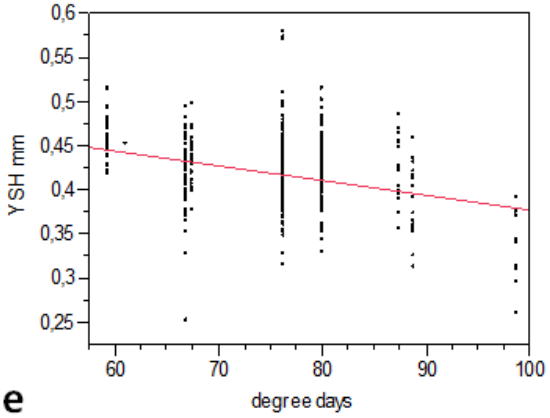
Fig. A4. Correlation between YSL of newly hatched Ballan wrasse larvae and time of incubation at 33‰ salinity (degree day, n=6 replicates) (YSL mm = 1,156 - 0,002*degree days).



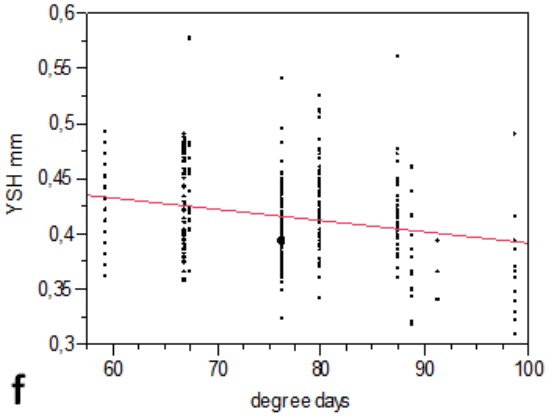
Appendix 13

Fig. A5. Correlation between YSH of newly hatched Ballan wrasse larvae and time of incubation at three different temperatures and three different salinities (degree day, n=6 replicates) a- 10°C (YSH mm = 0,673 - 0,003*degree days), b- 15°C (YSH mm = 0,537 - 0,002*degree days), c- 20°C (YSH mm = 0,46 - 0,001*degree days), d-26 ‰ (YSH mm = 0,562 - 0,002*degree days), e-30‰ (YSH mm = 0,54 - 0,002*degree days), f-33‰ (YSH mm = 0,496 - 0,001*degree days).




YSH mm
0,6
0,55
0,5
0,45
0,4
0,35
0,3
0,25
60
70
80
90
100
degree days

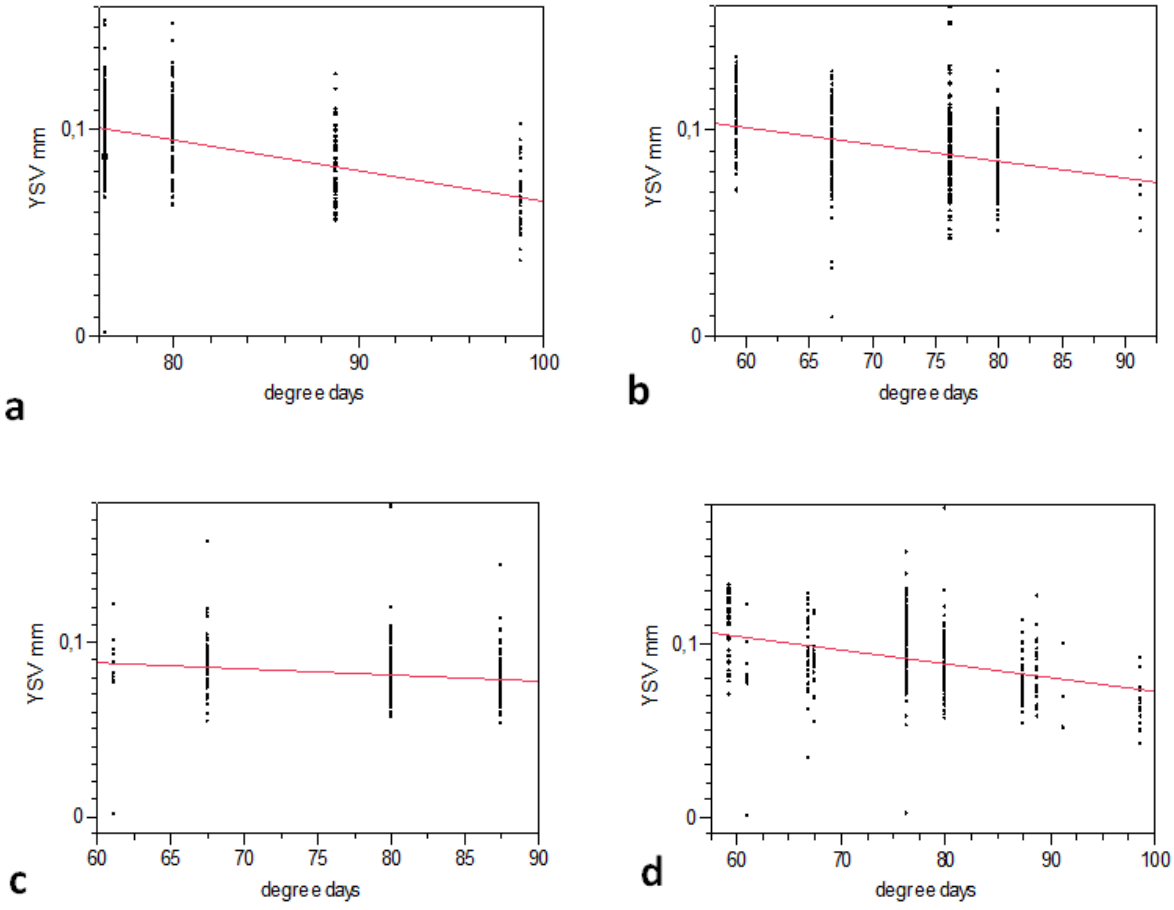
e

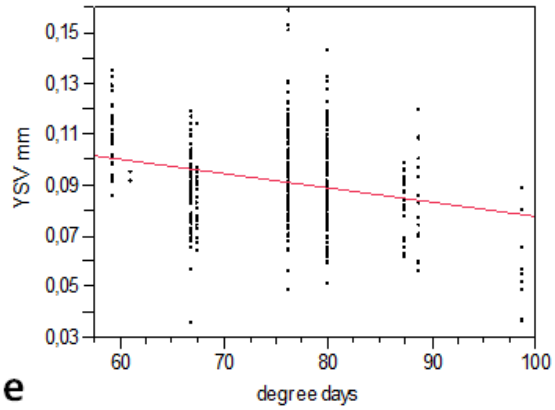

YSH mm
0,6
0,55
0,5
0,45
0,4
0,35
0,3
60
70
80
90
100
degree days

f

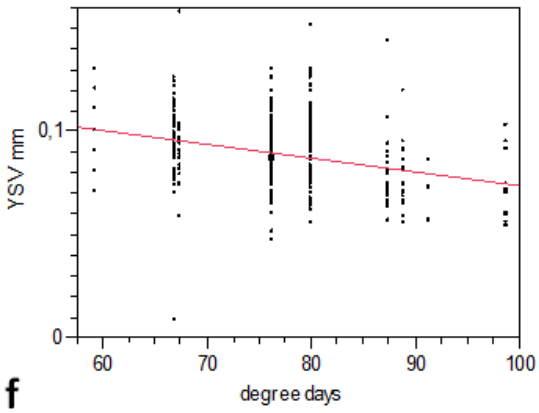
Appendix 14

Fig. A6. Correlation between YSV of newly hatched Ballan wrasse larvae and time of incubation at three different temperatures and three different salinities (degree day, n=6 replicates) a- 10°C (YSV mm^3 = 0,212 - 0,001*degree days), b- 15°C (YSV mm^3 = 0,15 - 0,001*degree days), c- 20°C (YSV mm^3 = 0,108 - 0,001*degree days), d-26 ‰ (YSV mm^3 = 0,153 - 0,001*degree days), e-30‰ (YSV mm^3 = 0,134 - 0,001*degree days), f-33‰ (YSV mm^3 = 0,14 - 0,001*degree days).




0,15
0,13
0,11
0,09
0,07
0,05
0,03
YSV mm
60
70
80
90
100
degree days

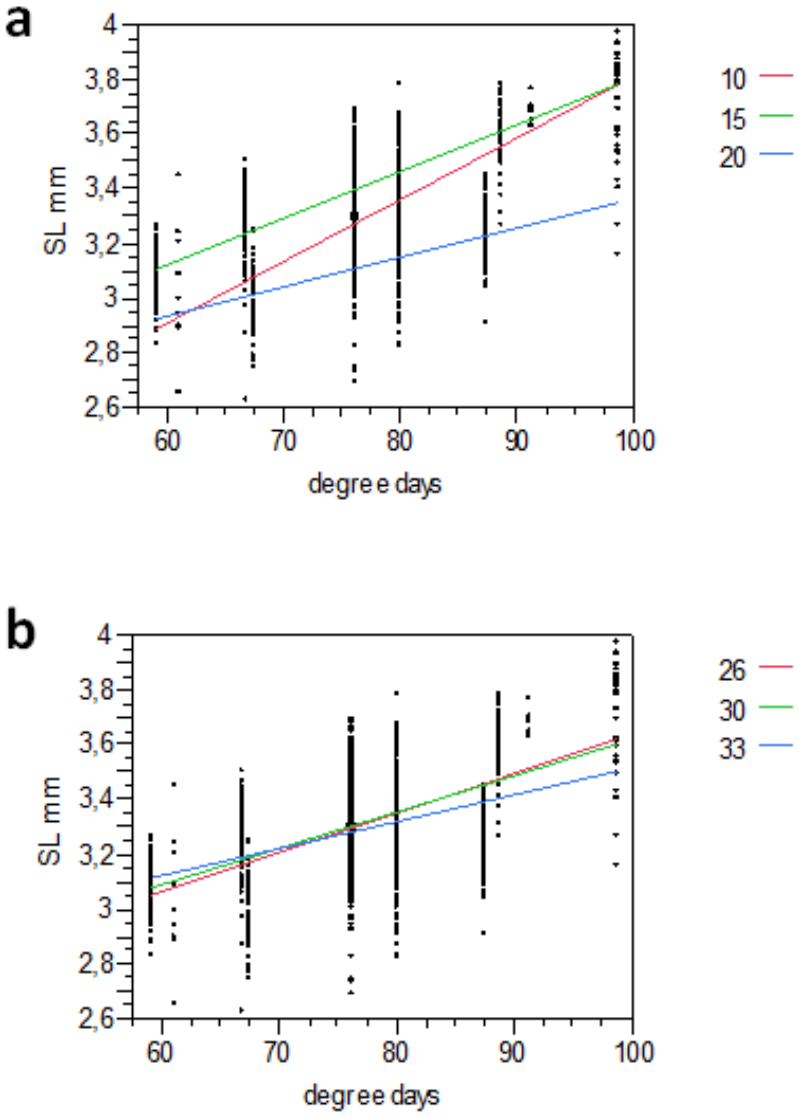
e

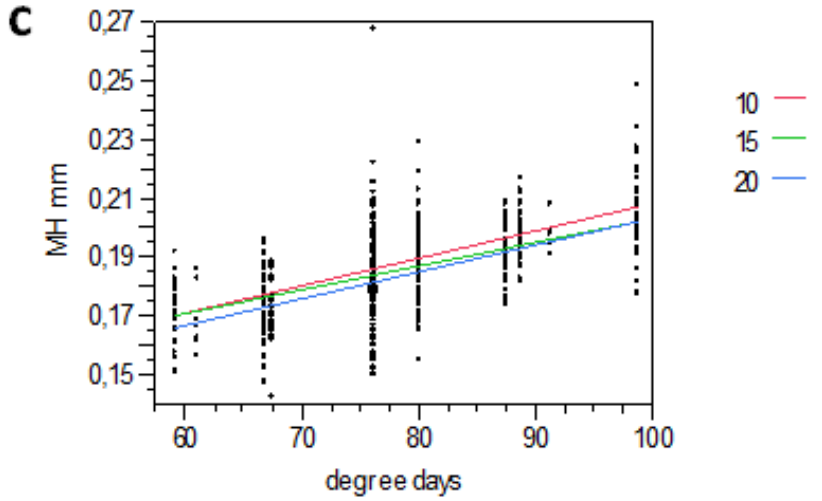

0,1
0
YSV mm
60
70
80
90
100
degree days

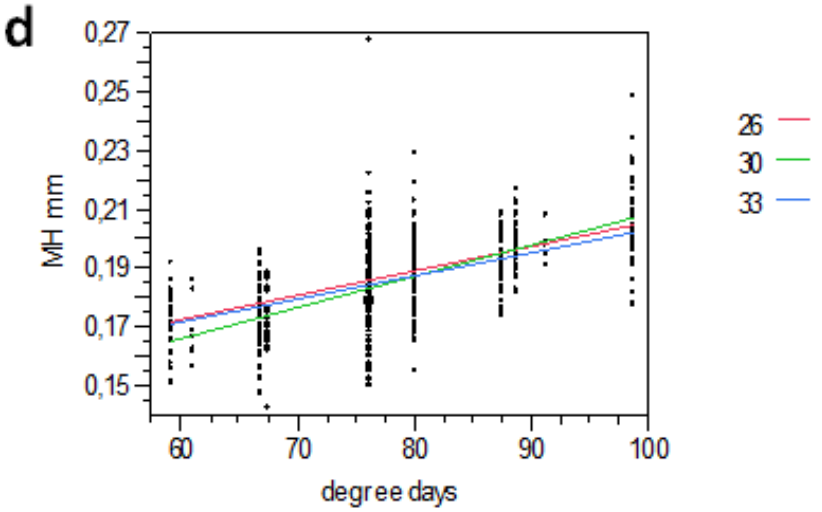
f

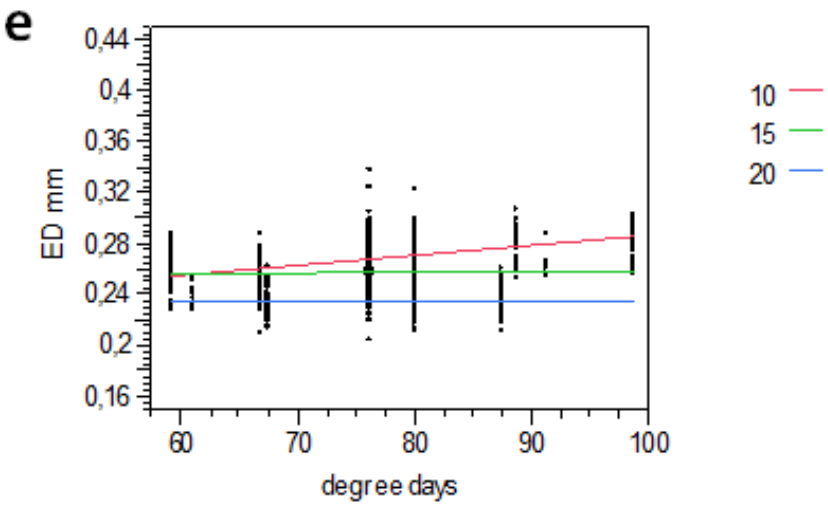
Appendix 15

Fig. A7. Correlation between morphometric parameters of newly hatched Ballan wrasse larvae , time to hatch and three different temperatures and three different salinities (degree day, n=6 replicates) a- SL at temperatures °C (10°C, 15°C, 20°), b- SL at salinities (26 ‰, 30‰, 33‰), c- MH at temperatures °C (10°C, 15°C, 20°), d- MH at salinities (26 ‰, 30‰, 33‰), e-ED at temperatures °C (10°C, 15°C, 20°), f-MH at salinities (26 ‰, 30‰, 33‰), g-YSL at temperatures °C (10°C, 15°C, 20°), h- YSL at salinities (26 ‰, 30‰, 33‰), i -YSH at temperatures °C (10°C, 15°C, 20°), j-YSH at salinities (26 ‰, 30‰, 33‰), k- YSV at temperatures °C (10°C, 15°C, 20°), l-YSV at salinities (26 ‰, 30‰, 33‰).

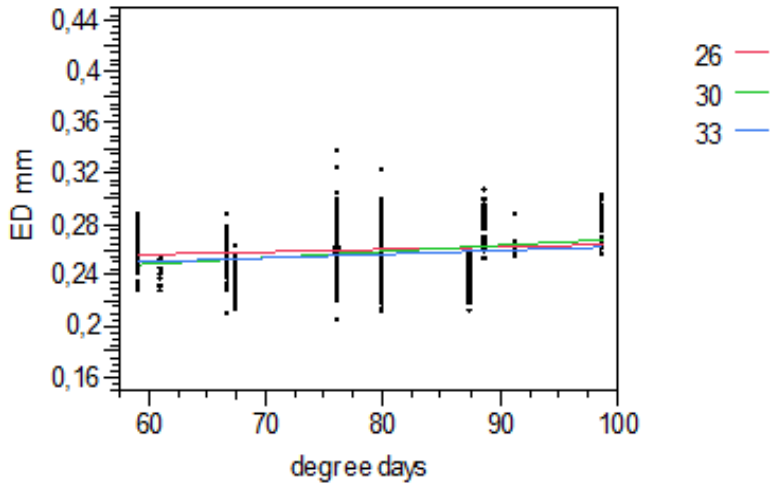



c
0,27
0,25
0,23
0,21
0,19
0,17
0,15
MH mm
60
70
80
90
100
degree days
10
15
20

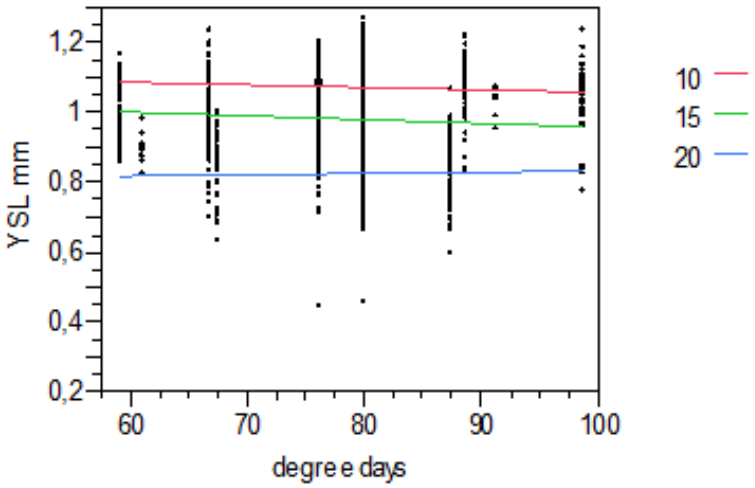

d
0,27
0,25
0,23
0,21
0,19
0,17
0,15
MH mm
60
70
80
90
100
degree days
26
30
33


e
0,44
0,4
0,36
0,32
0,28
0,24
0,2
0,16
ED mm
60
70
80
90
100
degree days
10
15
20

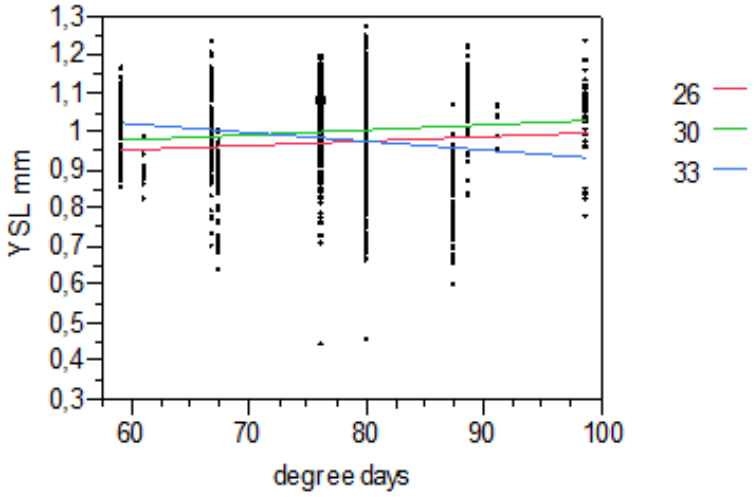
f


0,44
0,4
0,36
0,32
0,28
0,24
0,2
0,16
ED mm
60
70
80
90
100
degree days
26
30
33

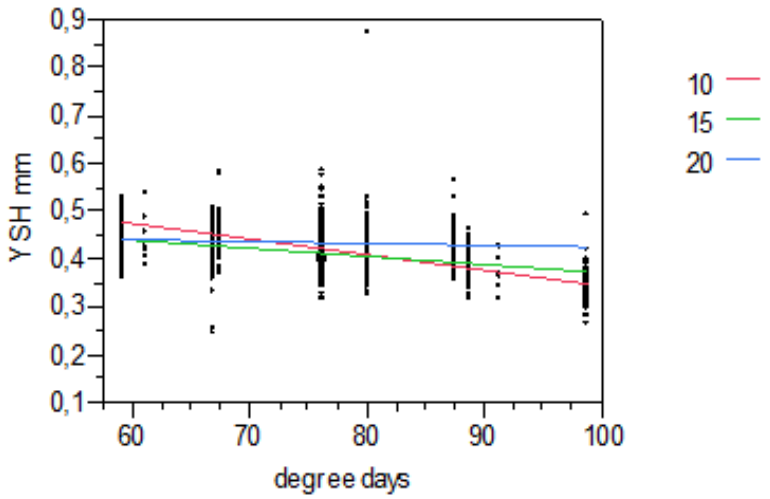
g


1,2
1
0,8
0,6
0,4
0,2
YSL mm
60
70
80
90
100
degree days
10
15
20

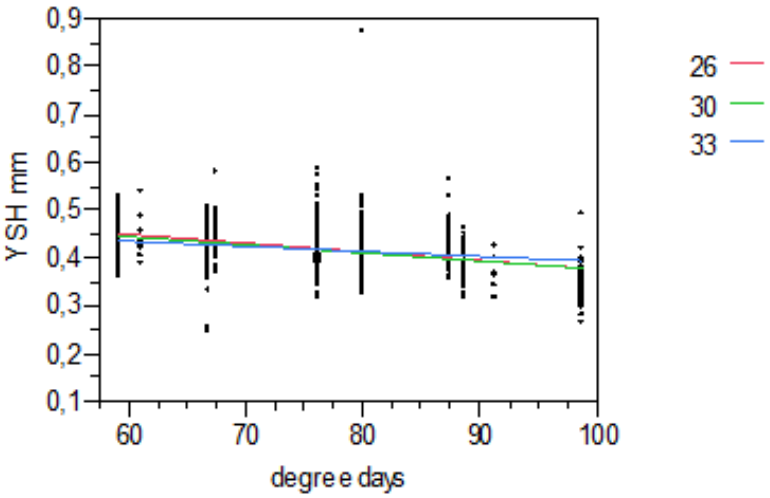
h


1,3
1,2
1,1
1
0,9
0,8
0,7
0,6
0,5
0,4
0,3
YSL mm
60
70
80
90
100
degree days
26
30
33

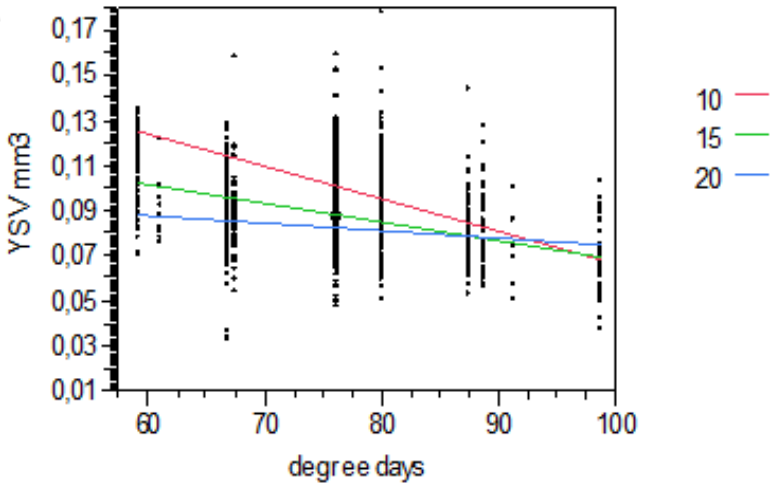
i


0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
YSH mm
60
70
80
90
100
degree days
10
15
20

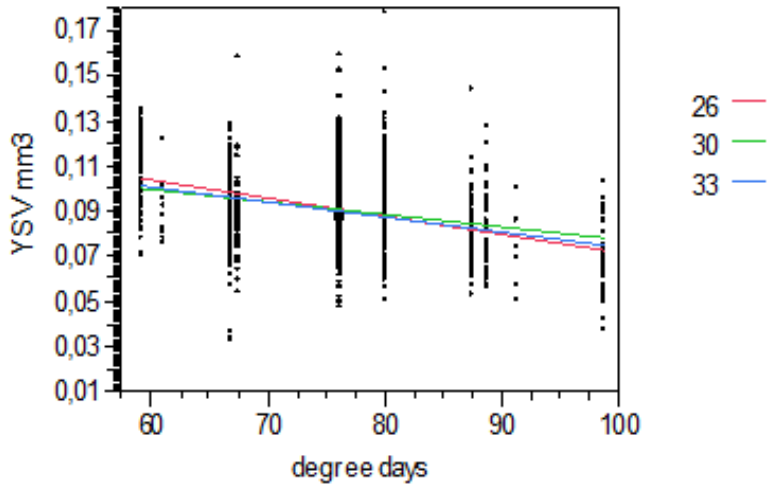
j


0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
YSH mm
60
70
80
90
100
degree days
26
30
33

k

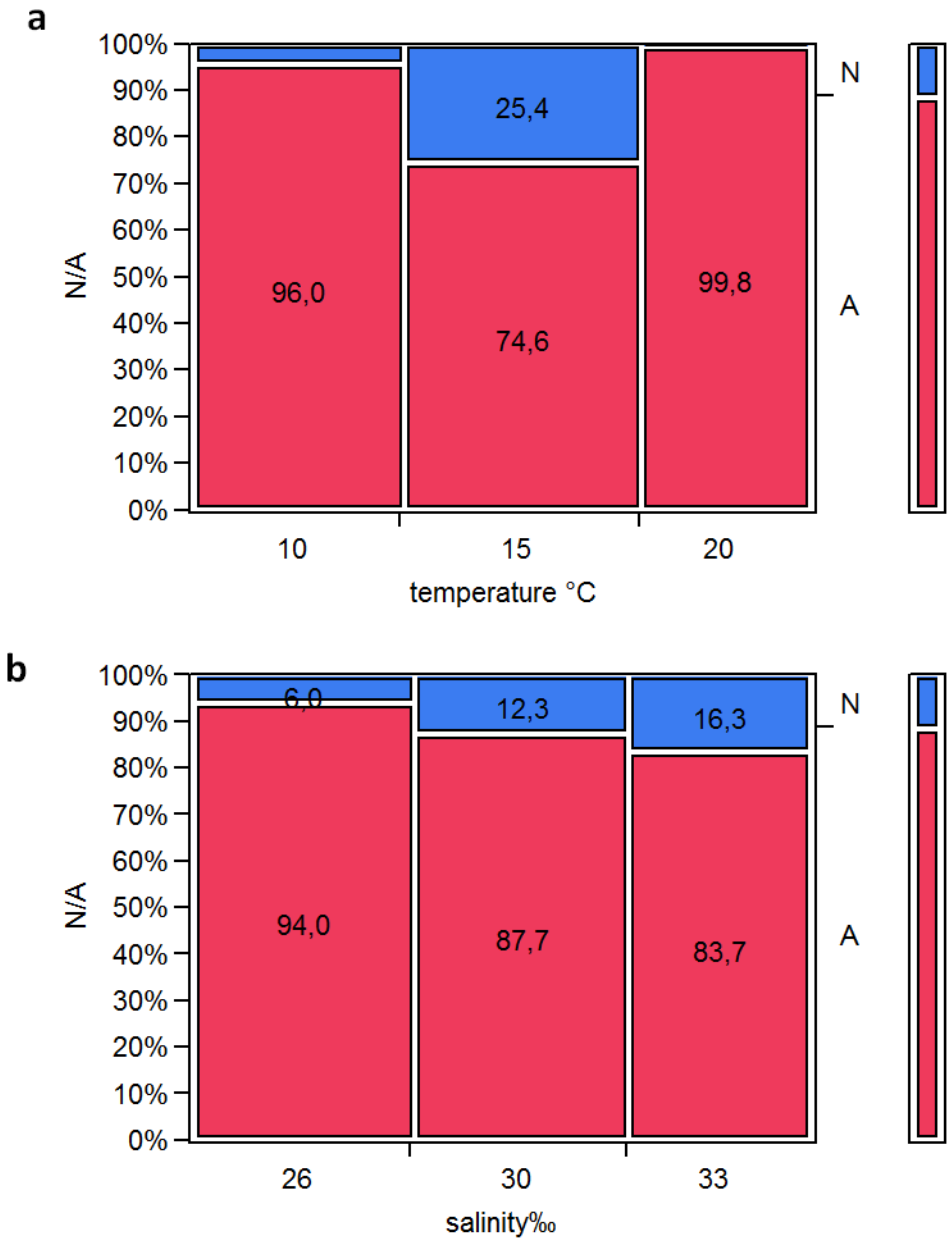

0,17
0,15
0,13
0,11
0,09
0,07
0,05
0,03
0,01
YSV mm3
60
70
80
90
100
degree days
10
15
20

l


0,17
0,15
0,13
0,11
0,09
0,07
0,05
0,03
0,01
YSV mm3
60
70
80
90
100
degree days
26
30
33

Appendix 16

Fig. A8. Normality/abnormality proportion (%) in newly hatched larvae of Ballan wrasse incubated at (a) different temperatures; (b) at different salinities (N-normal, A-abnormal larvae).



Appendix 17

Fig. A9 Occurrences of newly hatched larvae abnormality (%) depending on hatch-time (early – on first day of hatching, mid-second day of hatching, late-third day) (description of types see in Chapter 3.8.) (N-normal).