Reproductive Biology and Growth of Ballan Wrasse *Labrus bergylta* in West of Ireland Waters

Thesis submitted to the National University of Ireland, Galway in candidate for the degree of Doctor of Philosophy

By

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DECLARATION

This thesis is exclusively the result of my own research work. Larval size data presented in one of the experiments analysed in the present thesis (Chapter four) were different individuals from the individuals presented by D'Arcy, 2013. Larval otoliths' data from 0-49 days post hatch (Chapter four) is shared with D'Arcy, 2013; however these data were statistically analysed and written-up seperately.

The work was done under the guidance of Dr Anne Marie Power and Dr Richard Fitzgerald at the Martin Ryan Institute, National University of Ireland Galway.

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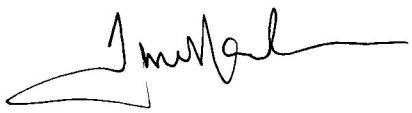
[Mohd Faizzi Bin Mohd Yusof]
Date:

In my capacity as supervisor of the candidate's thesis, I certify that the above statements are true to the best of my knowledge.



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Abstract

The research undertaken is very relevant to the aquaculture industry and fisheries management as it addresses natural cycle of the reproductive parameters including sex hormones, gonads development and nutritional background. Highest concentrations of circulatory estradiol and testosterone in the plasma coincided with the preponderance of vitellogenic follicles in the ovary and also had a sharply increased GSI. Manipulation of reproduction by induced breeding was successfully undertaken from captive and wild *Labrus bergylta*. Using hormonal agonist injections clearly demonstrated that hCG can advance, synchronize ovulation and spawning in *L. bergylta* without affecting egg quality. Egg and larval growth from artificial spawning from two different batches of *L. bergylta* broodstock were compared on the weaning onto formulated feed of two different diets to describe growth. Growth was positive followed an exponential curve between batches and diets of larvae, with varying changes in growth rates at different points in the development from hatching to 112 dph. In spawning batch II, larvae fed Diet B (micropellet + polychaetes) gave significant growth comparable to Diet A (micropellet alone) in term of dry weight and wet weight for the period when diets actually differed (49-112 dph). Mean standard lengths or myotome heights of larvae showed no effect of diet but significant effects according to spawning batch. This underlines the fact that spawning batch had a greater effect than diet, while mean specific growth rates (SGRs) declined over time with growth slowing down as larvae got older on all growth measures. Micro and macro-incremental counts of the sagittal otoliths from laboratory reared larvae and monthly sampled juveniles and adults were undertaken as a means to validate the number of rings deposited daily and yearly for ageing *L. bergylta*. Most relationships between the growth variables for *L. bergylta* were best described by regression with a quadratic fit or curvilinear relationship. The modelled maximum length from von Bertalanffy growth function (VBGF) in the present study gave L_{∞} = 42.48±0.884 cm and growth rates (k) of 0.16±0.013 $year^{-1}$ for all *L. bergylta* (male plus female). The relationship between fish length with otolith length, otolith weight and age (in years) for *L. bergylta* indicated strong positive correlations. Overall, the results suggest that fish length was a good predictor of age.

Chapter one
General Introduction

1.1 Ballan wrasse biology and distribution

Ballan wrasse *Labrus bergylta* Ascanius 1767 belongs to the labrid (wrasse) family (division: Teleostei, order: Perciformes, family: Labridae). In its native range, *L. bergylta* is recorded on eastern Atlantic coasts from Morocco to Norway (Darwall *et al*., 1992) and in the Azores (Figure 1.1, Fishbase.org). It is found in the North Sea and western parts of the Baltic, but is rare or even absent from Mediterranean areas (Treasurer, 1994; D'Arcy *et al*., 2013). In the Mediterranean Sea, there are some questionable records from the Adriatic and Marmara Seas and elsewhere (Treasurer, 1994). Meanwhile, it is absent from the Levant (Golani, 2006).

L. bergylta is the largest of the northern European wrasses, attaining a total length of between 300 mm and 600 mm (Sayer *et al*., 1996) and a body weight reaching 3.5 kg (Kvenseth *et al*., 1996). This species lives for between 17 and 25 years (Darwall *et al*., 1992) and some observations stated that individuals can live up to 29 years for males and 25 years for females (Dipper *et al*., 1977). *L. bergylta* possesses a deep-set body, a large head, and a small mouth with protractible thick fleshy lips. Cryptic colouration is displayed, i.e. adult colour is variable but this is not dependent on sex or season; instead, colouration changes according to unknown factors. The body, head and fins are often brownish red or reddish with numerous small white spots, but these can also be greenish with white spots or can display irregular large vertical dark stripes (Sayer *et al*., 1996). Over the range of small body lengths, from 80 to ~120 mm, colouration is often a light green colour (Sayer *et al*., 1996) (Figure 1.2). This species is found mainly in inshore waters (10-20 m depth) around rocks, offshore reefs and amongst seaweeds. Juveniles are occasionally found in seaweed-covered rock pools in the intertidal and shallow sub-littoral zone whereas adult distribution may extend to 30 m depth (Sayer *et al*., 1996).

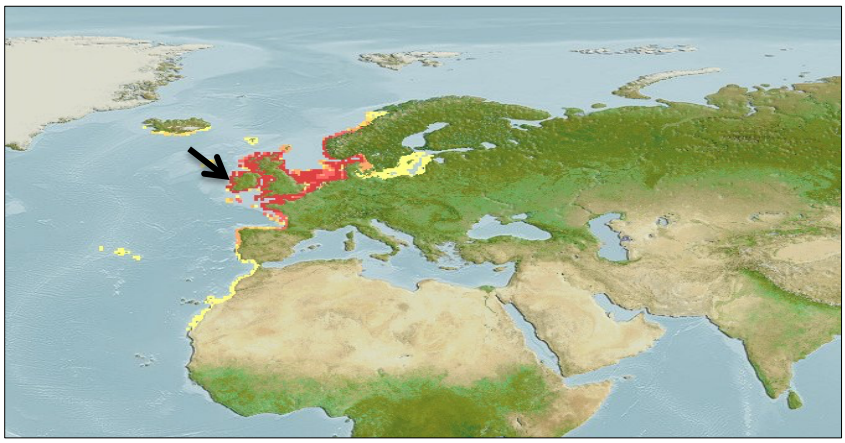


Figure 1.1: Native range of ballan wrasse *L. bergylta* predicted from OBIS and GBIF records on the eastern Atlantic seaboard with relative probabilities of occurrence (red: 0.80-1.00; orange: 0.40-0.59; yellow: 0.01-0.19). An arrow indicates the location of the study population in Galway on the west of Ireland.
[Map adapted from (*http://www.aquamaps.org/receive.php#)*]

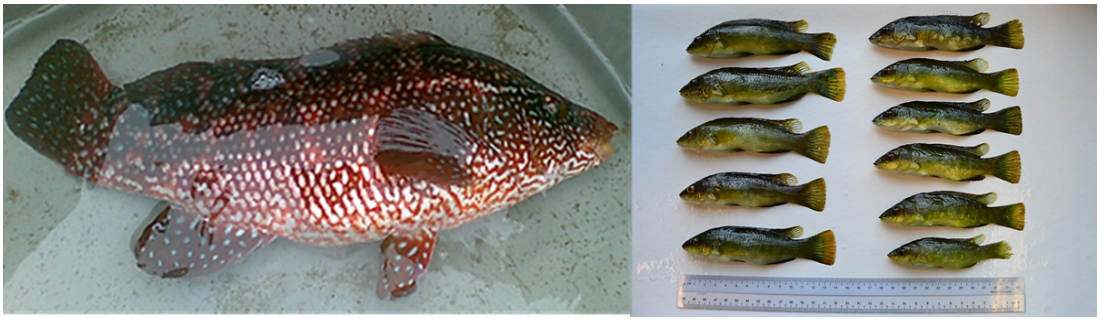


Figure 1.2: Ballan wrasse *Labrus bergylta* Ascanius 1767. Adult (left) and juveniles (right); the individuals shown were sampled in Carna, Co. Galway, Ireland in 2011.

L. bergylta of the north-eastern Atlantic and the Mediterranean sea preys upon a great variety of benthic invertebrates, including crustaceans and molluscs (Quignard and Pras, 1986). A study of stomach contents of *L. bergylta* sampled in the Faial island of the Azores archipelago showed that feeding was mainly on echinoderms (sea-urchins), gastropods and decapods. Decapods, mainly true crabs (Brachyura), were the most important food items accounting for 26.7% by weight of all identified food items (Figueiredo *et al*., 2005). Amongst decapods the most important species were *Xanthoincisus, Percnongibbesi* and *Scyllarusarctus*. Echinoderms such as the sea-urchins, *Arbacia lixula, Paracentrotus lividus* and *Sphaerechinus granularis* were the second most important prey group accounting for 45.1% by weight of all identified food items. Gastropods such as *Columbella adansoni, Haliotis* sp., *Calliostoma lusitanicum* and *Nassarius incrassatus* were the third most important prey group. Algae, bivalves, worms and other invertebrates

were also preyed by the *L. bergylta* in this study, but to a lesser extent (Figueiredo *et al*., 2005).

In another study, Deady and Fives (1995) found that decapods and bivalves were the major prey categories in the gut contents of 99 *L. bergylta* sampled between April and July in Galway Bay, Ireland. Algae and gastropods were also frequently eaten but in much smaller amounts. The volume of the major food categories did not vary significantly with fish length. However, fish longer than 20 cm did consume a greater volumes of bivalves than smaller fish. An increase in dietary diversity during the breeding season was also observed.

1.2 Reproduction and life cycle

Most wrasse species are known to undergo sexual inversions of the gonads (Nakamura *et al*., 1989; Koulish *et al*., 2002). *L. bergylta* displays protogynous hermaphroditism, whereby individuals function first as females and some individuals later become males. Protogyny is hypothesised to be a feature which has the effect of increasing the reproductive output of males (Muncaster *et al*., 2013), since this allows greater size to be attained before male reproduction. Greater size may be a particular advantage as *L. bergylta* males keep a harem of females, hence males show territorial aggressive behaviour associated with the defence of a breeding territory during the breeding season (Darwall *et al*., 1992). Female *L. bergylta* change sex when they are between 4 and 14 years old (Muus and Nielsen, 1999; Froese and Pauly, 2012). One (or more) females spawn in a nest built by the male, generally comprised of algae in a rocky crevice. The male guards the nest for one to two weeks until the eggs hatch (Muus and Nielsen, 1999). Sex identification is not particularly straightforward, as colouration is highly variable in both sexes, it is possible to differentiate the sex of adult *L. bergylta* by ultrasound or biopsy of the gonad. A recent analysis found that male-biased sexual size dimorphism was significant and the most effective morphometric method for sexing *L. bergylta* outside of the species spawning window used body mass (M_B in g), total body length (L_T in mm) and Fulton's condition factor (K) as discriminant variables to predict gender with 91% accuracy. The discriminant score of a specimen calculated according to the relationship $S_D = 0.01\ M_B - 0.016\ L_T - 3.835\ K + 6.252$ predicted female or male gender respectively when S_D is < 1.459 or S_D is > 1.504, (Leclercq *et al*., 2014). Another study found that sex change occurred most often in fish ranging

from 34 to 41 cm and the median size of fish in the female/male overlap was 36 cm total length (Muncaster *et al*., 2013). Muncaster *et al*. (2013) found that sex change in *L. bergylta* took place after the breeding season off Western Norway. From July to September, fish were most often in an early state of gonadal transition, characterized by degenerating previtellogenic oocytes and pockets of proliferating spermatogonia in the germinative epithelia (Muncaster *et al*., 2013).

L. bergylta spawns from late spring onwards, depending on latitude. In the southerly parts of its range on the Atlantic coast, spawning commences in April; whereas on the western coast of Sweden, spawning takes place from late May to late June (Darwall *et al*., 1992). However, spawning can occur as late as August in northern Norway (Muncaster *et al*., 2010). Data regarding the spawning season in Ireland has not previously been published, however this might be assumed to be similar to the United Kingdom, where spawning is between April and June (Sayer *et al*., 1996). *L. bergylta* are batch spawners and individuals may spawn several times over an extended breeding period of up to two months (Muncaster *et al*., 2010). Demersal eggs are sticky and males give parental care to the offspring. After a given batch of eggs hatch, the male moves to another territory (Darwall *et al*., 1992). The eggs are spherical with a diameter of between 0.70 and 1.15 mm, they appear creamy and white coloured and have small oil globule. The pre-larval (yolk sac period) of *L. bergylta* have big, ovoid eggs with an unsegmented yolk sack and hatching occurs at a body length of 2.7±0.2 mm (Dunaevskaya *et al*., 2012). The larvae hatch at 123 hours post-fertilization, which is equivalent to 62.5 degree days at 12.2±1.10°C at a length of 3.64±0.05 mm (D'arcy *et al*., 2012). The newly-hatched larvae are heavily pigmented with chromatophores from the posterior of the head to approximately the eighth post-anal segment (Dunaevskaya *et al.*, 2012).

1.3 Uses of *Labrus bergylta* in aquaculture

The rapid expansion of finfish farming in Northern Europe has focused attention on problems caused by a variety of pest organisms and one of the most serious of these is sea lice (family *Caligidae*). Ecto-parasites, especially from the species *Lepeophtheirus salmonis* and *Caligus elongatus,* have been associated with high infestation rates on juvenile pink salmon (*Oncorhynchus gorbuscha*) and chum salmon (*Oncorhynchus keta*) in the Northern Hemisphere (Morton *et al*., 2004; Costello, 2006; Figure 1.3). These parasites are also responsible for enormous

economic losses in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) aquaculture, costing hundreds of millions of euros annually and this issue is of particular concern to the largest producer in the world of Atlantic salmon, Marine Harvest ASA (Skiftesvik *et al*., 2013). Marine Harvest co-developed a commercial-scale wrasse hatchery project in Scotland with various partners, beginning in 2010. The project focuses on the production of farmed *L. bergylta* as cleaner fish for the Scottish Salmon industry (Meritxell Diez-Padrisa, 2013). The latest aggregated sea lice data published by the Scottish Salmon Producers Organisation (SSPO) shows that in the first quarter of 2014, sea lice numbers on farmed salmon were still "out of control" in a number of regions (*Undercurrent News,* 2014a). In Norway, sea lice mitigation and extraordinary mortality reduced Marine Harvest's profits by NOK 156.5 million ($23.7m) in the third quarter of 2014. Marine Harvest also incurred NOK 64.7 million in costs from 'exceptional mortality' in Norway, Scotland, Canada and Chile (*Undercurrent News*, 2014b).

Figure 1.3: A typical heavy infestation of an Atlantic salmon by the sea louse *Lepeophtheirus salmonis*, showing removal of skin over the head (Costello, 2006).

For the last two decades, lice-infested Atlantic salmon (*Salmo salar*) has been treated with organophosphate pesticides (Bjordal, 1992; Grave *et al*., 2004), with several types of pesticides including pyrethrins, cypremethrin, dichlorvos and azamethiphos being applied in bath treatments (Ernst *et al*., 2001). Other examples of pesticides are medicated feeds treatments containing ivermectin, emamectin benzoate, diflubenzuron, or teflubenzuron; these have been used to treat sea lice in New Brunswick, Canada, (Ohalloran and Coombs 1993; Haya *et al*. 2001; Stone *et al*., 2002; Treasurer *et al*., 2002; Saksida *et al*., 2013). Unfortunately, conventional

chemical treatment methods result in waste that is also known to have an adverse environmental impact (Ernst *et al*., 2001). For example, chemicals used in the treatment of sea-lice infestations are also lethal to species of shrimp and lobster in the vicinity of fish farms (Egidius and Moster, 1987; Samuelsen *et al*., 2015). In laboratory studies involving arthropod species, exposure to the azamethiphos concentrations used in bath treatments (100µg/l) for less than one hour, exhibited a 50% mortality rate (Burridge *et al*., 2000). Apart from these case studies, there is growing concern that lice are becoming resistant to chemical treatments (McHenery *et al*., 1991; Torrissen *et al*., 2013). In addition, there are several negative side-effects of chemical treatments: Bjordal (1990) found that chemical delousing was a major stressor for farmed salmon, expressed in increased heart rate and cortisol, while dichlorvos may cause mortality in salmon (Salte *et al*., 1987). If not properly used, the chemicals also represent a health risk to farm workers. The use of these chemicals is one of the most criticized aspects of salmon farming by environmental agencies, and proposals have been made to ban their use (Ross and Horsman, 1988).

The sea lice problem associated with salmon farms has recently been a controversial issue among local stakeholders and state agencies in Galway who are concerned about the impact of sea lice on wild Irish salmon and trout stocks along the west coast of Ireland. Anglers, state angling agencies and tourism-related businesses have come out in opposition to the proposed 15,000 tonne salmon farm based in Galway Bay on the Irish west coast (Anonymous, 2011). There are concerns that increased infection of wild salmon on their outward migrations from the river Corrib and other important wild salmon rivers in counties Galway and Mayo will result from the proximity of these very large salmon farms (Galway Independent, 30 October 2013; Figure 1.4). As this proposal involves organically-produced salmon, there are concerns about what this means for the potential to control sea-lice. Marine Harvest (Ireland) is the only producer of organic salmon in Ireland, produced by the Marine Harvest Group farms at Mulroy Bay, Inver Bay and Fanad in Co. Donegal, Clare Island in Clew Bay, Co. Mayo, Kilkieran Bay in Co. Galway and in Bantry and Kenmare Bays, in counties Cork and Kerry, respectively (marineharvestireland.com). The proposed fish farm development in Galway Bay has meant that there is a growing interest in farming *L. bergylta* in Ireland to reduce the environmental impact of chemical delousing agents.

Figure 1.4: Galway Independent newspapers dated 30th October 2013 and a petition by opponents to proposals for large salmon farms in Galway Bay, Ireland.

There is an urgent need for alternative, less harmful solutions to the sea lice problem and different approaches have been adopted; however, approaches such as capturing lice in light traps or repelling lice by sound or electrical stimuli have been attempted without promising results (Tully *et al*., 1996). Huse *et al*. (1990) found that shading of sea cages gave slightly reduced lice infestation, as did introductory trials with pyrethrum (an organic insecticide) mixed in an oil layer on the water surface (Jakobsen and Holm, 1990). Another approach is the use of deterrent techniques based on so-called semiochemical principles, which function like natural pheromones released by the sea lice; these can have a signaling affect to modify the behaviour of conspecifics (Costello, 2006). In a rather unusual application, a Norwegian innovator company, Stingray Marine Solutions AS, is collaborating with lazer development specialists (Jenoptik) to combat parasites with precision lazer treatments. In this case, a short laser impulse (using a JenLas D2.8 lazer) destroys the parasite tissue without damaging the fish (Peach, 2014; Ramsden, 2015). However, despite all these innovative solutions, utilization of cleaner-fish is, at present, the most promising alternative for lice control.

Wrasses have been recognized as important for the treatment of salmon lice for several decades (Kvenseth and Kvenseth, 1997, Leclerq *et al*., 2014) particularly in Norway (Muncaster *et al*., 2008; Skiftesvik *et al*., 2013). Based on previous trials, six different species of wrasse capable of cleaner activity; -(*Labrus bergylta*), goldsinny wrasse (*Ctenolabrus rupestris*), corkwing wrasse (*Symphodus melops*),

cuckoo wrasse (*Labrus bimaculatus*), rock cook (*Centrolabrus exoletus*) and scale-rayed wrasse (*Acantholabrus palloni*) (Treasurer, 1994; Deady *et al*., 1995; Tully *et al*., 1996; Samuelsen *et al*., 2003). Some specifics about the effectiveness of wrasse as biological cleaner-fish have emerged from a number of studies (Bjordal, 1990; Bjordal, 1992; Donnelly and Reynolds, 1994; Sayer *et al*., 1996; Deady *et al*., 1995; Tully *et al*., 1996; Treasurer, 1997; Treasurer *et al*., 2002 and Leclercq *et al*., 2014). Goldsinny (*Ctenolabrus rupestris*), corkwing (*Crenilabrus melops*), and rock cook (*Centrolabrus exoletus*) did not effectively clean larger hosts in one study due to significant losses of goldsinny and corkwing from cages - either due to escape or predation by larger post-smolts (Deady *et al*., 1995). But larger-bodied cleaner fish such as *L. bergylta* can circumvent this problem. *L. bergylta* also has the advantage of possessing a low temperature preference (≥3°C), which makes this species compatible for co-culture with salmon up to 1.3m in length (>2 kg) (Sayer *et al*., 1996). Control of sea lice using *L. bergylta* was first demonstrated to be effective by Kvenseth and Kvenseth (1997) in Norway. In this case, co-culture of *L. bergylta* with salmon of 3 - 7 kg body weight showed that there was a reduction from 60 lice per fish to 1 louse per fish over a 4 week period (Deady *et al*., 1995). In a more recent estimate, the mean number of mobile lice was maintained at <1 per salmon when using *L. bergylta* as cleaner-fish, with the ideal stocking ratio for these operations estimated at 5% wrasse to salmon (Skiftesvik *et al*., 2013). Tank-based trials performed by the University of Aberdeen at Machrihanish Marine Farm hatchery observed that infection levels declined rapidly from an initial infection of ∼12 lice per salmon at a ∼5% wrasse:salmon ratio (Leclercq *et al*., 2014).

Overall, the effectiveness of *L. bergylta* as a cleaner fish has been proven in experimental trials in sea cages, which has led to interest in the viable production of this species at a commercial scale (Skiftesvik *et al*., 2013). This means that a number of specific size grades of *L. bergylta* are required to be available in a predictable and controlled manner by the salmon farming industry. The *L. bergylta* that have been used in salmon farms over the past ten years have been caught directly from the wild in very large numbers, which has caused damage to wild stocks (unpublished reports suggest wild wrasse numbers in Norway have declined dramatically). Hatchery rearing has the advantage of reducing the environmental impacts on wild wrasse populations, as well as minimizing the labour and cost associated with cleaner fish acquisition (Darwall *et al*., 1992; Deady *et al*., 1995). Although several commercial

scale hatcheries for wrasse now exist outside of Ireland (including Labrus AS in Norway, plus two commercial hatcheries in U.K.; the SAIC at University of Stirling and the Scottish Sea Farms), wild wrasse continue to be captured in Ireland, towards research and development of cleaner fish technologies in this country (personal communication with industry). Developments in Scotland on using wrasse as biological controls for lice have been supported by the salmon farming industry (Hjul, 2015). Research into lumpfish as a cleaner fish has also begun more recently, due to its appetite for the ectoparasitic copepod (*Lepeophtherius salmonis*) (Imsland *et al*., 2014a), although this is several years behind wrasse in its development. The Scottish Aquaculture Innovation Centre (SAIC), established by the Scottish Government in 2014 at University of Stirling, began a £4 million applied research project to upscale the use of farmed wrasse (*L. bergylta*) and lumpfish (*Cyclopterus lumpus*) in commercial salmon farms. Lumpfish grow faster to a suitable size of 5-6 cm within 4-5 months (Imsland *et al*., 2014a). The lumpfish are reportedly easier to farm and can be used at a greater density compared with wrasse (15% compared to 4-5% relative to salmon density, respectively), as well as being less susceptible to *Vibrio* spp. infection (Imsland *et al*., 2014b). It may also present other advantages such as targeting both species of lice (*Lepeophtheirus salmonis* and *Caligus elongates*) as they consume a wide variety of invertebrates (Daborn and Gregory, 1983; Imsland *et al*., 2015).

A Northern Periphery Project ('Ecofish'; 2008-2011) involving researchers in Norway, Scotland and Ireland conducted linked research modules towards the development of commercial scale *L. bergylta* production. Researchers from Ireland and Scotland in this project faced problems getting captive broodstock to spawn (Ottesen, 2009). Marine Harvest also reported no spawning in 2011 because cold winter temperatures (~6°C) were suspected to have 'switched off' the spawning cue (Meritxell Diez-Padrisa, 2013). These problems may be caused by various factors in captive fish, which fail to spawn even though the oocytes appear to be at the right stage to ovulate (Patino *et al*., 2005). There is usually a strong correlation between spawning behaviour and elevated levels of gonadal steroids in fish plasma – this has been shown in a huge number of fish species - demoiselles, *Chromis dispilus* (Pankhurst and Barnett, 1993), common dentex, *Dentex dentex* (Pavlidis *et al*., 2004), female senegalese sole, *Solea senegalensis* (Guzman *et al*., 2009), curimata-pacu, *Prochilodus argenteus* (Arantes *et al*., 2010), seaperch, *Psammoperca*

waigiensis (Hung *et al*., 2010), spotted rose snapper, *Lutjanus guuatus* (Boza-Abarca *et al*., 2011), senegal sole, *Solea senegalensis* (Oliveira *et al*., 2011), caspian brown trout, *Salmo trutta caspius* (Hajirezaee *et al*., 2011), characiform fish, *Prochilodus lineatus* (Perini *et al*., 2013) and catfish, *Heteropneustes fossilis* (Chaube *et al*., 2014). Generally, increased levels of sex hormones in plasma are correlated with oocyte development and gonadosomatic index (GSI) changes. Uglem *et al*. (2002) found plasma testosterone and 17β-estradiol levels shown in the corkwing wrasse *Symphodus melops* correlates positively with the relative gonad size. Only a few papers have been published on the reproductive biology of *L. bergylta*, mainly by Muncaster *et al.* (2008, 2010 and 2013), which evaluated the circulating testosterone and 17β-oestradiol levels with the stages of ovarian development over two years in Norway. No record has been published on the natural circulating sex steroids and maturation of *L. bergylta* in the west coast of Ireland. Since the understanding of endocrinology is not so extensive compared to other areas of *L. bergylta* broodstock management, this represents an information gap in understanding annual egg and larvae production; therefore this aspect was examined in Chapter two of this thesis. This chapter focusses on the natural circulating sex steroids and maturation of *L. bergylta* and it also incorporates data on the nutritional background of wild *L. bergylta*, in order to compare between captive and wild broodstocks.

Sex hormones undoubtedly play a major role in egg maturation and spawning, and sex hormone manipulation could become a powerful tool for promoting or synchronising spawning. Suitable spawning agents, e.g. various types of gonadotropin hormones, were implemented singly and in combination to assess their effect on spawning of *L. bergylta* broodstocks in Chapter three. Gaining an element of control over spawning is an important factor in the effort to co-ordinate captive breeding in *L. bergylta*. This control might allow spawning to be synchronised and planned at a convenient time of the year, or to co-ordinate peaks and troughs of production.

Poor larval performance and mortality around the weaning stage are bottlenecks identified in the aquaculture production of *L. bergylta* (D'arcy *et al*. 2012; Dunaevskaya *et al*. 2012). Abnormal development often occurs during embryo and larval stages, including problems with swim bladder inflation, which is most likely related to suboptimal environmental conditions and feeding regimes. Most larval feeding studies have used a combination of live and dried diets, which have

resulted in improved feeding behavior and growth in larval fish (Ronnestad *et al*., 2013). This approach has shown promising results in a variety of species including angelfish, *Pterophyllum scalare* (Cichlidae) (Farhadian *et al*., 2014), clinid fish, *Myxodes viridis* (Ochoa-Munoz *et al*., 2013), winter flounder, *Pseudopleuronectes americanus*, Atlantic cod, *Gadus morhua* (Von Herbing, 2001) and yellowtail flounder, *Pleuronectes ferrugineus* (Rabe and Brown, 2000). Previous studies on *L. bergylta* larvae relied exclusively on *Artemia* until 50 days post hatch (dph), which resulted in poor growth and survival (Dunaevskaya *et al*., 2012). Thus, the study of larval growth needs to be advanced beyond 50 dph, in order to gain better growth and survival data, as a function of various diet formulations. Chapter four focused on this area, comparing different diets in co-feeding regimes up to 112 dph. The influence of spawning batch on growth was also considered in this chapter.

Analysis of growth and aging is fundamental to understanding growth rates, the speed of production in aquaculture and the longevity of aquacultured species, particularly those such as *L. bergylta*, which have heavily harvested from the wild in recent times. No information is available about the age structure of *L. bergylta* in the west of Ireland and its growth rates remain unknown, apart from data taken from larvae and juveniles (D'Arcy, 2013). Chapter five addresses this by examining age validation in *L. bergylta* using sagittal otoliths from known-age larvae, as well as wild juveniles and adults. Growth functions were also examined which will provide better insight into the life history of this species.

To summarise, the objectives of this thesis were; (i) to determine reproductive steroids and nutritional parameters in an annual cycle of wild *L. bergylta*; (ii) to establish a spawning induction protocol for artificial spawning; (iii) to establish larval weaning in co-feeding trials and examine any effects of two diets on growth performance to 112 day post hatch; and (iv) to determine age and growth from otolith microstructure in larval and adult otoliths.

References

Anonymous (2011). Submission by Inland Fisheries Ireland on the environmental impact statement for a deep sea fish farm development in Galway Bay. 16 pp.

Arantes, F. P., Santos, H. B., Rizzo, E., Sato, Y., & Bazzoli, N. (2010). Profiles of sex steroids, fecundity, and spawning of the curimata-pacu *Prochilodus argenteus* in the Sao Francisco River, downstream from the Tres Marias Dam, Southeastern Brazil. *Anim Reprod Sci, 118*(2-4), 330-336.

Bjordal, A. (1990). Sea lice infestation of the farmed salmon: possible use of cleaner-fish as an alternative method for delousing. *Canadian Technical Reports in Fisheries and Aquatic Sciences* 1761, 85–89.

Bjordal, A. (1992). Cleaning symbiosis as an alternative to chemical control of sea lice infestations of Atlantic salmon. *In*: Thorpe, J.E., Huntingford, F.A. (Eds.), The importance of feeding behaviour for the efficient culture of salmonid fishes. World Aquaculture Workshops. *World Aquaculture Society*, pp. 53–60.

Boza-Abarca, J., Valverde-Chavarria, S., Calvo-Vargas, E., Ramirez-Alvarado, M., & Rodriguez-Gomez, E. (2011). Hormone-induced spawning of wild and captive-grown spotted rose snapper *Lutjanus guuatus* using carp pituitary suspension and human chorionic gonadotropin. *Ciencias Marinas, 37*(2), 125-139.

Brown, J. A., Somerton, D. C., Methven, D. A., & Watkins, J. R. (1992). Recent advances in lumpfish *Cyclopterus lumpus* and ocean pout *Macrozoarces americanus* larviculture. *Journal of the World Aquaculture Society*, *23*(4), 271-276.

Burridge, L. E., Haya, K., Waddy, S. L., & Wade, J. (2000). The lethality of anti-sea lice formulations Salmosan (R) (Azamethiphos) and Excis (R) (Cypermethrin) to stage IV and adult lobsters (*Homarus americanus*) during repeated short-term exposures. *Aquaculture, 182*(1-2), 27-35.

Chaube, R., Singh, R. K., & Joy, K. P. (2014). Effects of ovaprim, a commercial spawning inducer, on vasotocin and steroid hormone profiles in the catfish *Heteropneustes fossilis*: In vivo and in vitro studies. *Gen Comp Endocrinol, 195*, 190-200.

Costello, M. J. (2006). Ecology of sea lice parasitic on farmed and wild fish. *Trends in parasitology*, *22*(10), 475-483.

Daborn, G. R., & Gregory, R. S. (1983). Occurrence, distribution, and feeding habits of juvenile lumpfish, *Cyclopterus lumpus* L. in the Bay of Fundy. *Canadian Journal of Zoology*, *61*(4), 797-801.

D'Arcy, J. Studies on the biology of the Ballan wrasse, *Labrus bergylta*: Macro- and micro-structure analysis of larval ballan wrasse otoliths, including age validation [dissertation]. Galway: National University of Ireland, Galway; 2013.

D'Arcy, J., Dunaevskaya, E., Treasurer, J. W., Ottesen, O., Maguire, J., Zhuravleva, N., Karlsen, A., Rebours, C., & FitzGerald, R. D. (2012). Embryonic development in ballan wrasse *Labrus bergylta*. *J Fish Biol, 81*(3), 1101-1110.

D'Arcy, J., Mirimin, L., & FitzGerald, R. (2013). Phylogeographic structure of a protogynous hermaphrodite species, the ballan wrasse *Labrus bergylta*, in Ireland, Scotland, and Norway, using mitochondrial DNA sequence data. *Ices Journal of Marine Science, 70*(3), 685-693.

Darwall, W. R. T., Costello, M. J., Donnelly, R., & Lysaght, S. (1992). Implications of Life-History Strategies for a New Wrasse Fishery. *J Fish Biol, 41*, 111-123.

Deady, S., & Fives, J. M. (1995). Diet of Ballan Wrasse, *Labrus bergylta*, and Some Comparisons with the Diet of Corkwing Wrasse, *Crenilabrus melops*. *Journal of the Marine Biological Association of the United Kingdom, 75*(3), 651-665.

Deady, S., Varian, S. J. A. & Fives, J. M. (1995). The use of cleaner-fish to control sea lice on two Irish salmon (*Salmo salar*) farms with particular reference to wrasse behaviour in salmon cages. *Aquaculture* 131, 73-90.

Dipper, F.A., Bridges, C.R., & Menz, A. (1977). Age, growth and feeding in ballan wrasse *Labrus bergylta* Ascanius 1767. *Journal of Fish Biology* 11, 105-120.

Donnelly, R. E., & Reynolds, J. D. (1994). Occurrence and distribution of the parasitic copepod *Leposphilus labrei* on corkwing wrasse (*Crenilabrus melops*) from Mulroy Bay, Ireland. *J Parasitol, 80*(2), 331-332.

Dunaevskaya, E., Amin, A. B., Ottesen, O. H. (2012) Organogenesis of Ballan Wrasse *Labrus Bergylta (Ascanius 1767)* Larvae. *J Aquacult Res Dev,* 3, 142

Egidius, E., & Moster, B. (1987). Effect of Neguvon and Nuvan Treatment on Crabs (*Cancer pagurus*, *Cancer Maenas*), Lobster (*Homarus gammarus*) and Blue Mussel (*Mytilus edulis*). *Aquaculture, 60*(2), 165-168.

Ernst, W., Jackman, P., Doe, K., Page, F., Julien, G., Mackay, K., & Sutherland, T. (2001). Dispersion and toxicity to non-target aquatic organisms of pesticides used to treat sea lice on salmon in net pen enclosures. *Marine Pollution Bulletin, 42*(6), 433-444.

Farhadian, O., Kharamannia, R., Soofiani, N. M., & Dorche, E. E. (2014). Larval feeding behaviour of angel fish *Pterophyllum scalare* (Cichlidae) fed copepod *Eucyclops serrulatus* and cladoceran *Ceriodaphnia quadrangula*. *Aquaculture Research, 45*(7), 1212-1223.

Figueiredo, M., Morato, T., Barreiros, J. P., Afonso, P., & Santos, R. S. (2005). Feeding ecology of the white seabream, *Diplodus sargus*, and the ballan wrasse, *Labrus bergylta*, in the Azores. *Fisheries Research, 75*(1-3), 107-119.

Froese, R., & Pauly, D. (eds.) (2012). FishBase. World Wide Web electronic publication. Available at: www.fishbase.org, version (10/2012). Accessed 20 January 2012.

Golani, D. (2006). The Indian scad (*Decapterus russelli*), (Osteichthyes : Carangidae), a new Indo-Pacific fish invader of the eastern Mediterranean. *Scientia Marina, 70*(4), 603-605.

Grave, K., Horsberg, T.E., Lunestad, B.T., Litleskare, I. (2004). Consumption of drugs for sea lice infestations in Norwegian fish farms: methods for assessment oftreatment patterns and treatment rate. *Dis. Aquat. Organ*, 60, 123–131.

Guzman, J. M., Ramos, J., Mylonas, C. C., & Mananos, E. L. (2009). Spawning performance and plasma levels of GnRHa and sex steroids in cultured female Senegalese sole (*Solea senegalensis*) treated with different GnRHa-delivery systems. *Aquaculture, 291*(3-4), 200-209.

Hajirezaee, S., Amiri, B. M., Mehrpoosh, M., Nazeri, S., & Niksirat, H. (2011). Gonadotropin releasing hormone-analogue (GnRHa) treatment improves the milt production and sperm motility of endangered Caspian brown trout, *Salmo trutta caspius*, over the course of a spawning season. *Aquaculture Research, 42*(12), 1789-1795.

Haya, K., Burridge, L. E., & Chang, B. D. (2001). Environmental impact of chemical wastes produced by the salmon aquaculture industry. *Ices Journal of Marine Science, 58*(2), 492-496.

Hjul, J. (2015). Wrasse project boost for salmon industry. *FISHupdate*, [online] 25 May. Available at: http://www.fishupdate.com/wrasse-project-boost-for-scottish-salmon-industry/ [Accessed 28 October 2015].

Hongchao, S. X. R., & Guifen, H. (1981). The development of the egg larval stages of the lumpfish, *Inimicus Japonic*. *Oceanologia Et Limnologia Sinica*, *4*, 010.

Hung, Q. P., Anh, T. N., Mao, D. N., & Arukwe, A. (2010). Sex steroid levels, oocyte maturation and spawning performance in Waigieu seaperch (*Psammoperca waigiensis*) exposed to thyroxin, human chorionic gonadotropin, luteinizing hormone releasing hormone and carp pituitary extract. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology, 155*(2), 223-230.

Huse, I., Bjordal, A., Ferno, A., & Furevik, D. (1990). The Effect of Shading in Pen Rearing of Atlantic Salmon (*Salmo salar*). *Aquacultural Engineering, 9*(4), 235-244.

Huse, I., Bjordal, A., Ferno, A., & Furevik, D. (1990). The Effect of Shading in Pen Rearing of Atlantic Salmon (*Salmo salar*). *Aquacultural Engineering, 9*(4), 235-244.

Imsland, A. K., Reynolds, P., Eliassen, G., Hangstad, T. A., Foss, A., Vikingstad, E., & Elvegård, T. A. (2014a). The use of lumpfish (*Cyclopterus lumpus* L.) to control sea lice (*Lepeophtheirus salmonis* Krøyer) infestations in intensively farmed Atlantic salmon (Salmo salar L.). *Aquaculture*, *424*, 18-23.

Imsland, A. K., Reynolds, P., Eliassen, G., Hangstad, T. A., Nytrø, A. V., Foss, A., Vikingstad, E., & Elvegård, T. A. (2014b). Assessment of growth and sea lice infection levels in Atlantic salmon stocked in small-scale cages with lumpfish. *Aquaculture*, *433*, 137-142.

Imsland, A. K., Reynolds, P., Eliassen, G., Hangstad, T. A., Nytrø, A. V., Foss, A., Vikingstad, E., & Elvegård, T. A. (2015). Feeding preferences of lumpfish (*Cyclopterus lumpus* L.) maintained in open net-pens with Atlantic salmon (Salmo salar L.). *Aquaculture*, *436*, 47-51

Jakobsen, P. J., & Holm, J. C. (1990). Promising trials with a new drug (Pyrethrum) to control salmon lice. *Norske Fiskeoppdrett,* 1 (1990), 16-18.

Koulish, S., Kramer, C. R., & Grier, H. J. (2002). Organization of the male gonad in a protogynous fish, *Thalassoma bifasciatum* (Teleostei: Labridae). *J Morphol, 254*(3), 292-311.

Kvenseth, P. G., Bjelland, R. M. & Kvenseth, A. M. (1996). Berggylte som lusespiser hos stor laks. *Norsk Fiskeoppdrett* **22**, 38-42.

Kvenseth, P. G. & Kvenseth, A. M. (1997). Practical experiences in the use of cleaner-fish. Results from a questionaire to Norwegian salmon farms in 1997 and 1999. *Caligus. A newsletter on the biology and control ofsea lice for researchers, aquaculture and fisheries industry, educators, consultants, and management authorities.*

Leclercq, E., Davie, A., & Migaud, H. (2014). Delousing efficiency of farmed ballan wrasse (*Labrus bergylta*) against *Lepeophtheirus salmonis* infecting Atlantic salmon (*Salmo salar*) post-smolts. *Pest Manag Sci*, 70(8), 1274-1282.

Leclercq, E., Grant, B., Davie, A., & Migaud, H. (2014). Gender distribution, sexual size dimorphism and morphometric sexing in ballan wrasse *Labrus bergylta*. *J Fish Biol, 84*(6), 1842-1862.

Mchenery, J. G., Saward, D., & Seaton, D. D. (1991). Lethal and Sublethal Effects of the Salmon Delousing Agent Dichlorvos on the Larvae of the Lobster (*Homarus gammarus* L) and Herring (*Clupea harengus* L). *Aquaculture, 98*(4), 331-347.

Meritxell Diez-Padrisa. (2013). The Scottish Wrasse Projects, 4th Sea lice multination. Trondheim, November 2013 [online] Available at: (http://www.fhf.no/media/67864/scottish_wrasse_project_-_meritxell_diez_padrisa_-_marine_harvest.pdf [Accessed 28 October 2015].

Morton, A., Routledge, R., Peet, C., & Ladwig, A. (2004). Sea lice (*Lepeophtheirus salmonis*) infection rates on juvenile pink (*Oncorhynchus gorbuscha*) and chum (*Oncorhynchus keta*) salmon in the nearshore marine environment of British Columbia, Canada. *Canadian Journal of Fisheries and Aquatic Sciences, 61*(2), 147-157.

Muncaster, S., Andersson, E., Kjesbu, O. S., Taranger, G. L., Skiftesvik, A. B., & Norberg, B. (2010). The reproductive cycle of female Ballan wrasse *Labrus bergylta* in high latitude, temperate waters. *J Fish Biol, 77*(3), 494-511.

Muncaster, S., Andersson, E., Skiftesvik, A. B., Opstad, I., Taranger, G. L., & Norberg, B. (2008). Seasonal reproductive cycle of Ballan wrasse (*Labrus bergylta*) in Norway. *Cybium, 32*(2), 199-199.

Muncaster, S., Norberg, B., & Andersson, E. (2013). Natural sex change in the temperate protogynous Ballan wrasse *Labrus bergylta*. *J Fish Biol, 82*(6), 1858-1870.

Muus, B.J., Nielsen, J.G. (1999). Sea fish. Scandinavian Fishing Year Book. Hedehusene, Denmark. 340 pp.

Nakamura, M., Hourigan, T. F., Yamauchi, K., Nagahama, Y., & Grau, E. G. (1989). Histological and Ultrastructural Evidence for the Role of Gonadal-Steroid Hormones in Sex Change in the Protogynous Wrasse *Thalassoma duperrey*. *Environmental Biology of Fishes, 24*(2), 117-136.

Ochoa-Munoz, M. J., Valenzuela, C. P., Toledo, S., Bustos, C. A., & Landaeta, M. F. (2013). Feeding of a larval clinid fish in a microtidal estuary from southern Chile. *Revista De Biologia Marina Y Oceanografia, 48*(1), 45-57.

Ohalloran, J., & Coombs, K. (1993). Treatment of Sea Lice on Atlantic Salmon with Ivermectin. *Canadian Veterinary Journal-Revue Veterinaire Canadienne, 34*(8), 505-505.

Oliveira, C., Mananos, E., Ramos, J., & Sanchez-Vazquez, F. J. (2011). Impact of photoperiod manipulation on day/night changes in melatonin, sex steroids and vitellogenin plasma levels and spawning rhythms in Senegal sole, *Solea senegalensis*. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology, 159*(3), 291-295.

Ottesen, O. H. Environment friendly fish farming and use of cleaner fish. Presentation of EcoFish 2009 October 3. Available from: URL: http://www.eco-fish.org//

Pankhurst, N. W., & Barnett, C. W. (1993). Relationship of population density, territorial interaction and plasma levels of gonadal steroids in spawning male demoiselles *Chromis dispilus* (Pisces: Pomacentridae). *Gen Comp Endocrinol, 90*(2), 168-176.

Patino, R., Bolamba, D., Thomas, P., & Kumakura, N. (2005). Effects of external pH on hormonally regulated ovarian follicle maturation and ovulation in Atlantic croaker. *Gen Comp Endocrinol, 141*(2), 126-134.

Pavlidis, M., Greenwood, L., & Scott, A. P. (2004). The role of sex ratio on spawning performance and on the free and conjugated sex steroids released into the water by common dentex (*Dentex dentex*) broodstock. *Gen Comp Endocrinol, 138*(3), 255-262.

Peach, M. (2014). Laser technique combats sea parasites. Resolveoptics.com, [online] 28 May. Available at: http://optics.org/news/5/5/52 [Accessed 28 October 2015].

Perini, V. D., Paschoalini, A. L., da Cruz, C. K. F., de Rocha, R. D. G. A., Senhorini, J. A., Ribeiro, D. M., Formagio, P. S., Bazzoli, N., Rizzo, E. (2013). Profiles of sex steroids, fecundity and spawning of a migratory characiform fish from the Paraguay-Parana basin: a comparative study in a three-river system. *Fish Physiology and Biochemistry, 39*(6), 1473-1484.

Quignard, J. P. & Pras, A. (1986). Labridae. In *Fishes of the North-Eastern Atlantic and the Mediterranean* (Whitehead, P. J. P., Bauchot, M. L., Hureau, J. C., Nielsen, J. & Tortonese, E., eds.), pp. 919–942. Paris: UNESCO.

Rabe, J., & Brown, J. A. (2000). A pulse feeding strategy for rearing larval fish: an experiment with yellowtail flounder. *Aquaculture, 191*(4), 289-302.

Ramsden, N. (2015). Sea lice laser tech firm sees rapid sales on launch. *Undercurrent News*, [online] 10 March. Available at: http://en.ofweek.com/news/Sea-lice-laser-tech-firm-sees-rapid-sales-on-launch-26162 [Accessed 28 October 2015].

Ronnestad, I., Yufera, M., Ueberschar, B., Ribeiro, L., Saele, O., & Boglione, C. (2013). Feeding behaviour and digestive physiology in larval fish: current knowledge, and gaps and bottlenecks in research. *Reviews in Aquaculture, 5*, S59-S98.

Ross, A., & Horsman, P.V. (1988). The use of Nuvan 500EC in the salmon farming industry. Report of the Marine Conservation Society, Ross-on-Wye. HR9 5BU. England. 24pp.

Saksida, S. M., Morrison, D., McKenzie, P., Milligan, B., Downey, E., Boyce, B., & Eaves, A. (2013). Use of Atlantic salmon, *Salmo salar* L., farm treatment data and bioassays to assess for resistance of sea lice, *Lepeophtheirus salmonis*, to emamectin benzoate (SLICE (R)) in British Columbia, Canada. *J Fish Dis, 36*(5), 515-520.

Salte, R., Syvertsen, C., Kjonnoy, M., & Fonnum, F. (1987). Fatal Acetylcholinesterase Inhibition in Salmonids Subjected to a Routine Organophoshpate Treatment. *Aquaculture, 61*(3-4), 173-179.

Samuelsen, O. B., Kvenseth, P. G., Andreassen, J. H., Torkildsen, L., Ervik, A., & Bergh, O. (2003). The efficacy of a single intraperitoneal injection of oxolinic acid in the treatment of bacterial infections in goldsinny wrasse (*Ctenolabrus rupestris*) and corkwing wrasse (*Symphodus melops*) studied under field and laboratory conditions. *Journal of Veterinary Pharmacology and Therapeutics, 26*(3), 181-186.

Samuelsen, O. B., Lunestad, B. T., Hannisdal, R., Bannister, R., Olsen, S., Tjensvoll, T., Farestveit, E., Ervik, A. (2015). Distribution and persistence of the anti sea-lice drug teflubenzuron in wild fauna and sediments around a salmon farm, following a standard treatment. *Science of the Total Environment, 508*, 115-121.

Sayer, M. D. J., Treasurer, J. W. & Costello, M. J. (1996). Wrasse biology and aquaculture applications: commentary and conclusions. In *Wrasse: biology and use in aquaculture* (Sayer, M. D. J., Treasurer, J. W. & Costello, M. J., eds.), pp. 272-274. Oxford: Fishing News Books.

Skiftesvik, A. B., Bjelland, R. M., Durif, C. M. F., Johansen, I. S., & Browman, H. I. (2013). Delousing of Atlantic salmon (*Salmo salar*) by cultured vs. wild ballan wrasse (*Labrus bergylta*). *Aquaculture, 402*, 113-118.

Stone, J., Roy, W. J., Sutherland, I. H., Ferguson, H. W., Sommerville, C., & Endris, R. (2002). Safety and efficacy of emamectin benzoate administered in-feed to Atlantic salmon, *Salmo salar* L., smolts in freshwater, as a preventative treatment against infestations of sea lice, *Lepeophtheirus salmonis* (Kroyer). *Aquaculture, 210*(1-4), 21-34.

Torrissen, O., Jones, S., Asche, F., Guttormsen, A., Skilbrei, O. T., Nilsen, F., Horsberg, T. E., Jackson, D. (2013). Salmon lice-impact on wild salmonids and salmon aquaculture. *J Fish Dis, 36*(3), 171-194.

Treasurer, J. W. (1994). The Distribution, Age and Growth of Wrasse (Labridae) in Inshore Waters of West Scotland. *J Fish Biol, 44*(5), 905-918.

Treasurer, J. W. (1997). Parasites of wrasse (Labridae) in inshore waters of west Scotland stocked as cleaner fish of sea lice (Caligidae) on farmed Atlantic salmon. *J Fish Biol, 50*(4), 895-899.

Treasurer, J. W., Wallace, C., & Dear, G. (2002). Control of sea lice on farmed Atlantic salmon *Salmo salar* L. with the oral treatment emamectin benzoate (SLICE). *Bulletin of the European Association of Fish Pathologists, 22*(6), 375-380.

Tully, O., Daly, P., Lysaght, S., Deady, S., & Varian, S. J. A. (1996). Use of cleaner-wrasse (*Centrolabrus exoletus* (L) and *Ctenolabrus rupestris* (L)) to control infestations of *Caligus elongatus* Nordmann on farmed Atlantic salmon. *Aquaculture, 142*(1-2), 11-24.

Uglem, I., Mayer, I., & Rosenqvist, G. (2002). Variation in plasma steroids and reproductive traits in dimorphic males of corkwing wrasse (*Symphodus melops* L.). *Horm Behav, 41*(4), 396-404.

Undercurrent News. (2014a). Half of Scottish salmon farms report ongoing sea lice problems, 9 June. Available at: https://www.undercurrentnews.com/2014/06/09/half-of-scottish-salmon-farms-report-ongoing-sea-lice-problems/ [Accessed 28 October 2015].

Undercurrent News. (2014b). Marine Harvest takes hit from sea lice extraordinary mortality costs, 22 October. Available at: https://bantryblog.wordpress.com/2014/10/22/undercurrent-news-22-oct-2014-marine-harvest-takes-hit-from-sea-lice-extraordinary-mortality-costs/ [Accessed 28 October 2015].

Von Herbing, I. H. (2001). Development of feeding structures in larval fish with different life histories: winter flounder and Atlantic cod. *J Fish Biol, 59*(4), 767-782.

Chapter two

Sex Steroids and Nutritional Status on Maturation in an Annual Reproductive Cycle of *Labrus bergylta*

2.1 Introduction

2.1.1 Sex steroids and maturation

There are numerous studies that demonstrate the major role played by sex steroids in fish reproduction. For example, correlations have been shown between oocyte development and plasma levels of gonadal steroids in salmoniforms, cyprinids, tilapia (*Oreochromis niloticus*), catfish (*Heteropneustes fossilis*), goldeye (*Hiodon alosoides*) and walleye (*Stizostedion vitrum*) (Lee *et al.,* 2001). Vitellogenesis and final oocyte maturation in teleosts is regulated via steroids secreted by follicular cells surrounding oocytes. Estradiol, in particular, stimulates hepatic synthesis and secretion of vitellogenin which subsequently accumulates in the oocytes (Kobayashi *et al*., 1996).

In addition, 17α-hydroxy-20β-dihydroxyprogesterone (17α-20β-OHP) has been found to be effective in inducing oocyte final maturation in rainbow trout (Fostier *et al*., 1981a and 1981b); amago salmon, *Oncorhynchus rhodurus* (Nagahama *et al*., 1980); brook trout, *Salvenilus fontinalis* (Duffey and Goetz, 1980); yellow perch, *Perca flavescens* (Goetz and Duman, 1986) and freshwater perch, *Anabas testudineus* (Bhattacharyya *et al*., 2000). By contrast, deoxycorticosterone in zebrafish, *Brachydanio rerio*, (Wilson *et al*., 2013) and notably corticosteroids in catfish, *Heteropneustes fossilis* (Chourasia and Joy, 2010) and *Mystus vittatus* (Upadhyaya and Haider, 1986), were more effective in inducing maturation. Accordingly, there are various possibilities of sex steroids which act as precursor for maturity and spawning across a range fish species.

In many studies, estradiol was responsible for stimulating vitellogenesis and this steroid was secreted by female gonads during the pre-spawning period (Moncaut *et al*., 2003; Rawat *et al*., 2013). During vitellogenesis, many species have been observed exhibiting an increase in plasma estrogen levels, predominately 17β-estradiol, which was correlated with vitellogenic oocyte growth. To further clarify the fate of exogenous estradiol, a study in tilapias (*Oreochromis* sp.) administered high doses of dissolved estradiol (0.5mg/kg) in sesame oil to ovariectomized females

and sampled their plasma at intervals. The circulating levels of estrogen five hours after injection was extremely high. Further, a "physiological" level of approximately 1-10ng/mL was reached after 24 hours and maintained for 4 days (Yaron *et al*., 1977). This study showed increase the levels of estrogen via estradiol injection can compress and shorten the spawning season that permits optimization of time and resources. Thus hormonal induction has many beneficial downstream effects that are realized when spawning is confined to a predictable and defined period.

A number of studies have been published on the endocrinological aspects of reproduction in various wild and captive fish species. However, little endocrinological research has been published on *L. bergylta* with the exception of annual plasma estradiol and testosterone levels, egg development and the gonado somatic index of wild caught fish in west Norway (Muncaster *et al*., 2008; 2010). *L. bergylta* are group synchronous spawners with multiple spawning events occurring in spring, with activity peaking in May (Muncaster *et al*., 2010). The seasonal reproductive cycle of *L. bergylta* from west Norway demonstrated a sharp peak of 11 ketotestosterone (11 KT) in wild caught male fish reaching up to 12ng/ml during May 2005 and 2006. This coincided with a maximal gonadosomatic index (GSI) during the spawning season indicating that this steroid is the primary androgen (Muncaster *et al*., 2008). Another study showed that maximal mean values (±s.e.) of plasma testosterone (0·95±0·26ng/ml), estradiol (1·75±0·43ng/ml) and gonadosomatic index (I_G; 10·71±0·81) occurred between April and May 2005. These parameters decreased greatly in July 2005 when only post-spawned fish with atretic ovaries were captured. A similar pattern of annual reproductive measures was shown in 2006 (Muncaster *et al*., 2010).

2.1.2 Nutritional background and maturation

Broodstock nutrition is without doubt one of the most poorly understood and researched areas of finfish aquaculture. In particular, the influence of dietary nutrients on reproductive processes such as concentrations of hormones is poorly understood. The natural composition of fish diets has been identified as a major factor in determining successful reproduction and offspring survival (Smits *et al*., 2013; Peterson *et al*., 2012). There is plenty of evidence that gonadal development and fecundity are greatly affected by broodstock nutrition (Tandler *et al*., 1995;

Izquierdo *et al*., 2001; Watanabe and Vassallo-Agius, 2003; Morais *et al*., 2014). In gilthead seabream (*Sparus aurata*) both protein and lipid fractions in squid meal play an important role in improving fecundity and egg quality (Tandler *et al*., 1995). The same observation was made in yellow fin sea bream (*Acanthopagrus latus*), where fecundity, fertilization rate, hatchability and survival of larvae was significantly affected by broodstock diets (Zakeri *et al*., 2009). In fact, in Nile tilapia (*Oreochromis niloticus*), egg hatchability increased linearly with increasing dietary protein level (El-Sayed *et al*., 2003; El-Sayed and Kawanna, 2008).

While, carbohydrates can be used as a cost-effective ingredient to contribute to glycogen accumulation in the liver as well as providing other benefits in the broodstock diet (e.g. acting as binders and possibly playing a role in transport of nutrients in the hemolymph) (Hemre *et al*., 2002; Panserat and Kaushik, 2002), dietary protein may be even more critical. Gonadal maturation is a time of intense protein synthesis and is likely that the requirement for protein is higher at this time (Zakeri *et al*., 2009). Albumin and globulin are the major blood proteins which are synthesized in the liver. A protein-deficient diet causes a decrease in albumin synthesis as long as the deficiency is maintained. Albumin serves in the transport of hormones, drugs, fatty acids, bilirubin, metals, vitamins and cations (such as Ca^{2+}, Na^{+} and K^{+}) and one of its other main function is to regulate the colloidal osmotic pressure of blood. Albumin and globulin are also involved in the transport of sex steroids in plasma and their concentration is a major factor regulating steroid distribution between the protein-bound and free states. Sex hormone binding globulin (SHBG) is a glycoprotein possessing high affinity binding for 17 β-hydroxysteroid hormones such as testosterone and estradiol. Zeginiadou *et al*., (1997) found that estradiol binds to the globulin and albumin in a nonlinear manner with an exponential increase of the bound hormone as the protein concentration increased. The same relationship was observed with dihydrotestosterone (DHT) and albumin (though not with globulin in this case (Zeginiadou *et al.*, 1997). Albumin has been well studied in mammalian and avian species, but comparatively little is known about the presence and characteristics of albumin in lower vertebrates (Gingerich and Pityer, 1989; Bushnell *et al*., 1998).

As an example of knowledge gaps in fish: there is still debate over the presence of albumin in teleost species. It has been identified as present in the blood

of salmonid species (Byrnes and Gannon, 1990, 1992; Maillou and Nimmo, 1993a and 1993b; Gong and Hew, 1998; Metcalf *et al.,* 2007). However, albumin is absent from the blood of other teleosts studied to date, for example New Zealand long-finned (*Anguilla dieffenbachii*) and short-finned eel (*Anguilla australis schmidtii*) (Metcalf *et al*., 1999a) and Antarctic tooth fish (Metcalf *et al*., 1999b), carp (*Cyprinus carpio*) (De Smet *et al*., 1998; 2001) and cartilaginous fish (Metcalf and Gemmell, 2005). It might be predicted that albumin would be absent in less derived species and yet it is present in non-parasitic brook lamprey (*Lampetra appendix*) blood, although it is an enlarged seven-domain rather than three-domain protein form (Gray and Doolittle, 1992; Danis *et al.,* 2000).

Almost nothing is known about blood nutritional levels in *L. bergylta* and their interactions with sex steroids. With many gaps still existing, it is necessary to develop a fundamental knowledge of endocrine and nutritional physiology with respect to maturation to better understand the annual reproductive cycle in *L. bergylta,* particularly regarding information concerning the interaction of nutrition and reproductive measures. Improved knowledge could lead to formulation of standard nutritional value for broodstocks in order to achieve high gamete and larval production. The primary aim of this study is to examine some of the important nutritional requirements during a reproductive cycle of wild *L. bergylta* and their correlation with steroid levels and gonadal maturation. Specifically, this was carried out with a monthly evaluation of circulating plasma sex steroids and blood nutritional parameters, an examination of histological development of gonads and gonado-somatic and hepato-somatic indices in a reproductive cycle.

2.2 Materials and Methods

2.2.1 Sampling location

The Carna Research Station is the aquaculture research facility of the Martin Ryan Institute, National University of Ireland Galway (N.U.I.G.) and is located near the village of Carna on the country's west coast (53° 19' 50" North, 9° 49' 40" West - Figure 2.1). It is situated at the head of *Iorras Aithneach* Peninsula in Connemara, County Galway, approximately 50 km west of Galway city. The sampling area for *L. bergylta* was approximately 1 km^2 between Mweenish Island and Finnish Island approximately 53° 18' 18" North and 9° 52' 14" West (approximate position of nets indicated in Figure 2.1).

Figure 2.1: Carna Research Station and sampling locations between Mweenish and Finnish Islands, Carna, Galway, Ireland.

2.2.2 General sea temperatures and tides

The climate on the west coast of Ireland is maritime, characterized by mild conditions year-round, small annual variations in seawater temperatures and maximum precipitation from late autumn to early spring. Tides in the Carna area are semi-diurnal, ranging from 4.8 m to 1.5 m for mean spring and neap tides respectively. At Carna Research Station, daily water temperatures were obtained at 3 hour intervals using wireless data loggers (Tinytag) attached to the rearing tanks in the flow-through aquaculture system. Over the study period, maximum seawater temperatures were recorded in summer (July) of both years and minima were

recorded in December. Monthly mean sea temperature values from Carna Research Station are shown in Figure 2.2.; 2010 was generally warmer than 2011 (Figure 2.2).

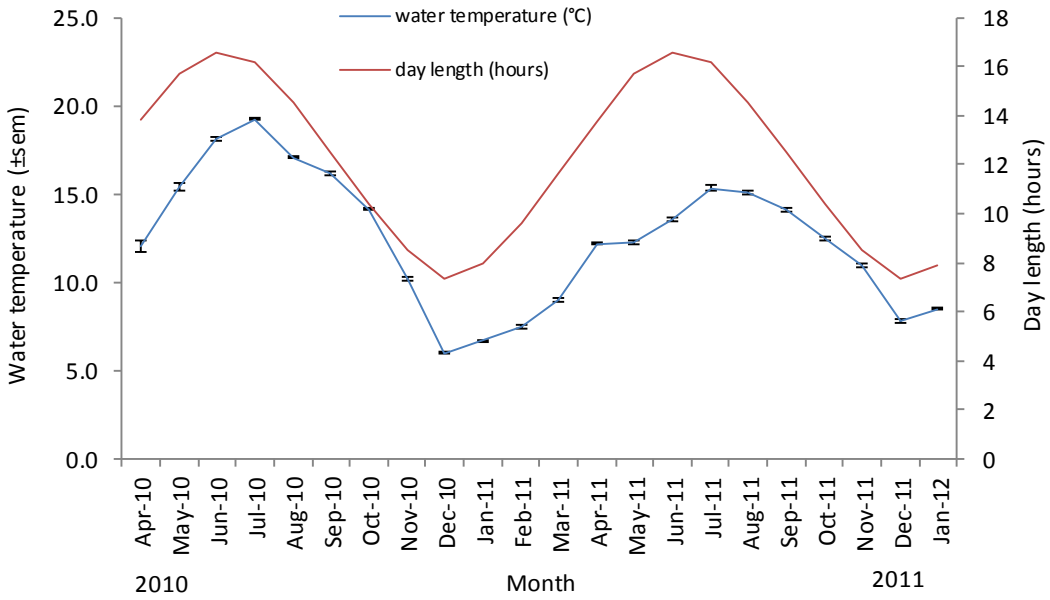


Figure 2.2: Sea water temperature (-) ± standard error of mean (SEM) and day length (-) from April 2010 to January 2012 at 53° 18' 18" North and 9° 52' 14" West.

2.2.3 Sampling strategy and procedure at sea

Adult *L. bergylta* were sampled monthly to give a maximum of 35 (summer season) and minimum of 8 (winter season) individuals each month from April 2010 to October 2011. Each sampling was performed over three days in the middle of each month (weather permitting). The fish were caught by trammel nets (mesh size 5 cm^2) and pots with crushed crab being used as bait. On each sampling date, a small boat was used to set the trammel nets in eight different areas between Mweenish and Finish Islands. All trammel nets were left for approximately seven daylight hours at 3 - 12 meters depth.

2.2.4 Physical data acquisition and biological sampling

All fish were subjected to sampling procedures according to the approval of Health Products Regulatory Authority in Ireland (HPRA) (see- "Scientific Animal Protection Legislation"). All fish were moved to rearing tanks on the trapping day and anaesthetised with MS222 (Tricaine Methanesulfonate) at 140 ppm (140mg/l) in sea water before physical data were taken. Fish were observed to lose responsiveness

within 2 to 5 minutes, after which time, body weight (g) and length measurements (cm) were recorded. Approximately 2 ml of blood was then sampled from individual fish using heparinised syringes through superficial venepuncture of the caudal vein. This method was used to avoid the blood from clotting as fresh plasma was needed for quantification of gonadal steroids and nutritional evaluations. Five to 10 fish were randomly euthanized from each sample via longer MS222 immersion until vital signs were lost. In these individuals, gonads, liver and both sagittal otoliths were extracted. Wet weight for gonads and livers were taken for the calculation of GSI and HSI respectively. The remaining live fish were all tagged using passive integrated transponder (PIT) tags to identify individual broodstock in the spawning trials. The fish were distributed according to selected sizes and maintained in 12 captive tanks with a continuous flow of sea water. They were fed a diet of mussel meats and chopped squid three times weekly. All the subsequent samples from these individuals of bloods, gonads and sagittal otoliths, were processed and labelled according to the PIT tag codes given to each sampled fish.

2.2.5 Samples processing and laboratory analyses

Fresh blood was immediately centrifuged to obtain plasma then stored at -80°C in advance of gonadal steroids and nutritional evaluations. Gonads were washed with freshwater to remove dirt and preserved in 75% ethanol prior to histological procedures. Extracted sagittal otoliths were washed with freshwater to remove soft tissue and stored dry in vials prior to mounting. All samples were transported to Martin Ryan Institute of National University of Ireland Galway for further processing and analysis. The methodology for processing these samples will be discussed further in the relevant thesis chapter.

2.2.6 Blood and tissue sampling

Overall, 169 wild caught mature *L. bergylta* from 400 g to 2000 g total body weight and approximately 11 fish were sampled each month between April 2010 and July 2011. The fish were netted and sedated by immersion in 6 litres of aerated salt water with a 100mg/L concentration of tricane methane sulfonate (MS222, Sigma) prior to blood sampling, to minimise tissue damage and handling stress. Fish were measured for length and weight using a standard fish sampling protocol as previously

described. Next, blood samples were collected to determine the levels of circulating steroids and nutrients in the blood. During blood sampling, 2 ml of blood was taken via puncture of the caudal vein (venepuncture) (Figure 2.3a and below) using a 5 ml heparinised (prepared solution from lithium heparin salt) sterile disposable syringe and needle (18GX1½). Heparin was used as an anticoagulant (5,000 IU) in which heparin sodium salt was prepared in 1ml buffered solution (Mohammadizadeh *et al*., 2012). In practice, after flushing, sufficient anticoagulant was retained within the needle to prevent the blood sample from clotting. The needle was inserted on the mid-ventral line behind the anal fin, by needle insertion under a scale then pushed through the musculature until the spinal vertebrae was reached. Puncture of the caudal vein could be physically felt through the needle and was confirmed by observing blood in the syringe. 2ml of blood was then collected from each fish. The wound commonly bleeds for several minutes after needle removal but blood loss was minimal.

Collected blood samples were transferred into 1.5 ml microtubes, then labelled appropriately before centrifugation (7691 relative centrifugal force (rcf) for 10 minutes) to separate blood cells and blue-green coloured plasma (Figure 2.3b). For assessment of sex steroid and biochemical indices of fish blood, analysis of blood plasma is preferred to that of blood serum (Hrubec and Smith 1999). Obtained plasma was separated equally into two 1.5 ml microtubes for separate steroid and nutrient analyses. Replicates of plasma were stored in a microtube box at -80°C. Plasma samples were evaluated for estradiol, testosterone, progesterone and cortisol via ELISA protocol (microtubes 1) meanwhile, nutrients for glucose, total protein, albumin and globulin was analysed via microplate reader and spectrometer (microtubes 2).

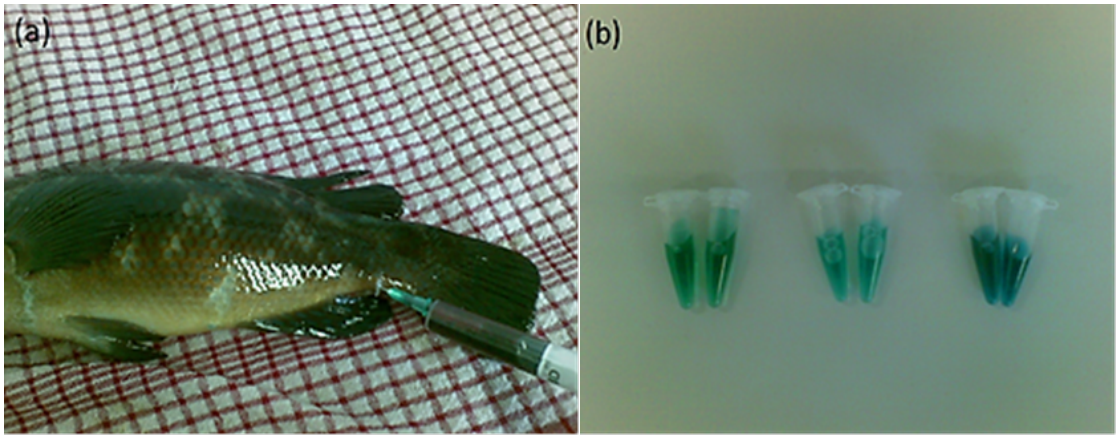


Figure 2.3(a-b): (a) Blood sampling through caudal vein and (b) Blue-green colouration of plasma.

Tissue samples such as gonads and liver were incised, removed and weighed (wet weight±0.01g) for gonadosomatic indices (GSI) and hepatosomatic indices (HSI) calculation. For this procedure, selected fish were humanely euthanized by administering an anaesthetic overdose of MS222 in solution. Gonads of all *L. bergylta* specimens lie in the posterior dorsal part of the body cavity and consist of a pair of elongate lobes separated anteriorly but united posteriorly before reaching the urogenital opening (Figure 2.4). The sex of an individual was determined by visual inspection of the gonads, which were subsequently removed, washed in phosphate buffered solution (PBS) and preserved in 75% ethanol. The same procedures were followed for liver tissue. The monthly gonadosomatic indices (GSI), hepatosomatic indices (HSI) and condition factor (K) were calculated according to the equations:

G.S.I = [Gonad Weight / Total Body Weight] x 100

H.S.I = [Liver Weight / Total Body Weight] x 100

K = [Body weight / (Body length)^3] x 100

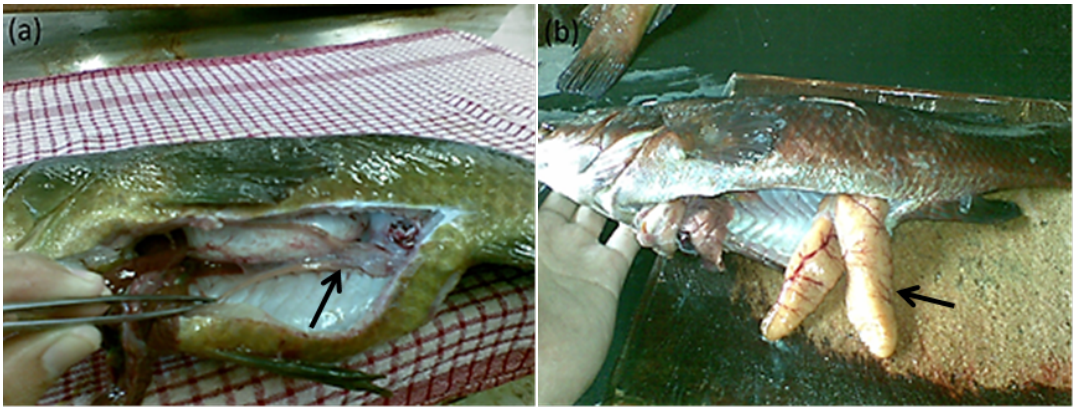


Figure 2.4(a-b): (a) Male gonads and (b) Female gonads.

2.2.7 Steroids analysis

Circulating plasma levels of sex steroids were measured by enzyme-linked immunosorbent assay (ELISA). Steroid titers were estimated in a 96 well test plate format using commercial 'EIA' kits, catalogue numbers #582251, #582701, #582601 and #500360 for estradiol, testosterone, progesterone and cortisol, respectively (Cayman Chemical Company 'www.caymanchem.com'). For each steroid, the kit included standards in the form of a solution in ethanol, antisera, acetylcholine esterase-labelled tracers and microplates precoated with monoclonal mouse anti-rabbit IgG**.** Standards were diluted to generate the standard curves consisting of nine concentrations ranging from 250 to 2pg/ml. These were run in duplicate on each 96 well test plate. Four blank and four maximum binding wells were run on each plate. Non-specific binding and total activity wells were run in triplicate. A dilution factor of 10 was used for all measured steroids via EIA protocol. Samples were run at two dilutions (a) 1:10 (40 µL plasma + 360 µL EIA buffer) and (b) 1:100 (40 µL of dilution (a) + 360 µL EIA buffer) with each dilution being run in triplicate. Serial dilutions of the standards and plasma samples produced parallel logit-log binding curves showing that the extracted plasma was suited to the assay condition for all steroids.

Inter assay variability was measured using aliquots of a pooled standard as follows: 7.5% (estradiol); 5.8% (testosterone); 10.8% (progesterone); 9.8% (cortisol). Two dilutions (1:10 and 1:100) of the pooled plasma extract were run in triplicate for each assay to provide a measure of inter-assay variation. The coefficient variation (CV) among replicates for each sample was generally less than 10%. All incubated plates were run using ELISA reader model LT-4000 (Labtech

International Ltd) at 2 wavelengths of 405 nm and 630 nm. All raw data generated from the ELISA reader were based on a standard concentration calculator supplied by the manufacturer [*www.caymanchem.com/analysis/eia*].

Assay detection limits in plasma were 0.02ng/ml for estradiol, 0.006ng/ml for testosterone, 0.01ng/ml for progesterone and 0.04ng/ml for cortisol. Values below the detection limit were treated as being equal to the detection limit. All steroid concentrations presented in the text and figures are expressed as the mean±s.e. nanogram/ml.

2.2.8 Nutritional analysis

L. bergylta blood plasma was assessed in terms of nutritional markers over a reproductive cycle. Plasma glucose, total protein, albumin and globulin were measured monthly in order to gain information on their relationship with changes in gonad and liver mass, as well as the circulating steroids. A total of 96 plasma samples collected between the months of April 2010 and July 2011 were removed from -70ºC storage and placed on ice and analysed as follows:

2.2.8.1 Glucose

Samples were defrosted in an ice bath before 50 µL of blood plasma sample was added to each test tube and labelled appropriately. Colour reagent was kept on ice and covered in tin foil (to maintain dark conditions) before 3 ml of the reagent was added to each sample. The samples were then placed in a preheated water bath at 37°C for 15 minutes. Then, the samples were placed on ice to stop further reaction. Samples absorbance was checked in a spectrophotometer at 505 nm.

A known amount of dry D-glucose (Sigma Aldrich, Wicklow, Ireland) was dissolved in distilled water in a 100 ml volumetric flask to create a glucose standard solution. The standard solution was then added in triplicate to labelled eppendorf tubes in volumes (µL) 1.0, 0.75, 0.5, 0.25 and 0.0, giving a total of fifteen labelled eppendorf tubes and the same procedure was carried out for fifteen test tubes. 750 µL of the prepared standard solution was added into the first three eppendorf tubes labelled 0.75 along with 250 µL of distilled water. 500 µL of prepared standard solution was added to the next three eppendorf tubes, labelled 0.5, along with 500 µL of distilled water; and so on up the concentration gradient. 50 µL from each

eppendorf tube was then pipetted into the corresponding test tube. 3 ml of the prepared colour reagent was then added to each test tube and each sample was placed in a water bath at 37°C for 15 minutes. The samples were then put into an ice bath before place it into a cuvette to stop the reaction. Samples were immediately tested for absorbance in a spectrophotometer (Model UVD-2950) against a reagent blank at 505 nm.

2.2.8.2 Total protein

Protein assays and analysis was adapted from Thermo Fisher Scientific protocols. Bradford reagent was produced by dissolving 100 mg Coomassie Brilliant Blue G-250 (Applichem, Darmstaot, Germany) in 50 ml of 95% concentration ethanol and 100 ml 85% (w/v) phosphoric acid, to form a dye. After the dye had dissolved completely, the solution was diluted to 1 litre volume before being filtered through Whitman #1 filter paper prior to use. To make a standard, 5.0 mg of bovine serum albumin (-BSA, Sigma Aldrich, Wicklow, Ireland) was added to 10 ml of distilled water in a test tube and dissolved by inverting several times. Serial dilutions were then made by adding 750 µL, 500 uL and 250 uL in triplicate into 9 tubes before diluting each sample to 1 ml volume with distilled water. Each sample was then vortexed for 3 seconds. A serial dilution of the total protein was then performed by adding 50 µL of plasma sample in a tube with 950 µL of distilled water. Then a 96-well test plate was labelled to indicate the various concentrations of stock solution. 4 µl of diluted stock solution was then added in triplicate to the appropriate test wells on the test plate. 200 µl of Bradford reagent was then added to each test well and kept dark for 10 minutes. After which time the resulting sample was then measured and analysed by using LT-4000 microplate reader at 595 nm absorbance.

2.2.8.3 Albumin

A dye solution was prepared by first adding 0.7 g of bromocresol green (BCG) dye in 30-40 ml of distilled water followed by 1 ml of 1N NaOH and mixing to dissolve the BCG. The resulting volume was then made up to 100 ml using distilled water in a conical flask. Brij-35 solution was dissolved by warming 12.5 g Brij-35 in 20 ml of distilled water before increasing the volume to 50 ml with distilled water. Buffered dye solution was then prepared by dissolving 5.9 g of succinic acid together with 1 g

of sodium hydroxide in 500 ml of distilled water. The pH of the resulting solution was then adjusted to 4.1-4.2 by adding 2.5 ml of Brij-35 solution, 100 mg of sodium azide and 8 ml of the dye solution and mixing before checking the pH. The resulting solution was made up to 1 litre and stored in minus temperature.

Samples were removed from minus storage (-70°C) and placed on ice to defrost. Samples were tested in triplicate. 20 µL of the test serum was added to 4 ml of buffered dye reagent before adding albumin reactant solution to each sample. Each test tube was vortexed for three seconds before being kept dark at 25°C for 10 minutes. The solution was then measured in a spectrophotometer (Thermo, Genesys 100v) at 630 nm absorbance.

To prepare an albumin standard, 0.0020 g of human serum albumin was added into a test tube. 10 ml of distilled water was then added and inverted several times to mix it. 750 µL of this solution was then added into three labelled tubes, 500 µL of solution was added into a further three tubes and 250 µL of solution was added into the final three test tubes. Distilled water was added to each tube to make a 1 ml volume solution. Samples were then vortexed for three seconds and placed on ice and allowed to stand for 10 minutes. The absorbance was then tested against the reagent blank using a spectrophotometer at 630 nm absorbance.

2.2.8.4 Globulin & albumin-globulin ratio

Calculation of the globulin levels in plasma samples was carried out by default -the albumin concentration was subtracted from the total protein concentration to give the globulin concentration. To calculate the A:G ratio, the albumin concentration was by dividing the albumin by the globulin concentration. The mean, standard deviation and standard error were also calculated for each monthly sample lot.

2.2.9 Histology of gonads

Histological procedures were employed to determine the sex and stage of gonadal maturity for at least 10 individuals of the total number of fish sampled each month. The preserved gonads in the 75% ethanol were incised into pieces (1 cm^3 cross section) then fixed in either alcohol-acetic-formalin (AAF) or 'Carnoy's solution' (Chloroform-acetic-alcohol) for 4 hours and stored in 70% alcohol. Prepared samples were then embedded in paraffin wax and sectioned at 5 to 7 µm using a

Leica-Jung Biocut 2035. A glass slide was then coated with chrome gelatine to ensure section attachment and was stained with Heidenhain's AZAN (The histological protocol for staining procedure with AZAN can be found in Appendix 1). Cross sections of the ovaries were then examined for egg development using a stereomicroscope fitted with a digital camera Olympus (Olympus, Tokyo, Japan). Cell A software (Olympus, Tokyo, Japan) was used to capture images and make digital measurements. The diameters of between 15 to 25 oocytes were randomly measured to the nearest 0.01 μm taken from the mid-cross section of the ovary from each female gonad with mean values being calculated. Classification of gonad maturity was labelled from Stage 1 to Stage 6 based on microscopical and histological examination of the ovaries. Increases in egg diameter indicated advancement in egg maturation as follows:- female *L. bergylta* were categorised as being either at the previtellogenic stage (oocyte diameter <100 μm), vitellogenic stage (oocyte 350-500 μm) or post-vitellogenic stage (oocyte >500 μm) (Muncaster *et al*., 2010). All mean egg diameters collected from a given month were then combined to give a monthly average egg diameter. For males, no ovarian cells were observed and testis had germ cells undergoing active spermatogenesis.

2.2.10 Data analysis

All analyses were performed using SPSS Statistics version 21.0. Analysis of variance (ANOVA) tested the effects of month on the concentrations of estradiol, testosterone, progesterone and cortisol; measurements of body weight and length were used as covariates. When a factor was found to be significant, least-square means were extracted from the analyses and Duncan's New Multiple Range tests for *pos hoc* testing ($p<0.05$) were performed to compare whether there were significant differences between the levels of the factor. ANOVA tests have been presented because of the utility of means comparison tests, the ability of the tests to handle a decrease in sample numbers, especially in winter months, and the common use of these tests elsewhere. The statistical model for anova analysis was:

$y_{ijk} = \mu + m_i + b_1X_1 + b_2X_2 + \varepsilon_{ijk}$

where y_{ijk} is the observation, μ is the overall mean, b_1X_1 is the regression of the observation on body weight, b_2X_2 is the regression of the observation on body length and ε_{ijk} is the random error term.

In the effect of month, the null hypothesis (Ho) stated that the different months have the same effect on estradiol, testosterone, progesterone, cortisol, oocyte diameter, gonad weight, GSI, hepatic weight, HSI and nutrients levels whereas the alternative hypothesis (H_A) stated that the different months do not have the same effect on the parameters stated. The relationship between the steroids hormones and all the respective variables were analysed by using Pearson's correlation analysis. The levels of significance selected was $p<0.05$. Where necessary, data were log-transformed to satisfy homogeneity of variance requirements, especially for plasma steroid and nutrient concentrations. In some instances, variances were still heterogeneous, however, the data were also assessed by repeated measures ANOVA and, in most cases, the outcomes were unchanged. For all statistical tests, values were expressed as mean±s.e. (standard error of the mean) and considered significantly different at $p<0.05$.

2.3 Results

2.3.1 Fish and body condition

A total of 169 mature fish were caught between April 2010 and July 2011 for monthly steroid evaluation. Within this 16-month period, various sizes of fish weighing from 404.00 g to 1801.00 g and measuring 29.00 cm to 45.50 cm in length were captured. Mean body weight of the sampled fish was 634.39±12.56g while mean body length was 33.37±0.18cm. Figure 2.5 shows the scatter plot of body length-weight of the sampled fish. The quadratic model equation is $y = 21.22 + 0.02x - 5.33E^{-6}x^2$ was fitted to the plot had a coefficient of determination (R^2) of 0.902, F = 761.76 and $p<0.01$.

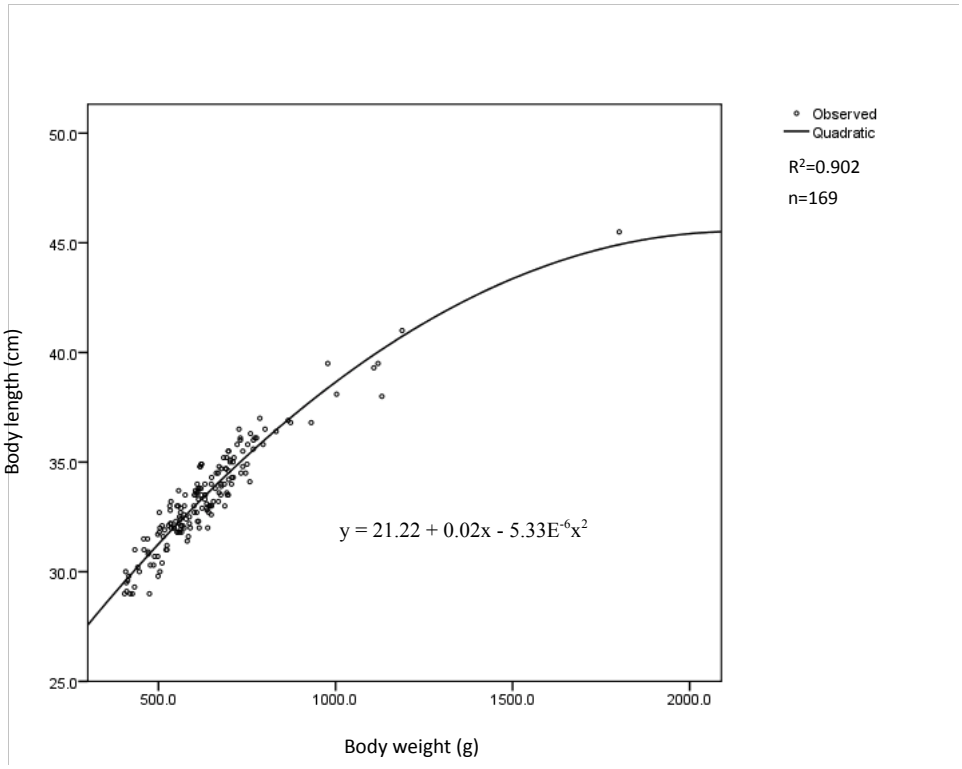


Figure 2.5: Scatter plot of body length versus body weight for wild sampled *L. bergylta* at Carna from April 2010 and July 2011 (n=169).

Figure 2.6 shows monthly changes in body condition factor K of wild *L. bergylta*. Condition factors were highest from Summer, through the Autumn and early Winter months (July-November). From December onwards condition declined, and the greatest loss of condition was recorded after the spawning period which occurs in April / May (K was lowest in April 2010 or June 2011).

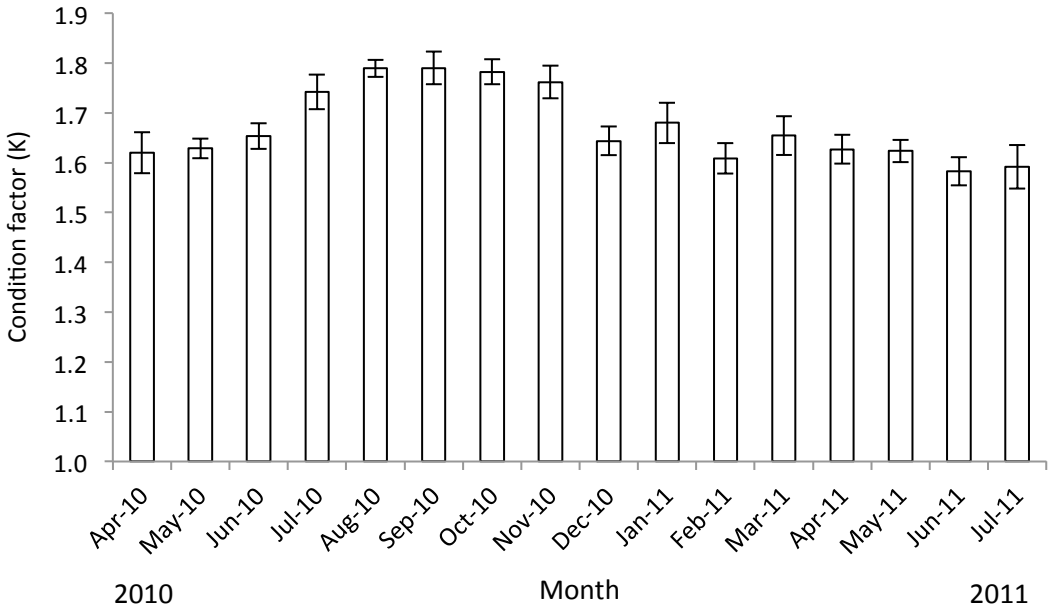


Figure 2.6: Mean condition factor (K) for monthly captured *L. bergylta* from April 2010 to July 2011 (n=169).

2.3.2 Effects of month and body size (body weight and body length) on estradiol, testosterone, progesterone and cortisol concentrations in a reproductive cycle

Table 2.1 shows the mean squares from ANOVA for the effect of month on estradiol, testosterone, progesterone and cortisol concentrations with body weight and body length used as covariates. Fish displayed significant monthly changes in plasma concentrations of sex steroids. The effect of month on estradiol and testosterone concentrations was highly significant ($p<0.01$) and on progesterone and cortisol concentrations was significant ($p<0.05$). However, the covariates body weight and body length had no significant effect on concentration of the steroids ($p>0.05$).

Table 2.1: Mean squares from ANOVA for the effect of month on estradiol, testosterone, progesterone and cortisol concentrations (ng/ml) with body weight and body length used as covariates (n=169).

		Mean squares			
Source of variation	d.f	Estradiol	Testosterone	Progesterone	Cortisol
Month	15	4476.12**	449.81**	574286.03*	3174448.77*
Body weight (g)	1	$202.46^{n.s}$	$35.95^{n.s}$	$69197.86^{n.s}$	$115217.53^{n.s}$
Body length (cm)	1	$407.03^{n.s}$	$1.64^{n.s}$	$251827.4^{n.s}$	$651995.88^{n.s}$
Error	151	1780.78	195.20	296428.13	1785388.51
Total (corrected)	168				

**($p<0.01$) highly significant
*($0.01<p<0.05$) significant
$^{(n.s)}$ not significant

Table 2.2 shows the monthly means between April 2010 and July 2011 for concentrations of sex steroids in *L. bergylta*. Estradiol concentrations were significantly higher ($p<0.05$) in April 2010 compared with the other months -apart from May of that year. Also, April and May of 2011 were significantly higher than most of the months. The corresponding values were 2.03±0.52ng/ml (in April 2010) and 1.17±0.20ng/ml (in May 2010); and 2.20±0.90ng/ml (in April 2011) and 2.00±0.68ng/ml (in May 2011). The lowest levels recorded were 0.35±0.09 and 0.44±0.06ng/ml in July of both years. The annual profile of testosterone concentrations followed a similar trend to estradiol although most values were not significantly different between months. The similarity with estradiol was in the fact that May (2010) or April/May (2011) maxima were significantly higher than July minima in both years; however most of the other months did not differ. The mean values for testosterone maxima in April and May were 0.67±0.10 and 0.95±0.13ng/ml, respectively in 2010 so that this steroid was roughly twice as high in May 2010 than the other months. In 2011, the monthly testosterone profile was a bit flatter so that the highest values were in March, April and May 2011 at 1.00±0.20, 1.01±0.14 and 1.03±0.20ng/ml, respectively, and these only differed from very low values in July but were otherwise not statistically significant. Like estradiol, the plasma testosterone concentration was significantly reduced in post spawning period in July of both years relative to peak spawning months. Overall, maximal estradiol values were approximately 4 fold greater than testosterone.

Progesterone concentration fluctuated regularly during the year. At the beginning of spring 2010, progesterone concentrations were high in April

(20.74±5.16ng/ml) and May (19.71±8.57ng/ml). The concentrations then dropped in June (9.88±4.65ng/ml) and July (7.92±2.69ng/ml). Following that, progesterone concentrations increased from August to November 2010 with values of 16.32±6.53, 15.86±2.88, 17.26±4.62 and 12.03±6.59ng/ml, respectively. The level decreased to a very low level in December 2010 (3.93±6.09ng/ml) and rose again in January 2011 (18.28±6.09ng/ml). Progesterone levels in 2011 followed pretty much the pattern shown in 2010 and there were very few months showing significant levels of this steroid apart from 2011 when there was a significant April peak relative to a lowpoints in February, March, June and July.

For cortisol, it shows high degree of variation and there were no significant differences in concentration between any of months across both of the years. Cortisol levels peaked in February 2011 at 92.22±14.09ng/ml. The cortisol concentrations were lowest in June 2010 (9.38±1.64ng/ml) and 2011 (20.15±4.67ng/ml).

To illustrate the monthly steroid changes in a yearly cycle, graphs have been plotted - Figure 2.7 (a) shows all four steroid concentrations in nanogram/ml and Figure 2.7 (b) shows estradiol and testosterone after being magnified as both of them have lower range of concentration compared with cortisol and progesterone. Estradiol and testosterone concentrations in *L. bergylta* showed a pronounced, repetitive seasonal cycling over the 16-month sampling period; estradiol sustained levels of 0.35±0.09 to 0.50±0.10ng/ml across most months of the study but these were exceeded by two annual surges in April/May of each year. Testosterone was similar, but surged less, with the maxima being more subtle and much lower in magnitude than estradiol. The highly fluctuating progesterone and cortisol concentrations are apparent in Figure 2.7 (a). In fact, the progesterone pattern shows weak maxima in April/May and minima in June/July. But there are other surges too in progesterone, in late Summer, Autumn and in January. Cortisol shows its highest level in the month of February 2011 prior to spawning, but apart from this and a minimum in June of both years, the cortisol is highly variable.

Table 2.2: Means and standard errors for the effect of month on estradiol, testosterone, progesterone and cortisol concentrations (ng/ml). Results of post-hoc testing are indicated by superscripts [a, b, c, d]. Means within a column with different superscripts are significantly different at $p<0.05$ (n=169).

Year	Month	Estradiol	Testosterone	Progesterone	Cortisol
2010	April	2.03±0.52 [b]	0.67±0.10 [abcd]	20.74±5.16 [ab]	43.77±17.46 [a]
	May	1.17±0.20 [ab]	0.95±0.13 [bcd]	19.71±8.57 [ab]	39.28±10.44 [a]
	June	0.50±0.10 [a]	0.53±0.08 [ab]	9.88±4.65 [a]	9.38±1.64 [a]
	July	0.35±0.09 [a]	0.45±0.05 [a]	7.92±2.69 [a]	41.23±7.05 [a]
	August	0.38±0.04 [a]	0.54±0.06 [abc]	16.32±6.53 [ab]	37.28±7.27 [a]
	September	0.43±0.03 [a]	0.62±0.04 [abcd]	15.86±2.88 [ab]	35.40±5.93 [a]
	October	0.48±0.06 [a]	0.66±0.05 [abcd]	17.26±4.62 [ab]	49.13±13.06 [a]
	November	0.46±0.02 [a]	0.52±0.10 [ab]	12.03±6.59 [ab]	42.89±11.85 [a]
	December	0.41±0.03 [a]	0.54±0.11 [abc]	3.75±0.86 [a]	25.41±16.70 [a]
2011	January	0.68±0.12 [a]	0.67±0.12 [abcd]	18.03±6.91 [ab]	50.13±12.22 [a]
	February	0.87±0.22 [ab]	0.70±0.09 [abcd]	6.25±1.18 [a]	91.28±20.66 [b]
	March	1.45±0.52 [ab]	1.00±0.20 [cd]	2.67±0.74 [a]	53.10±11.99 [a]
	April	2.20±0.90 [b]	1.01±0.14 [d]	28.70±5.44 [b]	45.05±10.92 [a]
	May	2.00±0.68 [b]	1.03±0.20 [d]	15.84±6.31 [ab]	44.50±20.63 [a]
	June	0.49±0.08 [a]	0.64±0.33 [abcd]	4.09±0.95 [a]	20.15±4.67 [a]
	July	0.44±0.06 [a]	0.50±0.07 [ab]	3.76±1.03 [a]	25.22±10.34 [a]

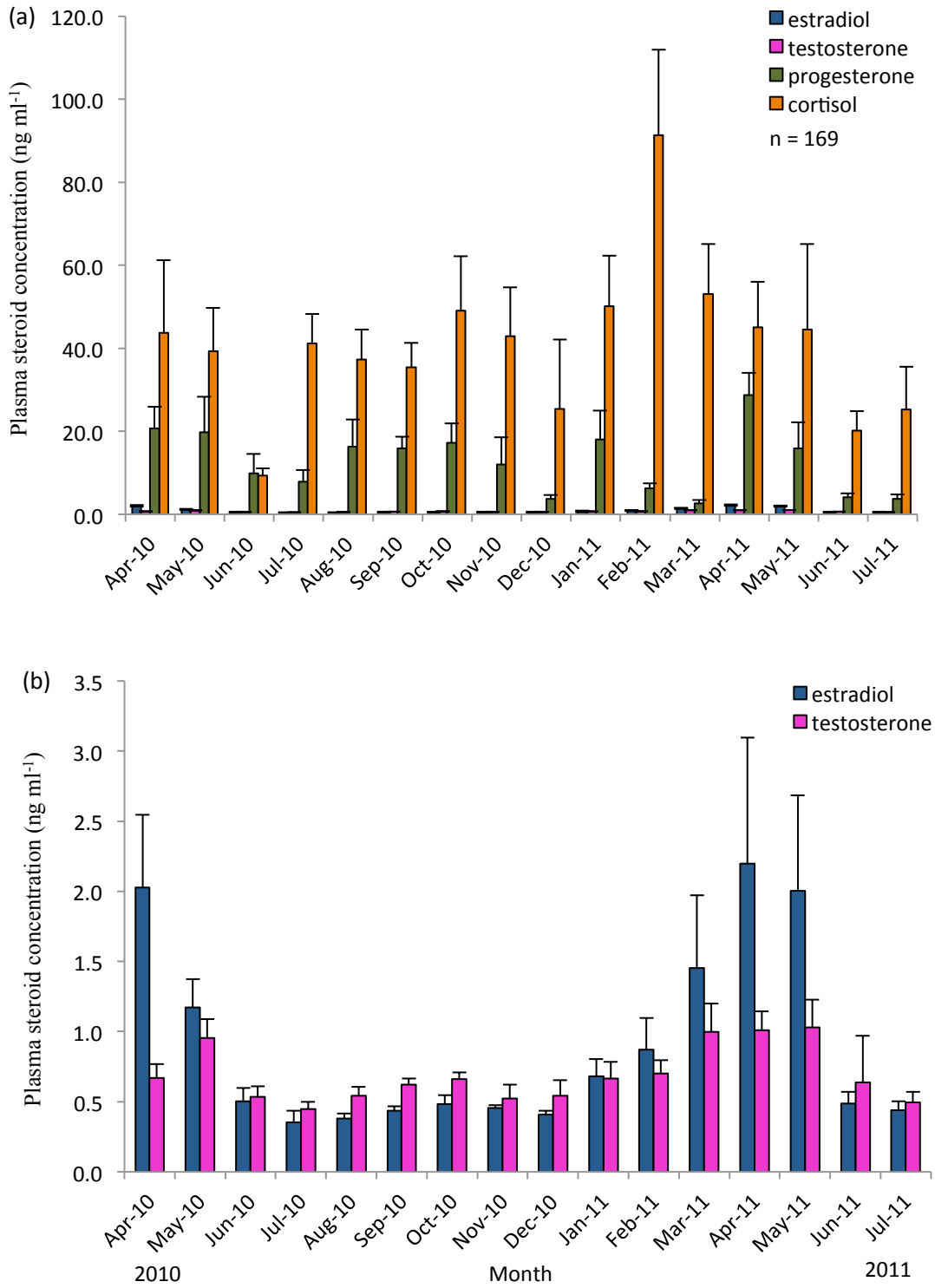


Figure 2.7(a-b): (a) Plasma steroid hormone levels in *L. bergylta* (mean±sem) between April 2010 and July 2011, shown to illustrate their relative concentration; (b) magnification of estradiol and testosterone levels from (a). See Table 2.2 for the respective statistical analysis.

The temperature of the shallow waters of Carna Bay near the study site showed annual variations associated with day length and season (Figure 2.2). The monthly mean of all daily mean water temperature lagged behind seasonal trends in day length, which reaches a maximum of 16.5 hours in June and a minimum of 7.4 hours in December. The mean of daily summer water temperatures of 19.2°C and 15.4°C were recorded in July of 2010 and 2011, respectively. After the summer peak, water temperature gradually dropped to December minima of 6.0°C (2010). Figure 2.8(a-b) shows mean monthly values of water temperature in relation to plasma steroids near the study site. Estradiol and testosterone gradually increased as water temperature increased from December 2010 to April 2011 (Figure 2.8(a)). Minimum concentrations of estradiol and testosterone were recorded as water temperatures decreased from July to December 2010 (Figure 2.8(a)). Figure 2.8(b) shows the progesterone and cortisol concentrations versus the mean of daily water temperature. Plasma levels of cortisol did not vary according to temperature; cortisol concentrations were lowest in June 2010 (9.38±1.64ng/ml), one month before water temperature reached a peak in July 2010 (19.2±0.1°C) and in 2011, cortisol dropped sharply from March-June, as water temperatures were rising. By contrast with this somewhat negative correlation between these parameters, cortisol concentrations dropped in December 2010 in a period when the water temperature was also at its lowest level. Plasma progesterone concentrations show variations throughout months, but these were fairly 'flat' without pronounced peaks. A peak, such as it was, took place in April 2011 as water temperature rose prior to the spawning period.

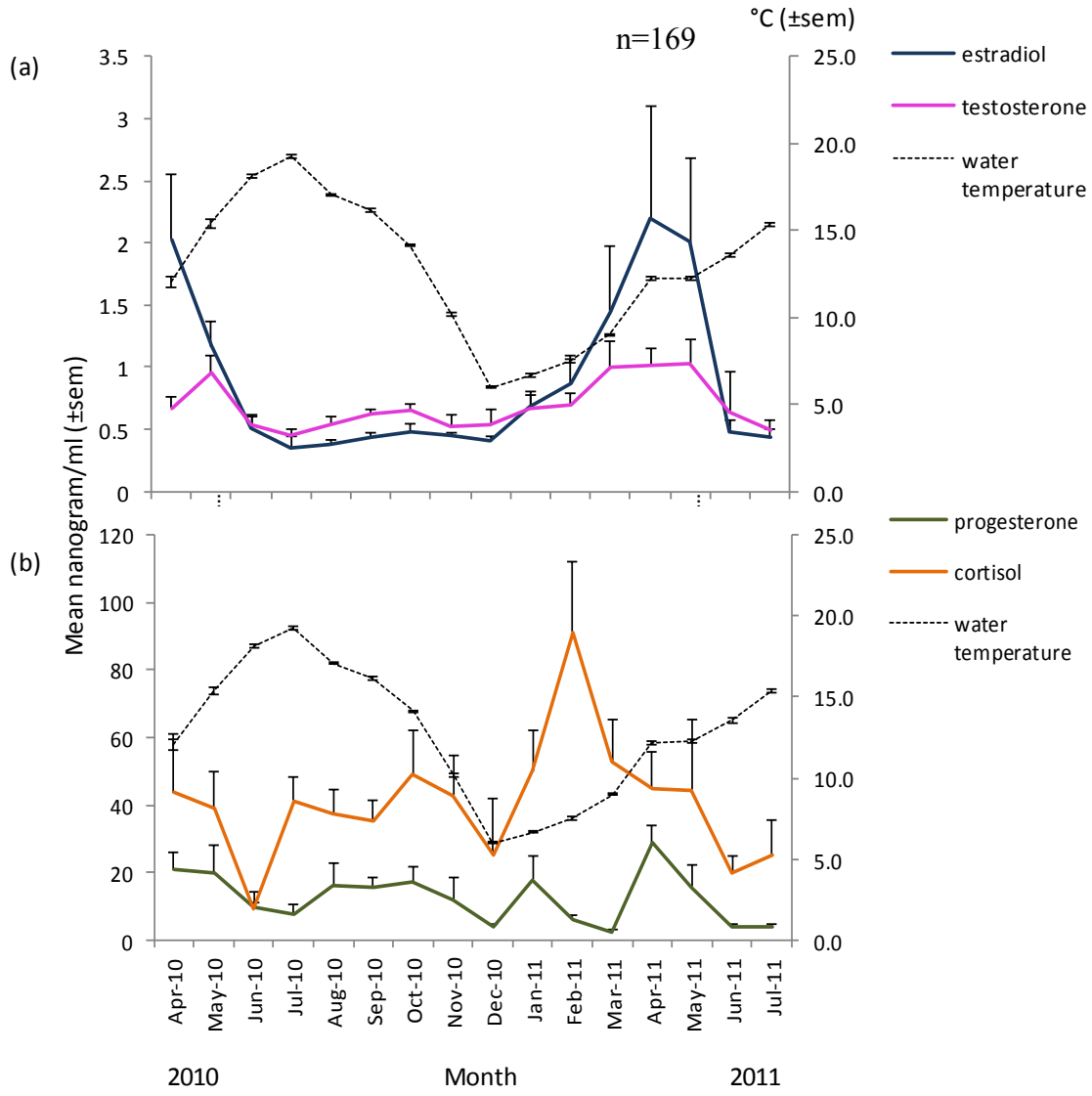


Figure 2.8(a-b): Relationship between mean of daily water temperature and average values per month of (a) estradiol and testosterone (b) progesterone and cortisol levels between April 2010 and July 2011.

2.3.3 Effects of month and body weight on concentrations (mg/dL) of glucose, total protein, albumin, globulin and ratio of albumin-globulin in a reproductive cycle

Adult *L. bergylta* of various body weights were examined for blood nutrients in a reproductive cycle: a total of 96 adult fish were sampled monthly between April 2010 and July 2011. Mean body weight of the sampled fish throughout the period was 651.17±210.14g. Table 2.3 shows the mean squares from analyses of variance for the effect of month on concentrations of glucose, total protein, albumin, globulin and albumin-globulin ratio (A:G) with body weight used as a covariate. The covariate body weight had no significant effect on concentrations of total protein, albumin and globulin ($p>0.05$) but showed highly significant effect on glucose concentrations ($F=21.14$; $p<0.01$).

Table 2.3: Mean squares from analyses of variance for the effect of month with body weight (g) as a covariate on glucose, total protein, albumin and globulin concentrations (mg/dL) (n=96).

		Mean squares				
Source of variation	d.f	glucose	total protein	albumin	globulin	A:G
Month	15	2231.60**	9.25**	0.139**	8.88**	8.15**
Body weight (g)	1	3677.12**	$0.007^{n.s}$	$0.001^{n.s}$	$0.00^{n.s}$	$0.027^{n.s}$
Error	79	577.54	0.15	0.015	0.153	0.150
Total (corrected)	95					

**($p<0.01$) highly significant
$^{(n.s)}$ not significant

Fish displayed highly significant monthly changes in plasma concentrations of all the respective plasma nutrients ($p<0.01$) (Table 2.3). Table 2.4 shows means concentrations of plasma glucose, total protein, albumin, globulin (all in mg/dL) and A:G ratio over the 16 months period. From April 2010 to September 2010, glucose concentrations show a range of 17 to 40mg/dL. Plasma glucose increased from September 2010 to its peak which was in the winter season in December 2010 (40.55±7.58 to 81.98±16.48mg/dL, respectively). Indeed, glucose concentrations were significantly higher in November, December and January than many of the other months. By February and March 2011 (28.59±5.70 and 24.51±7.76mg/dL, respectively) concetrations were decreased again. A brief increase ocurred in April 2011 at 42.56±14.55mg/dL before decreasing again in May and June 2011

(25.41±4.32 and 29.48±9.56mg/dL, respectively). The fluctuation of glucose concentrations continued with another increase in July 2011 (45.06±11.25mg/dL).

Total protein concentrations were significantly increased over a prolonged period from April and July 2010 and February 2011 relative to the other months ($p<0.05$). Fractioning the total protein into its two components (albumin + globulin) provided extra information as trends in albumin concentrations were distinct from total protein. Albumin peaked briefly in July of both years ($p<0.05$) with 0.98±0.08mg/dL in July 2010 and 1.40±0.13mg/dL in July 2011. Meanwhile, globulin concentrations were generally much higher than albumin concentrations in *L. bergylta*, demonstrating that these were the main constituents of the total protein concentrations in this species. Globulin trends were, unsurprisingly, very similar to total protein; with elevated concentrations in April to July 2010 and February 2011 ($p<0.05$). The monthly ratio of A:G followed the monthly globulin concentrations.

Table 2.4: Means and their standard errors for the effect of month on glucose, total protein, albumin and globulin concentrations (mg/dL). Results of post-hoc testing are indicated by superscripts [a, b, c, d, e]. Means within a column with different superscripts are significantly different at $p<0.05$ (n=96).

Year	Month	glucose	total protein	albumin	globulin	A:G
2010	April	17.32±1.78[a]	3.64±0.12[c]	0.81±0.03[a]	2.83±0.14[b]	2.02±0.16[b]
	May	45.31±10.13[abc]	5.19±0.13[d]	0.82±0.02[a]	4.37±0.11[c]	3.55±0.10[c]
	June	27.27±8.32[ab]	5.55±0.16[d]	0.83±0.02[a]	4.72±0.15[c]	3.89±0.15[cd]
	July	30.95±7.78[a]	6.16±0.26[e]	0.98±0.08[b]	5.17±0.25[d]	4.19±0.26[d]
	August	32.52±3.04[a]	2.69±0.11[ab]	0.78±0.01[a]	1.91±0.11[a]	1.14±0.11[a]
	September	40.55±7.58[abc]	2.98±0.09[b]	0.86±0.01[ab]	2.12±0.09[a]	1.25±0.09[a]
	October	61.28±12.52[bcd]	2.61±0.18[ab]	0.80±0.04[a]	1.81±0.18[a]	1.01±0.18[a]
	November	67.41±17.48[cd]	2.40±0.24[a]	0.77±0.05[a]	1.63±0.23[a]	0.86±0.23[a]
	December	81.98±16.48[d]	2.86±0.10[ab]	0.78±0.03[a]	2.07±0.10[a]	1.29±0.11[a]
2011	January	72.46±11.25[cd]	2.60±0.16[ab]	0.82±0.03[a]	1.77±0.14[a]	0.95±0.13[a]
	February	28.59±5.70[ab]	3.81±0.13[c]	0.81±0.03[a]	3.00±0.11[b]	2.19±0.10[b]
	March	24.51±7.76[a]	2.70±0.16[ab]	0.88±0.05[ab]	1.83±0.16[a]	1.03±0.12[a]
	April	42.56±14.55[abc]	2.49±0.14[ab]	0.82±0.05[a]	1.68±0.19[a]	0.92±0.20[a]
	May	25.41±4.32[a]	2.55±0.13[ab]	0.81±0.02[a]	1.74±0.13[a]	1.00±0.16[a]
	June	29.48±9.56[ab]	2.68±0.10[ab]	0.79±0.01[a]	1.89±0.10[a]	1.10±0.09[a]
	July	45.06±11.25[abc]	2.82±0.09[ab]	1.40±0.13[c]	1.84±0.16[a]	1.28±0.10[a]

2.3.4 Effects of month and body weight on oocyte diameter, gonad weight, gonadosomatic index (GSI), liver weight and hepatosomatic index (HSI) in a reproductive cycle

The maturity of ovary and hepatic mass was evaluated by gross measurements, histological parameters including oocyte diameter and indices. *L. bergylta* exhibits seasonal growth of the gonads in two phases, breeding and non breeding phases. The seasonal effect of month was highly significant on all of the parameters ($p<0.01$) meanwhile body weight showing a highly significant effect on liver weight and hepatosomatic index (HSI, $p<0.01$) (Table 2.5).

Table 2.5: Mean squares from analyses of variance for the effect of month with body weight (g) as a covariate on oocyte diameter (µm), gonad weight (g), gonadosomatic index (%) (GSI), hepatic weight (g) and hepatosomatic index (%) (HSI) (n=96).

		Mean squares				
Source of variation	d.f	oocyte diameter	gonad weight	GSI	liver weight	HSI
Month	15	158437.78**	325.74**	8.66**	15.72**	0.25**
Body weight (g)	1	3245.07 $^{n.s}$	97.14 $^{n.s}$	0.27 $^{n.s}$	83.36**	1.59**
Error	79	9676.95	33.81	0.911	5.09	0.11
Total (corrected)	95					

**($p<0.01$) highly significant
*($0.01<p<0.05$) significant
$^{(n.s)}$ not significant

Table 2.6 shows the means and standard errors of oocyte diameter, gonad weight, GSI, liver weight and HSI from April 2010 to July 2011. The oocyte diameter, gonad weight and GSI of *L. bergylta* reached significantly higher levels than most other months (apart from March and April 2011) during April of 2010 (524.08±17.46µm, 21.84±3.97g, 3.69±0.60, for the three parameters respectively). The same pattern ocurred in 2011 (548.57±23.31µm, 24.44±5.47g, 4.00±0.93, for ooctye diameter, gonad weight, and GSI, respectively). Oocyte diameter, gonad weight and GSI were also very high in March 2011. These parameters were greatly reduced for the rest of the months, however. Another point of note is that oocyte diameter began to increase slowly from September of the year before spawning the following Spring. For example ooctye diameter minima were 104.48±2.66µm in August 2010 but in September these increased to 113.44±3.34µm, by November these had reached

226.09±16.72µm. These values continued to rise through January – April of 2011 prior to spawning. Gonad weight and GSI fluctuated a bit more and only definitively started increasing from November 2010. The lowest gonad weight and GSI was recorded in September (1.42±0.13g and 0.30±0.03, respectively), while the minimum oocyte diameter was a month earlier in August (104.48±2.66µm).

HSI increased gradually from April 2010 and reached its maximum of 1.59±0.14 in September, thereafter decreasing gradually in October – December until reaching very low levels by February and March 2011 (0.77±0.13 and 0.98±0.13, respectively; Table 2.6). Moderate levels of HSI were seen in the period when spawning approached; HSI was 1.00±0.24 in January 2011 and 1.03±0.12 ($p>0.05$) in April 2011 and then levels gradually increased again to high levels by July 2011. There were few significant differences between months in HSI as all changes were so gradual. Liver weight was significantly higher mid Summer and mid Winter than at other times: maxima were reached in this parameter in July 2010 and 2011, but also in December 2010. Lowest liver weights were in May of each year (after spawning).

Figure 2.9 allows trends in HSI, GSI and oocyte diameter to be compared in a reproductive cycle and illustrates largely opposite trends between the HSI on the one hand and the GSI, on the other.

Table 2.6: Means and their standard errors for the effect of month on oocyte diameter (µm), gonad weight (g), gonadosomatic index (GSI), hepatic weight (g) and hepatosomatic index (HSI). [a, b, c, d, e] Means within a column with different superscripts are significantly different at $p<0.05$ (n=96).

Year	Month	oocyte diameter	gonad weight	GSI	liver weight	HSI
2010	April	524.08±17.46^{d}	21.84±3.97^{bc}	3.69±0.60^{c}	6.58±0.83^{ab}	1.14±0.12^{abcd}
	May	259.31±79.95^{bc}	4.48±1.71^{a}	1.09±0.49^{a}	5.25±0.91^{a}	1.19±0.16^{abcd}
	June	134.04±9.54^{a}	2.42±0.42^{a}	0.48±0.05^{a}	5.90±0.71^{ab}	1.21±0.12^{abcd}
	July	110.50±1.32^{a}	2.04±0.41^{a}	0.32±0.07^{a}	10.00±1.33^{cde}	1.50±0.17^{cd}
	August	104.48±2.66^{a}	2.17±0.21^{a}	0.36±0.03^{a}	8.96±0.94^{bcde}	1.46±0.12^{bcd}
	September	113.44±3.34^{a}	1.42±0.13^{a}	0.30±0.03^{a}	7.44±0.79^{abcd}	1.59±0.14^{d}
	October	125.43±4.76^{a}	2.71±0.55^{a}	0.44±0.05^{a}	8.91±1.66^{bcde}	1.20±0.22^{abcd}
	November	226.09±16.72^{ab}	2.31±0.25^{a}	0.33±0.03^{a}	8.54±0.35^{abcde}	1.25±0.12^{abcd}
	December	219.00±2.46^{ab}	4.62±0.31^{a}	0.57±0.05^{a}	10.44±0.26^{de}	1.29±0.09^{bcd}
2011	January	300.87±15.79^{bc}	9.04±2.66^{a}	1.02±0.26^{a}	8.12±1.01^{abcd}	1.00±0.24^{ab}
	February	358.59±14.34^{c}	9.39±2.06^{a}	0.94±0.20^{a}	7.45±0.79^{abcd}	0.77±0.13^{a}
	March	500.04±13.07^{d}	16.30±3.05^{b}	2.34±0.48^{b}	6.69±0.51^{abc}	0.98±0.13^{ab}
	April	548.57±23.31^{d}	24.44±5.47^{c}	4.00±0.93^{c}	6.29±0.67^{ab}	1.03±0.12^{abc}
	May	323.54±110.97^{bc}	5.75±3.75^{a}	0.89±0.47^{a}	5.86±0.73^{ab}	1.13±0.15^{abcd}
	June	103.25±6.51^{a}	3.34±0.18^{a}	0.54±0.05^{a}	8.46±0.57^{abcde}	1.36±0.11^{bcd}
	July	109.34±4.62^{a}	2.63±0.39^{a}	0.36±0.04^{a}	11.46±1.98^{e}	1.52±0.06^{d}

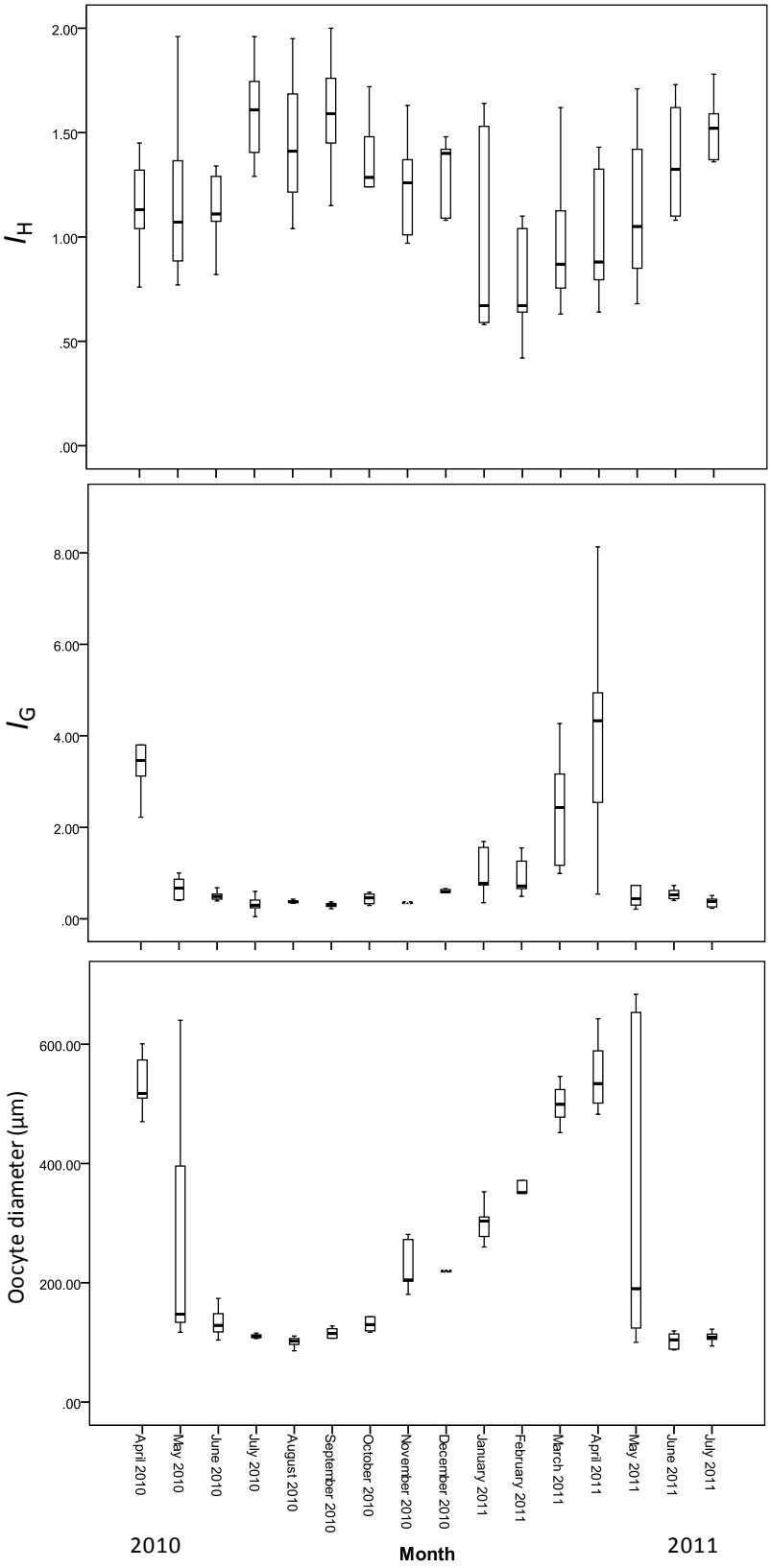


Figure 2.9: Boxplots showed development of hepatosomatic index (I_H), gonadosomatic index (I_G) and oocyte diameter between April 2010 and July 2011 (n=96). See Table 2.6 for the respective statistical analysis.

2.3.5 Summary of all parameters

Plasma estradiol and testosterone concentrations were positively related to oocyte diameters (Figure 2.10 and Figure 2.11 $p<0.01$) which peaked in April in both years, but no association was observed with progesterone and cortisol in which a general fluctuating trend was apparent across the months. Figure 2.10 shows that GSI levels closely followed seasonal cycles of estradiol and testosterone. However, glucose concentrations and HSI and levels did not approximate the patterns in estradiol and testosterone and showed rather the opposite cycle to these steroids. Both HSI and glucose showed a complementary / similar seasonality.

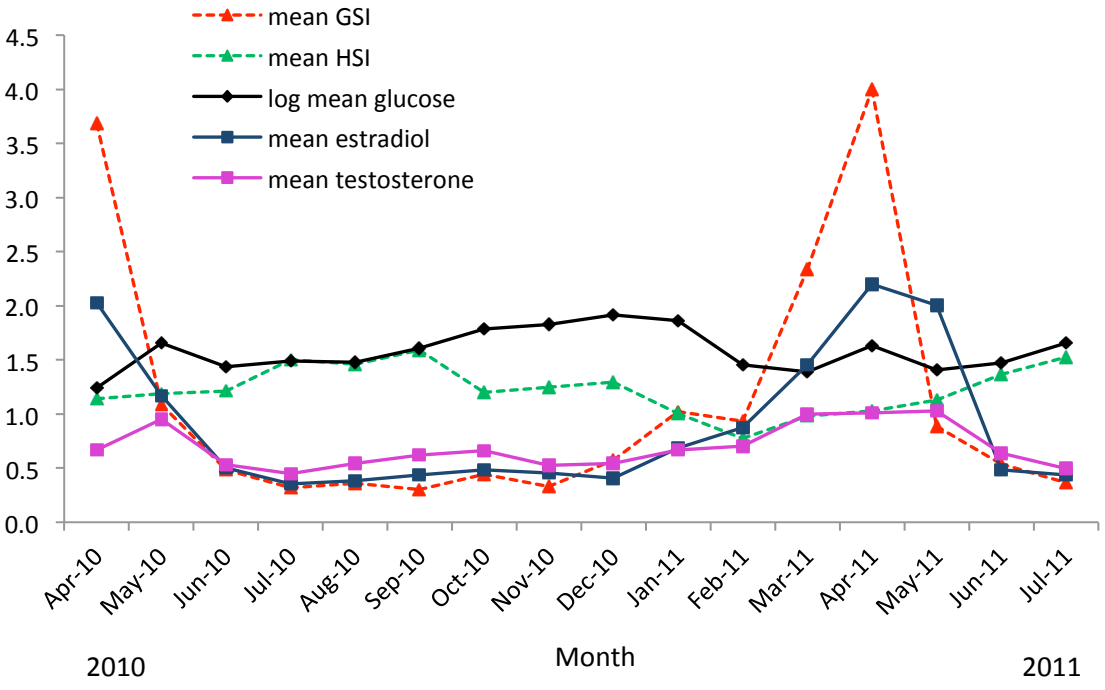


Figure 2.10: Overall relationships among GSI, HSI, plasma glucose, estradiol and testosterone concentrations for *L. bergylta* sampled from April 2010 to July 2011 (n=96). See Table 2.7 for the respective statistical analysis.

In Figure 2.11, oocyte diameter showed an opposite trend with glucose levels, while total protein, globulin and albumin showed minimal variations throughout sampling period. Cortisol levels decreased at a lowest state in June 2010 and then steadily increased on the following month. The trend of cortisol increment had stimulated blood glucose production to be increased until the glucose levels decreased in February 2011. This eventually encountered by surged of cortisol levels on that month to restore the glucose levels within the physiological range.

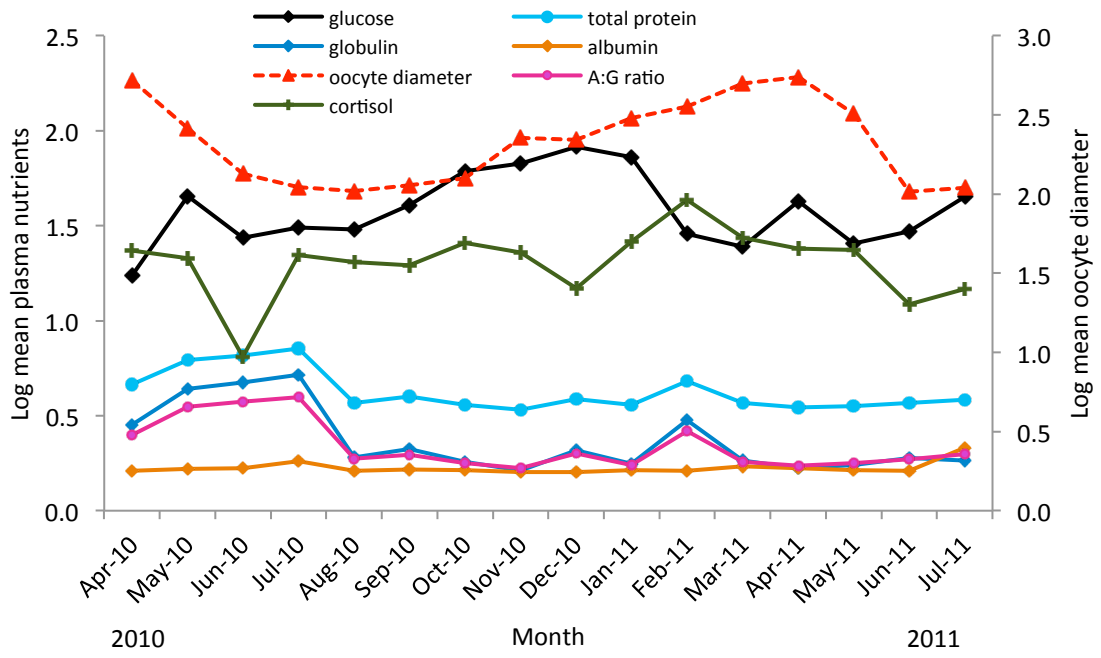


Figure 2.11: Oocyte diameter relationships with glucose, total protein, globulin and albumin concentrations between April 2010 and July 2011 (n=96). See Table 2.7 for the respective statistical analysis.

Overall, all steroids hormones including oocyte diameter and GSI showed negatively correlated ($p>0.05$) with glucose (exceptional to progesterone), total protein, albumin, globulin and A:G ratio. In contrary, HSI showed highly positive significant relationship to water temperature ($p<0.01$) and also positive relationships ($p>0.05$) to glucose, total protein, albumin, globulin and A:G ratio. Correlation matrices in Table 2.7 showed the relationships of all variables over 16 months period.

Table 2.7: Correlation matrices of the monthly means of all variables within 16 months (April 2010 – July 2011).

	estradiol	testosterone	progesterone	cortisol	oocyte diameter	GSI	HSI	CF	water temp	day length	glucose	total protein	albumin	globulin	A:G
estradiol	1	0.812**	0.528*	0.289	0.871**	0.854**	-0.547*	-0.763**	-0.226	0.166	-0.411	-0.149	-0.193	-0.144	-0.140
testosterone		1	0.397	0.334	0.691**	0.578*	-0.578*	-0.659**	-0.261	0.092	-0.255	-0.212	-0.268	-0.204	-0.205
progesterone			1	0.119	0.368	0.510*	-0.165	-0.540*	0.129	0.044	0.025	-0.092	-0.352	-0.076	-0.088
cortisol				1	0.487	0.237	-0.670**	0.023	-0.479	-0.454	-0.085	-0.131	-0.203	-0.128	-0.144
oocyte diameter					1	0.903**	-0.741**	-0.621*	-0.537*	-0.208	-0.221	-0.198	-0.248	-0.191	-0.195
GSI						1	-0.501*	-0.720**	-0.259	0.028	-0.303	-0.137	-0.164	-0.134	-0.136
HSI							1	0.444	0.662**	0.398	0.053	0.109	0.409	0.089	0.093
CF								1	0.000	-0.403	0.412	-0.126	0.206	-0.139	-0.147
water temp									1	0.796**	-0.444	0.529*	0.291	0.516*	0.523*
day length										1	-0.708**	0.446	0.350	0.433	0.454
glucose											1	-0.330	-0.067	-0.324	-0.328
total protein												1	0.085	0.999**	0.998**
albumin													1	0.040	0.079
globulin														1	0.998**
A:G															1

**($p<0.01$) highly significant
*($0.01<p<0.05$) significant
GSI (gonadosomatic index)
HSI (hepatosomatic index)
CF (condition factor)
A:G (albumin-globulin ratio)

2.3.6 Histology of ovaries and egg development in a reproductive cycle

The maturity stages of the ovaries were examined histologically in 110 fish between April 2010 to July 2011. A cross section of a late vitellogenic oocyte (Stages IV and V) is shown in Figure 2.12; here, the cytoplasm contains a large amount of lipid droplets and yolk globules. Also, the follicular layer is increased in thickness and is found together with pronounced outer and inner zona radiata, arranged as part of the oocyte membrane. The mature oocyte diameter ranges from 100.16 to 683.73 µm.

Over the entire reproductive cycle oocytes could be characterised as belonging to eight oogenetic stages. Figure 2.13 (A-F) shows the cycle of various oogenetic stages; beginning in April and May, which contained mature eggs (Stage IV and V) and post spawned ovaries (Stage VI) (Figure 2.13 A and B). Stage IV and V (Figure 2.13 A) oocytes were ready to spawn as the vitellogenic process had initiated in most of these oocytes to become late yolk and mature oocytes. In these stages, although the ovary contained all the stages of oocytes, most of the oocytes were transforming into vitellogenic oocytes. In other words, vitellogenesis (matured oocytes) occupied the most areas of the ovaries which were observed from April to May but all oocytes did not mature at the same time. Only the matured oocytes ovulated in the spawning period while immature oocytes gained maturation under way of vitellogenesis and released at the second spawning time in the months of April and May of another year.

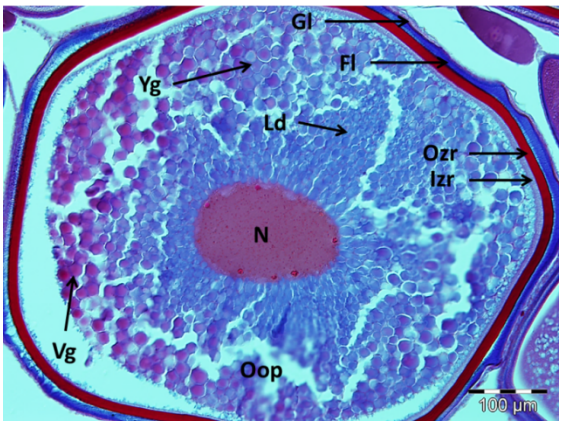


Figure 2.12: Cross section of late vitellogenic oocyte using AZAN stain. Ld-lipid droplet, N-nucleus, Yg-yolk globule, Oop-ooplasm, Ozr-outer zona radiata, Izr-inner zona radiata, Fl-follicular layer, Vg-vitellogenin globule, Gl-gelatinous layer (sticky properties).

Stage VI (Figure 2.13 B), post-spawned ovaries contained atretic and resorbing oocytes, which predominated in May and June. These months also coincided with the occurrence of postovulatory follicles (POF) of which the presence of unovulated oocytes in post spawning. In the post spawning phase, the ovary consisted of young oocytes which were mostly non-vitellogenic, along with some vitellogenic and some unspawned oocytes having intact germinal vesicles at the periphery. In Stage VII (Figure 2.13 C), occasionally there were proliferations or thickenings of the ovarian wall -these predominated in July. In the month of August to October were resting stages of immature or recovering oocytes (Stage VIII and I, Figure 2.13 D). Stage II (Figure 2.13 E), characterised early maturing oocytes. These young oocytes exhibited early vitellogenic activity which is characterised by the presence of vacuoles in the oocytes and an increase in the cytoplasm with distinctive differentiation of the follicular epithelium. Stage II occasionally predominated in November and were more prevalent in December and January. Meanwhile, a positive progress into early yolk oocytes Stage III (Figure 2.13 F) had taken place by February and late yolk oocytes were apparent in March. Thereafter oocytes continuously developed into mature oocytes (Stage IV to V) in which the vitellogenic oocytes contained a centrally placed nucleus containing a large number of nucleoli, ready for next ovulation in April and May.

To summarise, the annual ovarian cycle of *L. bergylta* can be divided into four phases, as follows: preparatory phase characterised by developing ooctytes (November to January), developed oocytes in the prespawning phase (February to March), ripe oocytes in the spawning phase (April and May) and regressed oocytes in the post spawning phase (June to October).

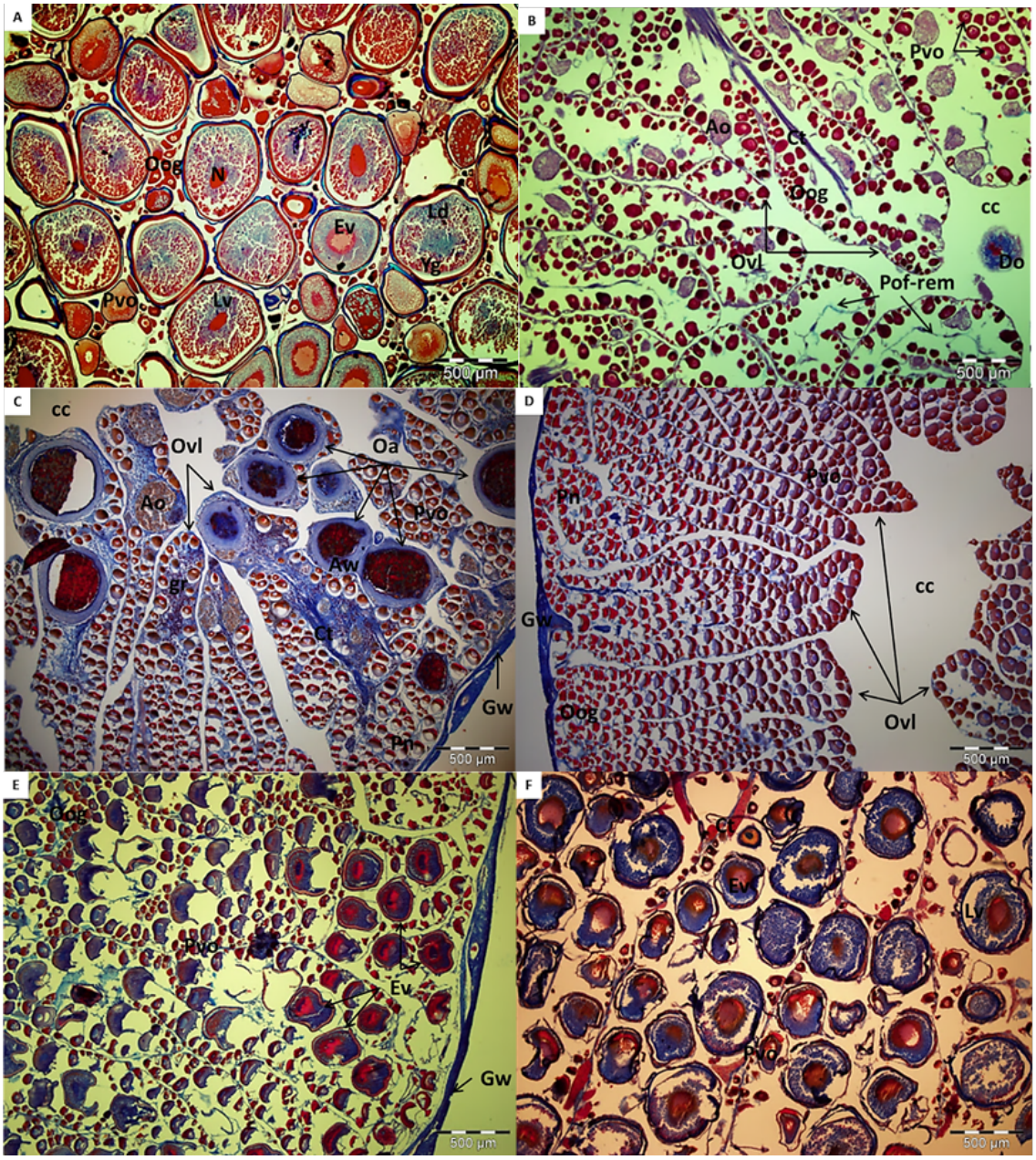


Figure 2.13(A-F): Light micrographs of cross sections of ovary with developing follicles in various stages of maturation in *L. bergylta* using AZAN stain. Ovaries were collected from monthly caught females between April 2010 and July 2011. (A) April; Stage IV and V, late yolk and mature oocytes (B) May and June; Stage VI, resorbing/postspawn (atretic eggs) (C) July; Stage VII, proliferation/thickening of the ovarian wall (D) September; Stage VIII and I, resting and immature oocytes (E) November; Stage II, early maturing oocytes and (F) March; Stage III, early yolk oocytes. Abbreviations: Ao-atretic degenerating oocyte, Ct-connective tissue, Oa-ovarian artery, Aw-arterial wall, Pn-perinucleolar oocytes, Ev-early vitellogenic oocyte, Lv-late vitellogenic oocyte, Pvo-previtellogenic oocyte, cc-central cavity, Gw-gonad wall, gr-granulocytes, Oog-oogonia, Ovl-ovarian villi, Do-degenerating oocytea, Pof-rem-postovulatory follicle remnants.

2.3.7 Histology of transitional gonads

Occasional histological observation of sex change was made in *L. bergylta* which is a protogynous species (females changing sex to males). Observation of transitional ovaries was occasional (n=12), but when it occurred, this was between June and September coinciding with the post spawning period, or the resting stages of ovaries. The process of sex change from ovary to testis was divided into four stages on the basis of changes in the structure of the germinal and somatic elements (Muncaster *et al*., 2013).

Figure 2.14 (A) illustrates the normal structure of a cross section of female *L. bergylta* ovary. In Stage 1, concurrent with gross structural development of the ovary was the appearance of primary oocytes formed from cell columns that developed into lamellae, which projected laterally into the ovarian cavity. The cells formed in the lamellar folds and basal tissue layers. Overall, the key structure of female ovaries was dominant ovarian villi-like lamellae protruding towards a central cavity filled with resting oocytes. These ovaries were filled with previtellogenic oocytes during the resting phase, but contained no spermatogenic tissue.

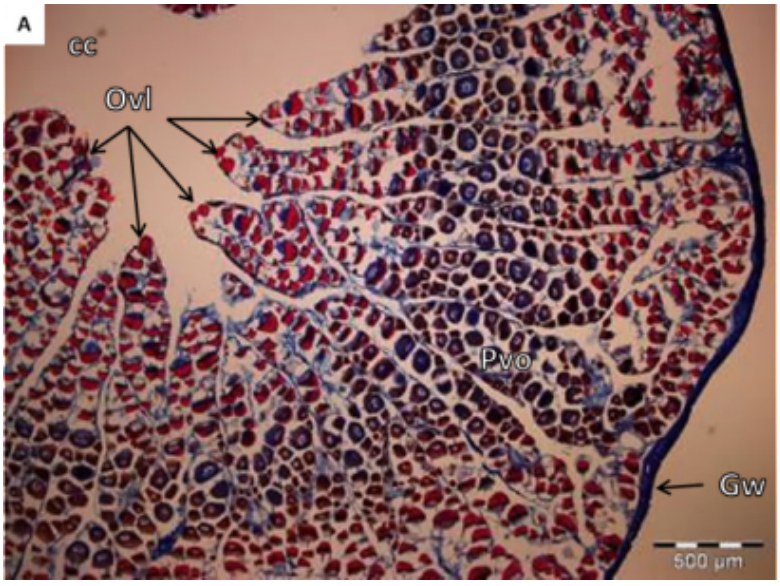


Figure 2.14(A): A typical cross section of a female *L. bergylta* ovary showing dominant ovarian villi protruding towards a central cavity filled with pre-vitellogenic oocytes. Abbreviations: Pvo-previtellogenic oocyte; cc-central cavity; Gw-gonad wall; Ovl-ovarian villi.

At the commencement of sex change in Stage 2, there was some evidence of cell lining alterations from female fish to a male gonad structure. In the initial part of the process, lamellar folds and basal tissue layers loosened and degenerated. Also, previtellogenic and vitellogenic oocytes began to degenerate. In the transitional ovary, we could observe the remaining unovulated eggs surrounded by cell differentiations and growth of the cell lining of the ovaries. These cell linings gradually changed into spermatogonia and supporting cells (leydig and sertoli cells) and eventually to the formation of lobuli testis (Figure 2.15: B1, B2 (10x magnification of B1) and C). The remained eggs here would eventually degenerate. Thereafter, aggregations of stromal tissue, granulocytes and loose connective tissue were prominent in the central region of the lamellae (Figure 2.15 (C)).

In Stage 3, steroid producing cells (Leydig cells) developed at the border of the loose connective tissues. Presumed spermatogonia proliferated on the periphery of the lamellae, and Leydig cells increased in size and number. At this stage, the ovarian villi remnants are still present and eventually changed into developing seminiferous lobules and tubules (D, E1 and E2). By Stage 4, proliferation and expansion of new basal cells and seminiferous lobules which specialized in support for sperm production had totally changed the internal structure of the gonads and there was an absence of lamellae. Spermatogonia formed cysts and underwent spermatogenesis. The presence of spermatozoa in seminiferous tubules was prominent at this stage. Finally, sex change to male was considered complete, with the beginning of active spermatogenesis and spermiation (F1 and F2 (60x magnification of F1)).

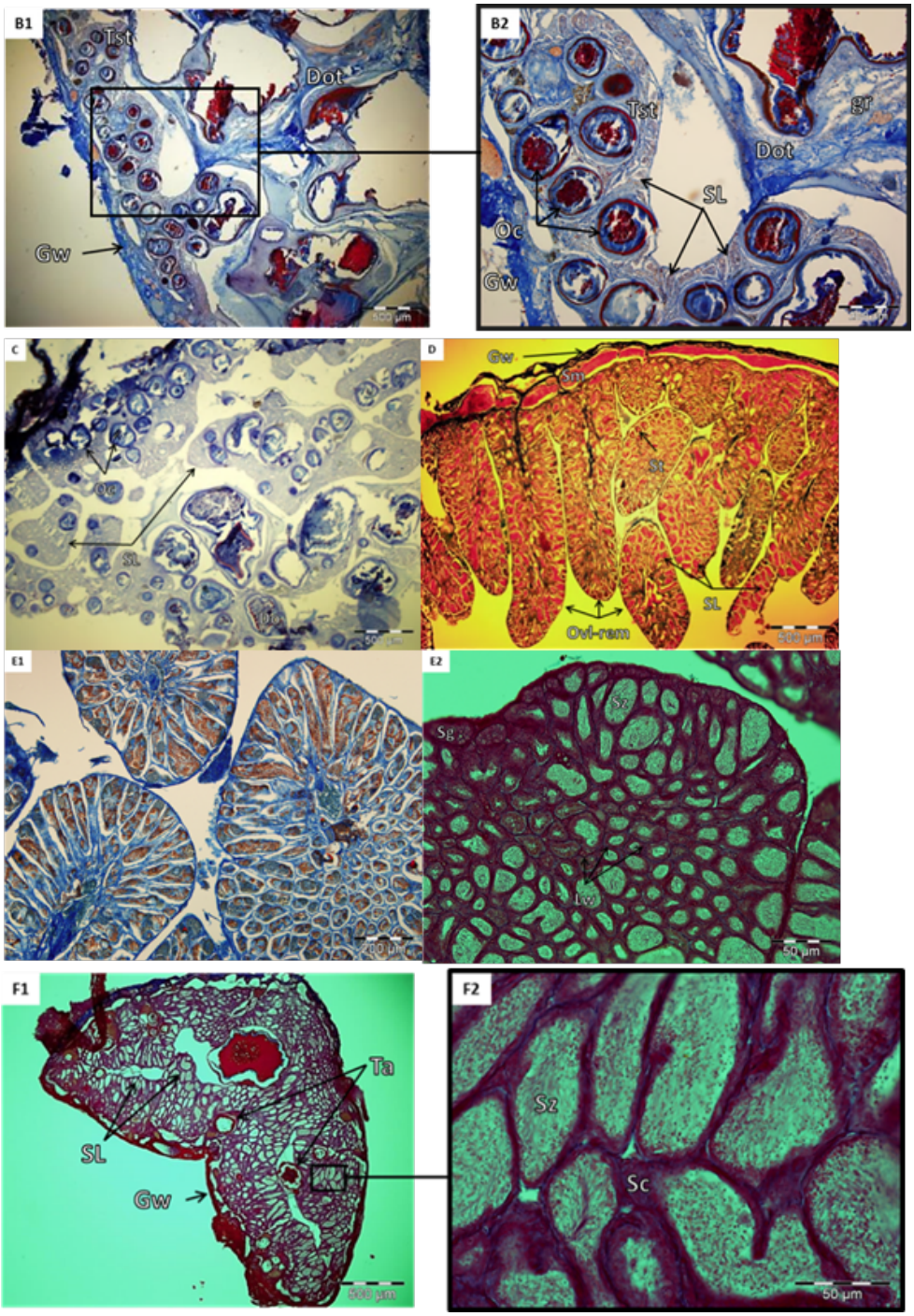


Figure 2.15(B1-F2): Histological sections of transitional ovaries in *L. bergylta* using AZAN stain; (B1 & B2): Oocytes mature testis filled with mainly spermatozoan cysts (sc) and a clearly formed sperm duct (sd). A small ovary (O) with ovarian lamella (ol) and central cavity (cc) is located dorsally, (C): Aggregations of stromal tissue, granulocytes (gr) and loose connective tissue were prominent in the central region of the lamellae, (D, E1 & E2): The ovarian villi remnants (Ovl-rem) are still present and eventually changed into developing seminiferous lobules (SL) and tubules (St) and (F1 & F2): Absence of lamellae and the presence of spermatozoa (Sz) in seminiferous tubules (St) was prominent.

Abbreviations: Sc-spermatocytes, Sg-spermatogonia, SL-seminiferous lobules, St-seminiferous tubules, Sz-spermatozoa, St-spermatids, Tst-testicular part, gr-granulocytes, Pvo-previtellogenic oocyte, Do-degenerating oocyte, Ovl-rem-ovarian villi remnants, Dot-degenerative ovarian tissue, Oc-oocyte, Sm-smooth muscle, Ta-testicular artery.

2.4 Discussion

Natural cycles in plasma steroids concentrations, plasma nutrients, gonad and hepatic indices and histological data were collected over 16-months in a reproductive cycle of wild *Labrus bergylta* at Carna, Co. Galway in the west of Ireland. By randomly sampling individuals (mainly adults) of various body sizes in local populations, the average maturity profiles were profiled in this protogynous species. With the notable exception of Muncaster *et al*., (2010; 2013), who provided monthly estradiol and testosterone profiles in western Norway, there is a lack of research focussed on sex steroids in *L. bergylta*, which may be regarded as fundamental drivers of maturation and spawning. Nutritional markers are also poorly studied in this species.

In the present study, consistent trends were apparent in some of the reproductive parameters towards the success of spawning in n=169 *L. bergylta* individuals, with estradiol, testosterone, oocyte diameter, Gonadosomatic index (GSI) all following the cyclical patterns in the lead up to spawning in April 2010 and April 2011. In estradiol, two threshold concentrations were evident in April 2010 and 2011, when this species spawns at Carna. The plasma concentrations of both estradiol and testosterone started to increase in January 2011 during the pre-spawning phase, peaking in April (estradiol) or May (testosterone) before falling sharply to their lowest levels in July of each year. Highest concentrations of circulatory estradiol and testosterone in the plasma coincided with the preponderance of vitellogenic follicles in the ovary. During this period, the females also had a sharply increased GSI. At peak levels, estradiol concentrations were 2.20±0.90ng/ml in April 2011 and these were lowest at 0.35±0.09 in July 2010. Meanwhile, testosterone concentrations were highest at 1.03±0.20ng/ml in May 2011 and lowest at 0.45±0.05ng/ml in July 2010. The four-fold increase in concentration of estradiol at its peak relative to testosterone could be due to the majority of female *L. bergylta* in the sampled population, as only a few males were sampled. The latter was clear on histological examination of gonads of all sampled individuals.

Given the timing of spawning season in April in the west of Ireland, it was unsurprising that the highest level of GSI was in April (4.00±0.93). But in fact, GSI was observed to begin increasing from December prior to spawning the following spring and this increase in GSI was closely correlated with increasing oocyte diameter ($p<0.01$). In

western Norway, Muncaster *et al.,* (2010) observed that, between 2005 and 2006, estradiol (1.75±0.43ng/ml), testosterone (0.95±0.26ng/ml) and GSI (10.71±0.81) were maximal in April and May and decreased greatly in July (0.14±0.01ng/ml, 0.07±0.01ng/ml and 1.35±0.11, respectively). The latitude of study populations of *L. bergylta* from Bergen, western Norway (60° 06' N; 5° 10' E) differs somewhat from Carna, western Ireland (53° 16 N; 9° 50' W), yet both studies have shown similar patterns of estradiol, tesosterone and oocyte diameter; with spawning occurring in spring and peaking between April and May. Correlations between sea temperature, estradiol, testosterone and GSI at Carna indicate that temperature may be significant in controlling gonadal processes and reproduction. Temperature has been suggested to fulfill a permissive role in teleost reproduction (Taranger *et al*., 2010). An increase in temperature can induce the reproductive cycle in percids and moronids (Wang *et al*., 2010). Further research into the relationship between the photothermal cues and the reproductive cycle of *L. bergylta*, e.g. effects of temperature on enzymatic processes in the gonads, will have important implications for controlling reproduction in this species. It would also be of interest to partition the importance of temperature and photoperiod as a cue for spawning, as the latter has been shown to be an environmental cue to a pre-ovulatory surge in hormonal secretion in cyprinids (Aida, 1988).

Defined reproductive cycles are typical of temperate and high latitude teleosts (Crim, 1982). In this context, an interesting observation at Carna was a mini surge in both estradiol and testosterone mid-way through the autumn months, centering around the month of October. Also interesting was the fact that the lowest GSI was not immediately after spawning, rather this was in September (0.30±0.03), as re-absorbing the gonads was delayed following steep steroid declines in June and July. The ovary also remained somewhat active with unspawned oocytes in May and June at Carna. This raises the question that prolonged spawning may occur in certain years, or in certain locations when conditions are suitable. Alternatively, when conditions are not suitable, a longer resting period may occur between mid-summer and autumn when previtellogenic oocytes predominate and steroid levels are minimal. The *L. bergylta* in this study appeared to enter a short quiescent period between August and October, when only previtellogenic oocytes and nests of oogonia were present in the ovary (Stage I). Atresia

of unspawned oocytes was only complete by September. This was also when oocyte diameter was lowest. Plasma concentrations of estradiol and testosterone were minimal in September and October, suggesting the existence of a brief quiescent period in late summer and autumn as photo period and sea temperature started to decrease (in August).

Estradiol is secreted by the cells of the ovarian follicles and promotes the development and maintenance of the female sexual characteristics (Ohta *et al*., 2002; Arroyo *et al*., 2008; Jamalzadeh *et al*., 2012; Peng *et al*., 2015). Estradiol remains high throughout the period of oocyte growth and an increase in plasma concentrations of this steroid have been reported once spawning commences (Nagahama and Yamashita, 2008). Almost the same profile of estradiol in this study and has been reported during the transition from vitellogenic to maturational stage in rainbow trout, *Oncorhynchus mykiss* (Verslycke *et al*., 2002; Barkataki *et al*., 2012). Similarly, according to Fostier *et al*., (1983) and Rinchard *et al*., (1993), in other teleosts such as gudgeon, (*Gobio gobio*), there was no decrease of estradiol level during oocyte maturation. Clearly estradiol does promote spawning in *L. bergylta* as shown in the present study. A conversion of testosterone to estradiol had been demonstrated in many studies. Kim *et al*., 2003 suggest that ethanol activates P450arom activity and promotes the conversion of testosterone to estradiol in the ovary of tilapia (*Oreochromis mossambicus*). In human study, testosterone therapy was partly converted to 17 beta-estradiol and 5 alpha-dihydrotestosterone (DHT) (Lakshman *et al*., 2010). For example, in gudgeon, *Gobio gobio*, a slight increase of testosterone levels during oocyte development can be related to its role as precursor of estradiol synthesis. The testosterone peak in 2010 and 2011 actually came a month later (in May), than estradiol (in April) suggested that the occurrence of sex change from female to male might influenced the levels of testosterone to be higher compared to estradiol in post-spawning *L. bergylta* in this study.

The annual profile of progesterone in *L. bergylta* in the present study was less clearly cyclical and this was more difficult to interpret in the context of spawning. Although concentrations increased in spawning periods i.e. showed a maximum of 20.74±5.16ng/ml and 28.70±5.44ng/ml respectively in April of both years, progesterone levels for *L. bergylta* were not generally significantly different across monthly samples.

Moreover, large fluctuations were observed that were out-of-phase with spawning, such as concentrations of ~20ng/ml in April or May 2010 decreasing to ~7-9ng/ml in June and back up to 15-17ng/ml in August, September and October. In 2011, concentrations of progesterone fluctuated between 2.67±0.74ng/ml in March to 28.70±5.44ng/ml in April and back to ~15ng/ml in May. Thus, the present study showed the same pattern as Snelson *et al*., (1997) who reported that progesterone levels varied irregularly over the course of their study during reproduction of Atlantic stingray, (*Dasyatis sabina*), in Florida. Availability of progesterone may be necessary for maintenance of oocytes within their vitellogenic state prior to ovulation, but it is unknown whether late summer and autumn progesterone levels, which were rather elevated, have any link with prolonged spawning in *L. bergylta* (-see estradiol above). Jalabert *et al*., (1991), Nagahama *et al*., (1994) and Haider, (1997) have reviewed hormonal control of oocyte maturation in catfish, (*Clarias batrachus*), showing that 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-DP), which is derived from progesterone, was the most effective maturation inducing compound. A similar observation was made by Ohta *et al*., (2002) on final oocyte maturation in red seabream, *Pagrus major*. In amphibia, Liu and Patino, (1993) showed that high-affinity binding of progesterone on the oocyte membrane induced maturation in *Xenopus laevis*. Competitive studies showed that progesterone affinity was higher in this case than estradiol, testosterone, cortisol and a range of other steroids (Liu and Patino, 1993). Furthermore, densities of progesterone receptors increased with oocyte development after stimulation of intact follicles with gonadotropin. The progesterone concentrations of the present study indicates an important role for progesterone in regulating gonad development and spawning in *L. bergylta*, however its precise role awaits experimental manipulations *in vivo*.

As well as being involved in the stress response, cortisol is important for generating energy for synthesizing and developing eggs during spawning (McCormick and Nechaev, 2002). The primary functions of cortisol are to increase blood sugar through gluconeogenesis, suppress the immune system and aid in fat, protein and carbohydrate metabolism (Shankar *et al*., 2007). In these ways, when blood glucose has been depleted, cortisol carefully regulates the level of glucose to ensure a steady supply of circulating glucose through the bloodstream via gluconeogenesis. Of all steroids

measured in the present study, the production of cortisol showed the least clear pattern of annual cycling. Plasma cortisol concentrations in *L. bergylta* fluctuated in a quasi random fashion, apart from a peak in February 2011 (91.28±20.66ng/ml, $p<0.05$). It is perhaps noteworthy that this elevated cortisol coincided with a small peak in concentrations of total protein, globulin and A:G ratio in 2011 and was at the beginning of a surge in GSI in March and April. In 2010 however, cortisol was not particularly high when these nutritional markers were elevated. This inter-annual variability between 2010 and 2011 confounds explanation of cortisol influence, somewhat. Since all *L. bergylta* individuals were handled to a similar extent during blood sampling, the February 2011 peak was not obviously related to stress, however we cannot rule-out increased capture stress (which investigators were unaware of) that month. As vitellogenesis begins, the oocytes begin to accumulate vitellogenin (yolk) which is a lipophospoglycoprotein; vitellogenin is produced in the liver in teleosts under receptor-mediated stimulation of estradiol (Menuet *et al*., 2001). An indirect role for cortisol might be related to the increased vitellogenic activity which requires an increase in metabolism. Molecular evidence of seasonal cortisol changes has been provided in the Pacific salmon, *Oncorhynchus* sp., in which both males and females at spawning experienced a 5 to 7-fold increase in plasma cortisol levels relative to juvenile non-spawning salmon (Westring *et al*., 2008).

Overall, plasma sex steroid concentrations in *L. bergylta* were found to be relatively low (<5ng/ml) like several other marine species such as the saddleback wrasse *Thalassoma duperrey* (Nakamura *et al*., 1989), black eye goby *Coryphopterus nicholsii* (Kroon and Liley, 2000), honeycomb grouper *Epinephelus merra* (Bhandari *et al*., 2003) and red porgy *Pagrus pagrus* (Kokokiris *et al*., 2006), which have sex steroid levels between 1 and 10ng/ml. By contrast, other fish species such as salmonids may have concentrations of >50ng/ml (Taranger *et al*., 1998). Low sex steroid production may be typical of fishes that spawn multiple times within a season. Pankhurst and Carragher (1991) suggest that fishes with low steroid levels tend to follow a semi-lunar spawning cycle while group-synchronous spawners such as plaice *Pleuronectes platessa* and winter flounder *Pseudopleuronectes americanus* often have higher sex steroid

levels. It would seem that *L. bergylta* also fits this trend as an apparent group synchronous multiple spawner with low steroid levels.

Regarding nutritional markers, it is widely accepted that carnivorous fish utilize dietary carbohydrates (CHO) ineffectively and instead they are highly dependent on gluconeogenesis from amino acids for sustaining endogenous glucose demands (Moon, 2001; Stone, 2003; Enes *et al.,* 2009; Polakof *et al*., 2012 and Viegas *et al*., 2013). Gonad development is one of the important reasons for higher concentration of glucose in fish blood plasma. For example, a biochemical profile study of blood plasma of tench (*Tinca tinca*) showed that higher values of plasma glucose were found for females after reproduction in June compared with two months prior to reproduction in April (Svoboda *et al*., 2001). This observation was suggested to be linked to metabolic rates in water temperatures that changed from lower levels in April to higher levels in June. However somewhat different results were observed in *L. bergylta,* where glucose levels were at their highest three months prior to spawning in December when water temperatures were lowest (6.0±0.1°C).

In addition to releasing glucose, most of the protein components are also synthesized in the liver (Haschemeyer *et al*., 1981), thus examination of the parameters hepatosomatic index (HSI), along with condition factor (K) and GSI can be helpful. There was clear evidence of GSI and hepatosomatic index (HSI) displaying opposing patterns. This interplay is almost certainly due to the mobilisation of energy reserves in the liver (resulting in decreased HSI) to feed the requirement of the ovary for the development of oocytes (giving rise to increased GSI). For example, HSI was highest in July/September, lowest in January/February. Meanwhile, release of highest concentrations of free glucose into the blood plasma steadily climbed throughout summer and autumn and reached maximum levels in January before dropping sharply in February/March, ahead of spawning. The levels of HSI and plasma glucose were low at spawning, presumably due to the energy consumption involved (and despite higher temperatures and presumably higher metabolic rates, contrary to Svoboda *et al*., 2001). It is suggested that, prior to the breeding period, the declines, first in condition factor and sequentially in HSI and plasma glucose concentrations, reflect energy investment in the pre-spawning period in *L. bergylta*. Thus, free glucose released from main storage

organ in the liver in December gave decreasing HSI towards December to provide energy and protein (vitellogenin) towards egg development in the ovaries. In February, the vitellogenin/yolk started to be transported actively into the developing eggs which were eventually ready to ovulate between April and May. By contrast, between July and September, there was a positive balance of blood sugar levels and larger relative liver size (HSI) was achieved. *L. bergylta* also showed K rising during May-September/October and declining during pre-spawning and spawning phases. Reduced winter feeding in the wild is assumed for *L. bergylta* (Hamre *et al*., 2013), which could have affected K. However, given the patterns in GSI, HSI and glucose, variability in K could also conceivably have been due to expenditure of energy for reproductive preparations in winter and reproduction itself in spring.

The histological data showed that ovarian recrudescence (renewal) started in November and December (Stage II ovaries) with the appearance of early maturing oocytes containing cortical alveoli accompanied by higher concentrations of estradiol and GSI values. Increases in estradiol, testosterone and GSI values continued towards January, along with the appearance of early vitellogenic oocytes (Stage III ovaries) in February. Oogenesis proceeded rapidly from February to April as estradiol concentrations increased, and by March, most of the fish contained Stage IV ovaries with late vitellogenic oocytes. Prevalence of Stage IV and mature Stage V ooctyes increased in April and May. Post spawned gonads (Stage VI ooctyes) were most common in May and June and resting Stage VII ooctyes were seen in July. By September ovaries contained mainly Stage VIII and I oocytes (the latter being primary ooctyes forming basal tissues). Some evidence of sexual transition from females to males was observed. Observation of transitional gonads was occasional, but when it occurred, this was between June and September coinciding with the post spawning period, or the resting stages of the gonads and low plasma concentrations of estradiol and testosterone.

In future research, greater understanding of the roles of sex steroid, temperature and other cues would be gained through captive manipulation of fish and the measurement of sex steroids across a finer time scale as estradiol concentrations can fluctuate between the spawning of egg batches (Navas *et al*., 1998; Methven *et al*.,

1992; Kjesbu *et al*., 1996). Further attention also needs to be applied to identifying final maturation-inducing steroids in *L. bergylta*, for example, maturation-inducing progestins (Matsuyama *et al*., 1998 and 2002; Ohta and Matsuyama, 2002) as this would provide valuable information regarding the frequency and timing of ovulation for improved husbandry of this species.

References

Aida, K. (1988). A Review of Plasma-Hormone Changes during Ovulation in Cyprinid Fishes. *Aquaculture, 74*(1-2), 11-21.

Arroyo, G. A. B., Sosa, I. D. B., Cartagena, T. M., Ramirez, C. M. R., & Figueroa, J. L. A. (2008). Association between ovarian development and serum concentrations of 17 beta-estradiol and 17 alpha-hydroxy-4-pregnen-3-one in first maturation females of the shortfin silverside fish, *Chirostoma humboldtianum* (Atheriniformes : Atherinopsidae). *Veterinaria Mexico, 39*(1), 67-80.

Barkataki, S., Aluru, N., Li, M., Vijayan, M. M., & Leatherland, J. F. (2012). Characteristics of ovarian follicle steroidogenesis during vitellogenesis in an asynchronously ovulating stock of rainbow trout *Oncorhynchus mykiss*. *J Fish Biol, 80*(4), 741-751.

Bhandari, R. K., Komuro, H., Nakamura, S., Higa, M., & Nakamura, M. (2003). Gonadal restructuring and correlative steroid hormone profiles during natural sex change in protogynous honeycomb grouper (*Epinephelus merra*). *Zoological Science, 20*(11), 1399-1404.

Bhattacharyya, S., Sen, U., Bhattacharyya, S. P., & Mukherjee, D. (2000). Identification of maturation-inducing steroid in a freshwater perch *Anabas testudineus* and differential responses of intact follicles and denuded oocytes to cyclic AMP in oocyte maturation. *Journal of Experimental Zoology, 287*(4), 294-303.

Bushnell, P. G., Conklin, D. J., Duff, D. W., & Olson, K. R. (1998). Tissue and whole-body extracellular, red blood cell and albumin spaces in the rainbow trout as a function of time: A reappraisal of the volume of the secondary circulation. *Journal of Experimental Biology, 201*(9), 1381-1391.

Byrnes, L., & Gannon, F. (1990). Atlantic Salmon (*Salmo salar*) Serum-Albumin - Cdna Sequence, Evolution, and Tissue Expression. *DNA and Cell Biology, 9*(9), 647-655.

Byrnes, L., & Gannon, F. (1992). Sequence-Analysis of a 2nd Cdna-Encoding Atlantic Salmon (*Salmo salar*) Serum-Albumin. *Gene, 120*(2), 319-320.

Chourasia, T. K., & Joy, K. P. (2010). Seasonal variation in tissue estrogen-2/4-hydroxylases (EH) and in vitro effects of steroids on ovarian EH activity in the catfish *Heteropneustes fossilis*. *Steroids, 75*(13-14), 1097-1105.

Crim, L. W. (1982). Environmental Modulation of Annual and Daily Rhythms Associated with Reproduction in Teleost Fishes. *Canadian Journal of Fisheries and Aquatic Sciences*, 39(1), 17-21.

Danis, M. H., Filosa, M. F., & Youson, J. H. (2000). An albumin-like protein in the serum of non-parasitic brook lamprey (*Lampetra appendix*) is restricted to preadult phases of the life cycle in contrast to the parasitic species *Petromyzon marinus*. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology, 127*(2), 251-260.

De Smet, H., Blust, R., & Moens, L. (1998). Absence of albumin in the plasma of the common carp *Cyprinus carpio*: binding of fatty acids to high density lipoprotein. *Fish Physiology and Biochemistry, 19*(1), 71-81.

De Smet, H., Blust, R., & Moens, L. (2001). Cadmium-binding to transferrin in the plasma of the common carp *Cyprinus carpio*. *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology, 128*(1), 45-53.

Duffey, R. J., & Goetz, F. W. (1980). The Invitro Effects of 17alpha-Hydroxy-20beta-Dihydroprogesterone on Germinal Vesicle Breakdown in Brook Trout (*Salvelinus fontinalis*) Oocytes. *Gen Comp Endocrinol, 41*(4), 563-565.

El-Sayed, A. F. M., & Kawanna, M. (2008). Effects of dietary protein and energy levels on spawning performance of Nile tilapia (*Oreochromis niloticus*) broodstock in a recycling system. *Aquaculture, 280*(1-4), 179-184.

El-Sayed, A. F. M., Mansour, C. R., & Ezzat, A. A. (2003). Effects of dietary protein level on spawning performance of Nile tilapia (*Oreochromis niloticus*) broodstock reared at different water salinities. *Aquaculture, 220*(1-4), 619-632.

Enes, P., Panserat, S., Kaushik, S., & Oliva-Teles, A. (2009). Nutritional regulation of hepatic glucose metabolism in fish. *Fish Physiology and Biochemistry, 35*(3), 519-539.

Fostier, A., Breton, B., Jalabert, B., & Marcuzzi, O. (1981a). Evolution of the Plasma-Levels of Glycoproteic Gonadotropin and 17-Alpha Hydroxy-20-Beta Dihydroprogesterone during Maturation and Ovulation in the Rainbow-Trout, *Salmo gairdnerii*. *Comptes Rendus De L Academie Des Sciences Serie Iii-Sciences De La Vie-Life Sciences, 293*(15), 817-820.

Fostier, A., Jalabert, B., Billard, R., Breton, B., & Zohar, Y. (1983). The Gonadal-Steroids. *Fish Physiology, 9*, 277-372.

Fostier, A., Jalabert, B., Campbell, C., Terqui, M., & Breton, B. (1981b). Invitro Liberation Kinetics of 17-Alpha Hydroxy-20-Beta Dihydroprogesterone by Rainbow-Trout (*Salmo gairdnerii*) Follicles. *Comptes Rendus De L Academie Des Sciences Serie Iii-Sciences De La Vie-Life Sciences, 292*(12), 777-780.

Gingerich, W. H., & Pityer, R. A. (1989). Comparison of Whole-Body and Tissue Blood Volumes in Rainbow-Trout (*Salmo gairdnerii*) with I125 Bovine Serum-Albumin and Cr-51 Erythrocyte Tracers. *Fish Physiology and Biochemistry, 6*(1), 39-47.

Goetz, F. W., & Duman, P. (1986). 17-Alpha, 20-Beta-Dihydroxy-4-Pregnen-3-One Stimulates Pgf Production in Invitro Incubations of Intrafollicular Yellow Perch (*Perca flavescens*) Oocytes. *American Zoologist, 26*(4), A109-A109.

Gong, Z. Q., & Hew, C. L. (1998). Two rainbow trout (*Oncorhynchus mykiss*) albumin genes are differentially regulated. *DNA and Cell Biology, 17*(2), 207-216.

Gray, J. E., & Doolittle, R. F. (1992). Characterization, Primary Structure, and Evolution of Lamprey Plasma-Albumin. *Protein Science, 1*(2), 289-302.

Haider, S. (1997). Hormonal control of oocyte maturation in fish. *In*: Advances in Fish Research, vol 2, pp 285–298. Ed BR Singh. Delhi: Narendra Publishing House.

Hamre, K., Nordgreen, A., Grotan, E., & Breck, O. (2013). A holistic approach to development of diets for Ballan wrasse (*Labrus bergylta*) - a new species in aquaculture. *Peerj, 1*.

Haschemeyer, A. E. V., Cohen, R., & Mathews, R. W. (1981). Liver Protein-Metabolism and Reproduction in Cold-Adapted Fish of Antarctica. *Federation Proceedings*, 40(3), 581-581.

Hemre, G. I., Mommsen, T. P., & Krogdahl, A. (2002). Carbohydrates in fish nutrition: effects on growth, glucose metabolism and hepatic enzymes. *Aquaculture Nutrition, 8*(3), 175-194.

Hrubec, T. C., & Smith, S. A. (1999). Differences between plasma and serum samples for the evaluation of blood chemistry values in rainbow trout, channel catfish, hybrid tilapias, and hybrid striped bass. *Journal of Aquatic Animal Health, 11*(2), 116-122.

Izquierdo, M. S., Fernandez-Palacios, H., & Tacon, A. G. J. (2001). Effect of broodstock nutrition on reproductive performance of fish. *Aquaculture, 197*(1-4), 25-42.

Jalabert, B., Fostier, A., Breton, B., & Weil, C. (1991). Oocyte maturation in vertebrates. *In*: Vertebrate Endocrinology: Fundamentals and Biomedical Implications (eds) Peter, K., Pang, T., and Schreibman, M. P. (New York: Academic Press) Vol. 4A, pp 1–396.

Jamalzadeh, H. R., Hajirezaee, S., Nazeri, S., Khara, H., & Mirrasuli, E. (2012). Identification of follicular vitellogenesis stage by monitoring of plasma calcium and estradiol-17 beta concentrations in the cultured Caspian brown trout, *Salmo trutta* caspius Kessler, 1877. *Biologia, 67*(4), 796-799.

Kim, B. H., Rahman, M. S., Kim, S. J., Lee, Y. D., & Takemura, A. (2003). Alcohol treatment promotes conversion of testosterone to estradiol-17 beta in female tilapia, *Oreochromis mossambicus*. *Fish Physiology and Biochemistry*, 29(4), 263-268.

Kjesbu, O. S., Kryvi, H., & Norberg, B. (1996). Oocyte size and structure in relation to blood plasma steroid hormones in individually monitored, spawning Atlantic cod. *J Fish Biol, 49*(6), 1197-1215.

Kobayashi, D., Tanaka, M., Fukada, S., & Nagahama, Y. (1996). Steroidogenesis in the ovarian follicles of the medaka (*Oryzias latipes*) during vitellogenesis and oocyte maturation. *Zoological Science, 13*(6), 921-927.

Kokokiris, L., Fostier, A., Athanassopoulou, F., Petridis, D., & Kentouri, M. (2006). Gonadal changes and blood sex steroids levels during natural sex inversion in the protogynous Mediterranean red porgy, *Pagrus pagrus* (Teleostei : Sparidae). *Gen Comp Endocrinol, 149*(1), 42-48.

Kroon, F. J., & Liley, N. R. (2000). The role of steroid hormones in protogynous sex change in the blackeye goby, *Coryphopterus nicholsii* (Teleostei : Gobiidae). *Gen Comp Endocrinol, 118*(2), 273-283.

Lakshman, K. M., Kaplan, B., Travison, T. G., Basaria, S., Knapp, P. E., Singh, A. B., LaValley, M. P., Mazer, N. A., & Bhasin, S. (2010). The Effects of Injected Testosterone Dose and Age on the Conversion of Testosterone to Estradiol and Dihydrotestosterone in Young and Older Men. *Journal of Clinical Endocrinology & Metabolism*, 95(8), 3955-3964.

Lee, Y. H., Du, J. L., Yen, F. P., Lee, C. Y., Dufour, S., Huang, J. D., Sun, L. T., & Chang, C. F. (2001). Regulation of plasma gonadotropin II secretion by sex steroids, aromatase inhibitors, and antiestrogens in the protandrous black porgy, *Ancanthopagrus schlegeli* Bleeker. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology, 129*(2-3), 399-406.

Liu, Z. M., & Patino, R. (1993). High-Affinity Binding of Progesterone to the Plasma-Membrane of Xenopus-Oocytes - Characteristics of Binding and Hormonal and Developmental Control. *Biology of Reproduction, 49*(5), 980-988.

Maillou, J., & Nimmo, I. A. (1993a). Albumin-Like Proteins in the Serum of Rainbow-Trout (*Salmo gairdnerii*). *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology, 104*(2), 387-393.

Maillou, J., & Nimmo, I. A. (1993b). Identification and Some Properties of an Albumin-Like Protein in the Serum of Prespawning Atlantic Salmon (*Salmo salar*). *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology, 104*(2), 401-405.

Matsuyama, M., Ohta, K., Morita, S., Hoque, M. M., Kagawa, H., & Kambegawa, A. (1998). Circulating levels and in vitro production of two maturation-inducing hormones in teleost: 17 alpha,20 beta-dihydroxy-4-pregnen-3-one and 17 alpha,20 beta,21-trihydroxy-4-pregnen-3-one, in a daily spawning wrasse, *Pseudolabrus japonicus*. *Fish Physiology and Biochemistry, 19*(1), 1-11.

Matsuyama, M., Onozato, S., & Kashiwagi, M. (2002). Endocrine control of diurnal oocyte maturation in the kyusen wrasse, *Halichoeres poecilopterus*. *Zoological Science, 19*(9), 1045-1053.

McCormick, M. I., & Nechaev, I. V. (2002). Influence of cortisol on developmental rhythms during embryogenesis in a tropical damselfish. *Journal of Experimental Zoology*, 293(5), 456-466.

Menuet, A., Anglade, I., Flouriot, G., Pakdel, F., & Kah, O. (2001). Tissue-specific expression of two structurally different estrogen receptor alpha isoforms along the female reproductive axis of an oviparous species, the rainbow trout. *Biology of Reproduction, 65*(5), 1548-1557.

Metcalf, V. J., Brennan, S. O., Chambers, G., & George, P. M. (1999). High density lipoprotein (HDL), and not albumin, is the major palmitate binding protein in New Zealand long-finned (*Anguilla dieffenbachii*) and short-finned eel (*Anguilla australis schmidtii*) plasma. *Biochimica Et Biophysica Acta-Protein Structure and Molecular Enzymology, 1429*(2), 467-475.

Metcalf, V. J., Brennan, S. O., & George, P. M. (1999). The Antarctic toothfish (*Dissostichus mawsoni*) lacks plasma albumin and utilises high density lipoprotein as its major palmitate binding protein. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology, 124*(2), 147-155.

Metcalf, V. J., & Gemmell, N. J. (2005). Fatty acid transport in cartilaginous fish: absence of albumin and possible utilization of lipoproteins. *Fish Physiology and Biochemistry, 31*(1), 55-64.

Metcalf, V. J., George, P. M., & Brennan, S. O. (2007). Lungfish albumin is more similar to tetrapod than to teleost albumins: Purification and characterisation of albumin from the Australian lungfish, *Neoceratodus forsteri*. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology, 147*(3), 428-437.

Methven, D. A., Crim, L. W., Norberg, B., Brown, J. A., Goff, G. P., & Huse, I. (1992). Seasonal Reproduction and Plasma-Levels of Sex Steroids and Vitellogenin in Atlantic Halibut (*Hippoglossus hippoglossus*). *Canadian Journal of Fisheries and Aquatic Sciences, 49*(4), 754-759.

Moncaut, N., Lo Nostro, F., & Maggese, M. C. (2003). Vitellogenin detection in surface mucus of the South American cichlid fish *Cichlasoma dimerus* (Heckel, 1840) induced by estradiol-17 beta. Effects on liver and gonads. *Aquat Toxicol, 63*(2), 127-137.

Moon, T. W. (2001). Glucose intolerance in teleost fish: face or fiction? *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology, 129*(2-3), 243-249.

Morais, S., Mendes, A. C., Castanheira, M. F., Coutinho, J., Bandarra, N., Dias, J., & Conceicao, L. E. C., & Pousao-Ferreira, P. (2014). New formulated diets for *Solea senegalensis* broodstock: Effects of parental nutrition on biosynthesis of long-chain polyunsaturated fatty acids and performance of early larval stages and juvenile fish. *Aquaculture, 432*, 374-382.

Mohammadizadeh, M., Afkhami, M., Bastami, K. D., Ehsanpour, M., Kazaali, A., & Soltani, F. (2012) Determination of some biochemical values in the blood of *Liza klunzingeri* from the coastal water of the Persian gulf. *Afr J Biotechnol*, 11(12), 2862-2868.

Muncaster, S., Andersson, E., Kjesbu, O. S., Taranger, G. L., Skiftesvik, A. B., & Norberg, B. (2010). The reproductive cycle of female Ballan wrasse *Labrus bergylta* in high latitude, temperate waters. *J Fish Biol, 77*(3), 494-511.

Muncaster, S., Andersson, E., Skiftesvik, A. B., Opstad, I., Taranger, G. L., & Norberg, B. (2008). Seasonal reproductive cycle of Ballan wrasse (*Labrus bergylta*) in Norway. *Cybium, 32*(2), 199-199.

Muncaster, S., Norberg, B., & Andersson, E. (2013). Natural sex change in the temperate protogynous Ballan wrasse *Labrus bergylta*. *J Fish Biol, 82*(6), 1858-1870.

Nagahama, Y., Kagawa, H., & Tashiro, F. (1980). The Invitro Effects of Various Gonadotropins and Steroid-Hormones on Oocyte Maturation in Amago Salmon *Oncorhynchus rhodurus* and Rainbow-Trout *Salmo gairdnerii*. *Bulletin of the Japanese Society of Scientific Fisheries, 46*(9), 1097-1102.

Nagahama, Y., & Yamashita, M. (2008). Regulation of oocyte maturation in fish. *Development Growth & Differentiation*, 50, S195-S219.

Nagahama, Y., Yamashita, M., & Tokumoto, T. (1994). Regulation of oocyte maturation in fish. *Curr. Topics Dev. Biol*, 30, 103–145.

Nakamura, M., Hourigan, T. F., Yamauchi, K., Nagahama, Y., & Grau, E. G. (1989). Histological and Ultrastructural Evidence for the Role of Gonadal-Steroid Hormones in Sex Change in the Protogynous Wrasse *Thalassoma duperrey*. *Environmental Biology of Fishes, 24*(2), 117-136.

Navas, J. M., Mananos, E., Thrush, M., Ramos, J., Zanuy, S., Carrillo, M., Zohar, Y., & Bromage, N. (1998). Effect of dietary lipid composition on vitellogenin, 17 beta-estradiol and gonadotropin plasma levels and spawning performance in captive sea bass (*Dicentrarchus labrax* L). *Aquaculture, 165*(1-2), 65-79.

Ohta, K., & Matsuyama, M. (2002). Steroidogenic pathways to 17,20 beta-dihydroxy-4-pregnen-3-one and 17,20 beta,21-trihydroxy-4-pregnen-3-one in the ovarian follicles of the bambooleaf wrasse *Pseudolabrus sieboldi*. *Fisheries Science, 68*(1), 41-50.

Ohta, K., Yamaguchi, S., Yamaguchi, A., Okuzawa, K., Gen, K., Kagawa, H., & Matsuyama, M. (2002). Biosynthesis of estradiol-17 beta in the ovarian follicles of the red seabream *Pagrus major* during vitellogenesis. *Fisheries Science, 68*(3), 680-687.

Pankhurst, N.W., & Carragher, J.F. (1991). Seasonal endocrine cycles in marine teleosts. *In*: Scott, A. P., Sumpter, J. P., Kime, D. E., and Rolfe, M. S. (eds) Reproductive Physiology of Fish, vol 91. Sheffield: Fish Symposium, pp 131-135.

Panserat, S., & Kaushik, S. (2002). Nutritional regulation of hepatic glucose metabolism in fish: example of a poor user of dietary carbohydrates, the rainbow trout. *Productions Animales, 15*(2), 109-117.

Peng, S. M., Gao, Q. X., Shi, Z. H., Zhang, C. J., Wang, J. G., Yin, F., & Zhang, Y. L. (2015). Effect of dietary n-3 LC-PUFAs on plasma vitellogenin, sex steroids, and ovarian steroidogenesis during vitellogenesis in female silver pomfret (*Pampus argenteus*) broodstock. *Aquaculture, 444*, 93-98.

Peterson, B. C., Booth, N. J., & Manning, B. B. (2012). Replacement of fish meal in juvenile channel catfish, *Ictalurus punctatus*, diets using a yeast-derived protein source: the effects on weight gain, food conversion ratio, body composition and survival of catfish challenged with *Edwardsiella ictaluri*. *Aquaculture Nutrition, 18*(2), 132-137.

Polakof, S., Panserat, S., Soengas, J. L., & Moon, T. W. (2012). Glucose metabolism in fish: a review. *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology, 182*(8), 1015-1045.

Rawat, V. S., Rani, K. V., Phartyal, R., & Sehgal, N. (2013). Vitellogenin genes in fish: differential expression on exposure to estradiol. *Fish Physiology and Biochemistry, 39*(1), 39-46.

Rinchard, J., Kestemont, P., Kuhn, E. R., & Fostier, A. (1993). Seasonal-Changes in Plasma-Levels of Steroid-Hormones in an Asynchronous Fish the Gudgeon *Gobio gobio* L (Teleostei, Cyprinidae). *Gen Comp Endocrinol, 92*(2), 168-178.

Shankar, D. S., Sudarashan, S., & Kulkarni, R. S. (2007). Role of cortisol on condition factor in the female freshwater fish, *Notopterus notopterus* during four reproductive phases. *Journal of Environmental Biology, 28*(2), 275-278.

Smits, R. J., Luxford, B. G., Mitchell, M., & Nottle, M. B. (2013). Embryo survival, but not first-parity litter size, is increased when gilts are fed diets supplemented with omega-3 fatty acids from fish oil. *Animal Production Science, 53*(1), 57-66.

Snelson, F. F., Rasmussen, L. E. L., Johnson, M. R., & Hess, D. L. (1997). Serum concentrations of steroid hormones during reproduction in the Atlantic stingray, *Dasyatis sabina*. *Gen Comp Endocrinol, 108*(1), 67-79.

Stone, D. A. J. (2003). Dietary carbohydrate utilization by fish. *Reviews in Fisheries Science, 11*(4), 337-369.

Svoboda, M., Kouril, J., Hamackova, J., Kalab, P., Savina, L., Svobodova, Z., & Vykusova, B. (2001). Biochemical profile of blood plasma of tench (*Tinca tinca* L.) during pre- and postspawning period. *Acta Veterinaria Brno, 70*(3), 259-268.

Tandler, A., Harel, M., Koven, W. M., & Kolkovski, S. (1995). Broodstock and larvae nutrition in gilthead seabream *Sparus aurata* - New findings on its mode of involvement in improving growth, survival and swimbladder inflation. *Israeli Journal of Aquaculture-Bamidgeh, 47*(3-4), 95-111.

Taranger, G. L., Carrillo, M., Schulz, R. W., Fontaine, P., Zanuy, S., Felip, A., Weltzien, F. A., Dufour, S., Karlsen, O., Norberg, B., Andersson, E., & Hausen, T. (2010). Control of puberty in farmed fish. *Gen Comp Endocrinol, 165*(3), 483-515.

Taranger, G. L., Haux, C., Stefansson, S. O., Bjornsson, B. T., Walther, B. T., & Hansen, T. (1998). Abrupt changes in photoperiod affect age at maturity, timing of ovulation and plasma testosterone and oestradiol-17 beta profiles in Atlantic salmon, *Salmo salar*. *Aquaculture, 162*(1-2), 85-98.

Upadhyaya, N., & Haider, S. (1986). Germinal Vesicle Breakdown in Oocytes of Catfish, *Mystus vittatus* (Bloch) - Relative Invitro Effectiveness of Estradiol-17-Beta, Androgens, Corticosteroids, Progesterone, and Other Pregnene Derivatives. *Gen Comp Endocrinol, 63*(1), 70-76.

Verslycke, T., Vandenbergh, G. F., Versonnen, B., Arijs, K., & Janssen, C. R. (2002). Induction of vitellogenesis in 17 alpha-ethinylestradiol-exposed rainbow trout (*Oncorhynchus mykiss*): a method comparison. *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology, 132*(4), 483-492.

Viegas, I., Rito, J., Gonzalez, J. D., Jarak, I., Carvalho, R. A., Meton, I., Pardal, M. A., Baanante, I. V., & Jones, J. G. (2013). Effects of food-deprivation and refeeding on the regulation and sources of blood glucose appearance in European seabass (*Dicentrarchus labrax* L.). *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology, 166*(3), 399-405.

Wang, N., Teletchea, F., Kestemont, P., Milla, S., & Fontaine, P. (2010). Photothermal control of the reproductive cycle in temperate fishes. *Reviews in Aquaculture*, 2(4), 209-222.

Watanabe, T., & Vassallo-Agius, R. (2003). Broodstock nutrition research on marine finfish in Japan. *Aquaculture, 227*(1-4), 35-61.

Westring, C. G., Ando, H., Kitahashi, T., Bhandari, R. K., Ueda, H., Urano, A., Dores, R. M., Sher, A. A., & Danielson, P. B. (2008). Seasonal changes in CRF-I and urotensin I transcript levels in masu salmon: Correlation with cortisol secretion during spawning. *Gen Comp Endocrinol, 155*(1), 126-140.

Wilson, K. S., Matrone, G., Tucker, C. S., Mullins, J. J., Kenyon, C. J., Hadoke, P. W. F., & Denvir, M. A. (2013). Glucocorticoids play a key role in maturation and structural organisation of the developing zebrafish cardiovascular system. *European Heart Journal, 34*, 275-275.

Yaron, Z., Terkatin-Shimony, A., Shahan, A. Y., & Salzer, H. (1977). Occurrence and biological activity of estradiol-17 in the intact and ovariectomized Tilapia aurea. *Gen. comp. Endocrinol*., 33, 45-52.

Zakeri, M., Marammazi, J. G., Kochanian, P., Savari, A., Yavari, V., & Haghi, M. (2009). Effects of protein and lipid concentrations in broodstock diets on growth, spawning performance and egg quality of yellowfin sea bream (*Acanthopagrus latus*). *Aquaculture, 295*(1-2), 99-105.

Zeginiadou, T., Kolias, S., Kouretas, D., & Antonoglou, O. (1997). Nonlinear binding of sex steroids to albumin and sex hormone binding globulin. *European Journal of Drug Metabolism and Pharmacokinetics, 22*(3), 229-235.

Chapter three

Hormone Induction and Blood Steroid Manipulation for the Artificial Spawning of *Labrus bergylta*

3.1 Introduction

3.1.1 Reproductive hormones and control of spawning

Poor breeding success in captivity is a common reason for failure to close the lifecycle for the production of finfish in aquaculture (Mylonas and Zohar, 2001a). Numerous studies have indicated failure of captured fish to spawn due to unsuccessful oocyte maturation and ovulation, with most of the developing oocytes eventually undergoing apoptosis (Wang *et al*., 2008; Podhorec and Kouril, 2009). Figure 3.1 shows how the reproductive processes of fish are regulated by the hypothalamo-pituitary-gonadal axis (Rosenfeld *et al*., 2012). Stimulation of this pathway can be due to external or internal inputs including temperature, presence of spawning grounds or food (Hamaguchi *et al*., 2002; Moore *et al*., 2007; de Lapeyre *et al*., 2009; Phelps *et al*., 2009; Li *et al*., 2015). Once the pathway is stimulated, the hypothalamus of the brain discharges gonadotrophin-releasing hormone (GnRH) or corticosteroid releasing hormone (CRH) in order to stimulate the function of gonadotrophs and corticotrophs in the anterior pituitary gland (Stuartkregor *et al*., 1981; Kime, 1993; Wootton and Smith, 2014). In response to stimulation, the anterior pituitary in turn secretes the gonadotropin hormones such as luteinizing hormone (LH) or follicle stimulating hormone (FSH) or adrenocortical tropic hormone (ACTH) into the blood. These tropic hormones stimulate the gonads and the adrenal cortex to synthesize and secrete sex steroids (estradiol, testosterone and progesterone) in order to prepare for gamete production which in turn induces final gonad maturation and ovulation (Peter *et al*., 1988; Tacon *et al*., 2000; Chaves-Pozo *et al*., 2008; Perini *et al*., 2013; Martins *et al*., 2014).

The function of the different reproductive steroids can generally be summarised as follows: estradiol is transported by the blood to the liver and stimulates the hepatic synthesis and secretion of the yolk protein vitellogenin which finally accumulates in the oocytes (Beresford *et al*., 2011; Nyina-Wamwiza *et al*., 2012; Baumann *et al*., 2013; Rawat *et al*., 2013; Oguz *et al*., 2015). Testosterone both

stimulates granulosa cells to produce estradiol and is a major hormone for male gonad development. In mammals, progesterone sustains a pregnancy but in fish, this hormone triggers the process of final maturation in eggs and sperm. Cortisol is released in response to stress and a low level of blood glucocorticoids or low blood glucose. Its primary function in spawning is to increase blood sugar by stimulating energy production through gluconeogenesis e.g. in the event of spawning. High levels of cortisol also result from the stress response, which may reduce spawning success (Espmark *et al*., 2008; Westring *et al*., 2008; Cook *et al*., 2011; Hoogenboom *et al*., 2012; McConnachie *et al*., 2012; Baker and Vynne, 2014; Ganesh, 2014). The mechanisms described may exhibit negative (inhibin) or positive (activin) feedback loops from the gonads which control the regulation of steroids, maintaining homeostasis.

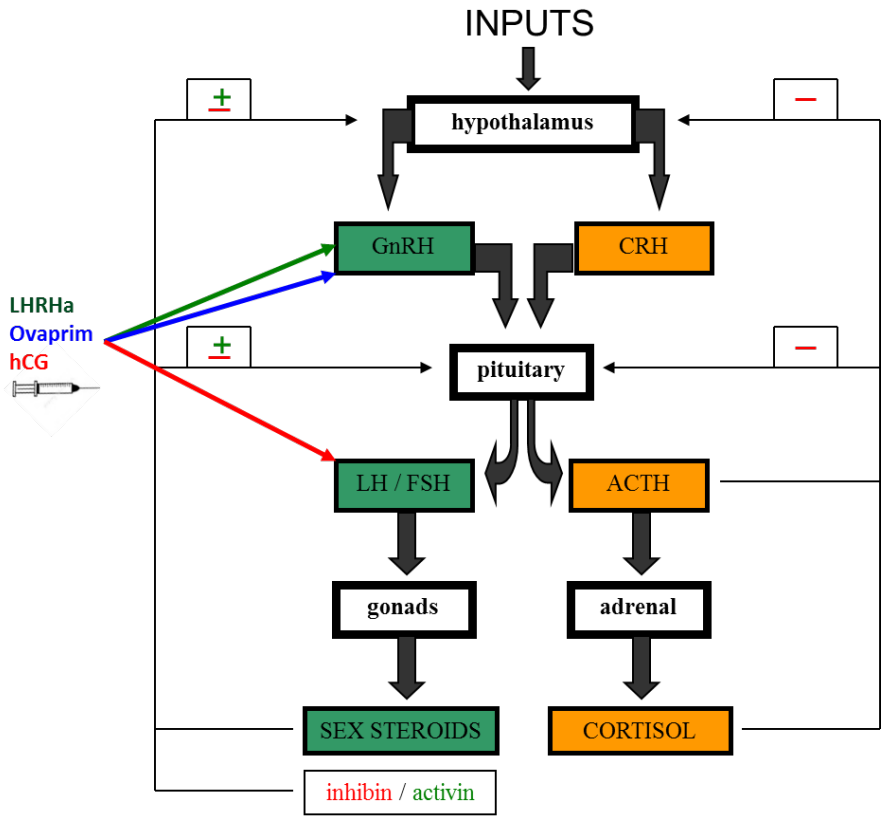


Figure 3.1: Schematic diagram of gonadotropins action on hypothalamo-pituitary-gonado axis in response to steroids production.

As can be seen in Figure 3.1, GnRH plays an important role in the regulation of gonadal development and ovulation, as does its mediation by endogenous neuroendocrine and endocrine changes in the teleost brain (Lethimonier *et al*., 2004; Abe and Oka, 2011; Gomes *et al*., 2013). This provides a basis for the artificial control of spawning by administration of synthetic agonists of gonadotropin-releasing hormones (GnRHa) or pituitary extracts, or purified fish gonadotropins like LH or human chorionic gonadotropin (hCG). Synthetic GnRHa has been extensively used in fish breeding to induce LH synthesis for final oocyte maturation, ovulation and spawning (Fornies *et al*., 2001). This is because studies have suggested that, in teleosts, the low occurrence of ovulation and spawning events in captivity could be a result of the failure to release pituitary LH (Planas *et al*., 2000).

3.1.2 Hormonal manipulation

GnRHa delivery systems mimic the function of GnRH in the hypothalamus and release GnRHa via neuroendocrine systems from a time period of a few hours (Pankhurst *et al*., 1986; Zanuy *et al*., 1986; Mylonas and Zohar 2001b; Prat *et al*., 2001), to several days (Mylonas *et al*., 1992) or over many weeks (Vazirzadeh *et al*., 2008). This stimulates the LH plasma levels together with FSH from the anterior pituitary gland into the blood circulation, inducing final oocyte maturation. Most of these strategies for spawning induction involve either implant (Morehead *et al*., 1998; Fornies *et al*., 2001; Berlinsky *et al*., 2005), intra-peritoneal injection (Hill *et al*., 2005; Vazirzadeh *et al*., 2008; Noori *et al*., 2010), intramuscular injection or even topical applications (Hill *et al*., 2005). Hence, exogenous gonadotropin-releasing hormones such as LHRHa (luteinizing hormone releasing hormone analogue) (Hassin *et al*., 1998), hCG (human chorionic gonadotropin) (Zairin, Asahina, Furukawa and Aida, 1992; Zairin, Furukawa and Aida, 1992a, 1992b) or Ovaprim® (salmon GnRHa + dopamine inhibitor) (DiMaggio *et al*., 2014; Chaube *et al*., 2014) may be administered into the blood by several routes.

LHRHa is a synthetic analogue of the naturally occurring GnRH hormone and is usually used in preference for experimental studies because of its longer-lasting effect (Noori *et al*., 2010; Su *et al*., 2013). In higher organisms, LHRHa supports estradiol production in females via an acute rise which triggers ovulation and corpus luteum

formation, while in males, it stimulates testosterone production. When compared to a control group, LHRHa treatments resulted in significant increase in egg volume in wild-caught female striped trumpeter (*Latris lineata*) (Morehead *et al*., 1998). Despite its apparently shorter activity relative to LHRHa, certain formulations of GnRHa e.g. FIA [Freund's incomplete adjuvant]-emulsified GnRHa can be effective in advancing the onset and synchronization of ovulation in rainbow trout within a two-week period, shortening the egg collection period without affecting broodstock survival and egg quality (Vazirzadeh *et al*., 2008).

Another agent that acts as a pituitary analogue is the human chorionic gonadotropin (hCG) [D-Ala6, Pro9-NEt mGnRHa ethylamide] which acts similarly to the luteinizing hormone (LH), with both hormones being capable of promoting egg maturation and inducing ovulation. In mammals, hCG is placental in origin (rather than from pituitary) and can also trigger ovulation and directly maintains pregnancy prior to progesterone. hCG has been an effective spawning agent in Asian catfish, *Clarias batrachus* (Sahoo *et al*., 2007; Sahoo *et al*., 2010) and in captive yellowfin bream, *Acanthopagrus australis* (Sparidae) (Black and Black, 2013). hCG significantly affects in-vitro steroidogenesis in gonadal fragments of a protogynous hermaphroditic wrasse (Morrey *et al*., 1995) and promoted gonad reversal in the bluehead wrasse, *Thalassoma bifasciatum* (Koulish and Kramer, 1989).

Artificial hormones have recently been developed to give better spawning results by synergistically formulating analogues of gonadotropins with a dopamine inhibitor to facilitate ovulation and spawning. Salmon gonadotropin releasing hormone analogue (sGnRHa [D-Arg6-Pro9-Net sGnRH]) together with a brain neurotransmitter inhibitor (dopamine antagonist, domperidone) was formulated as a commercial product called Ovaprim® (Syndel Laboratories, Ltd.). The GnRHa ingredient within Ovaprim® elicits the release of stored gonadotropins from the pituitary gland while domperidone serves to inhibit GnRH release. Domperidone is a blocker of dopamine receptors, therefore this ingredient is very important for species in which the reproductive cascade would be stopped due to stressors that lead to dopamine release, because dopamine will block GnRH activity. Ovaprim® comes in liquid form and is a common spawning inducer in numerous fish species used in aquaculture (Chaube *et al*., 2014). Successful captive

breeding performance of the Malaysian mahseer, *Tor tambroides* and *Tor douronensis* was achieved with 0.5ml/kg Ovaprim® (Ingram *et al*., 2007). Dopamine antagonists have also been used in conjunction with LHRHa treatments to improve spawning success in channel catfish, *Ictalurus punctatus* (Silverstein *et al*., 1999), senegalese sole, *Solea senegalensis* (Guzman *et al*., 2011) and streaked prochilod, *Prochilodus lineatus* (Characiformes) (Viveiros *et al*., 2013).

3.1.3 Optimisation of spawning induction protocols

Various hormonal formulations, dosages and combinations have been tested to compare performance in inducing a spawning event. For example, a successful spawning event occurred in *E. striatus* using hCG combined with LHRHa as priming or resolving injections in which it was suggested these hormones could be used interchangeably. The difference between them is that, hCG has a prominent role in exerting gonadotropic activity, whereas LHRHa, being a hypothalamic hormone stimulates the release of endogenous gonadotropins stored in the pituitary (Watanabe *et al.,* 1995). Ovaprim® and hCG used in combination can also produce spawning, with higher dosages being associated with increased fecundity (Haniffa and Sridhar, 2002). Timing of administration can also be critical. Research has shown that efficacy of hormonal treatments was guaranteed only when this was carried out straight away after capture from the wild; even a delay of 24 hours post capture reduced its efficacy (Haddy and Pankhurst, 2000). Capture and handling stress can reduce the responsiveness of fish to exogenous hormone treatment and the best results are obtained if hormonal treatment is administered at the time of capture (Chatakondi, 2014).

3.1.4 Induced spawning and successful egg and larval production

An important element of artificial spawning is the production of viable eggs with high fertilisation rates and good larval survival. This consideration may affect the choice of hormone and the administration protocol. For example, injected or implanted LHRHa was effective for inducing ovulation in black sea bass (*Centropristis striata*) with maximum oocyte diameters >500 μm, however the quality of eggs produced were highly variable (Berlinsky *et al*., 2005). In one study, Ovaprim® had better performance

than LHRHa or hCG in inducing higher spawning frequency, fecundity and viability of the eggs of *Channa punctatus* (Haniffa *et al*., 2004) but superiority of Ovaprim® was not found in other species (Legendre *et al*., 2000). The method of hormone administration may affect spawning success (Hill *et al*., 2005) and may also affect egg quality after spawning (Lee *et al*. 2000). Whether hormones are administered topically or by injection can affect latency period (i.e. time elapsed between hormone injection and ovulation), however faster action (lower latency) may result in lower quality eggs (Hill *et al*., 2005). Varying hormonal dosages used in spawning induction may also affect the fertilisation process and hatching rate e.g. in Korean spotted sea bass (*Lateolabrax maculates*) 1000 International Units (IU)/kg of hCG injection had a 5% better fertilisation and ~7% better hatching rate than 2000IU/kg of hCG injection (Lee and Yang, 2002). Indeed, higher doses of gonadotropin sometimes produced negative affects resulting in either premature ovulation or over-ripeness (Hill *et al*., 2005). Low fertilisation rates, in turn, may be due to asynchronicity between the process of maturation and ovulation (Patino and Sullivan, 2002). Increased deformity in larvae at lower or higher doses may be attributed to the fertilisation of unripe or overripe ova.

3.1.5 Artificial reproduction in *Labrus bergylta*

Seasonality of *Labrus bergylta* reproduction varies largely along latitudinal gradients in western Europe (Treasurer, 1994; Muncaster *et al*., 2008; Muncaster *et al*., 2010; Muncaster *et al*., 2013). Muncaster *et al*. (2010) found that, within a breeding season, there was a 60-day difference in the peak spawning period between Ireland and Norway. The use of *Labrus bergylta* as a cleaner fish in salmon aquaculture has stimulated interest in closing the lifecycle in this species and given rise to an international project on this topic ('EcoFish' http://www.northernperiphery.eu/en/projects/show/&tid=18). Of three partners (Ireland, Scotland and Norway), the natural spawning of captive *L. bergylta* was only achieved in Bukta Research Station, Norway. In comparable studies for yellowfin bream, *Acanthopagrus australis*, captive female fish fail not only to spawn naturally but also to complete vitellogenesis and oocyte maturation. In this case, the efficacy of two exogenous hormones (GnRHa and hCG) resulted in consistent spawning response (Black and Black, 2013). Cultured fish may fail to mature due to a lack of a

gonadotropin surge, probably due to a dissimilarity in environmental conditions between the culture system and the natural environment (Donaldson and Hunter, 1983; Mylonas *et al*., 1997; Kelley *et al*., 2006; Sarkar *et al*., 2006; Raizada *et al*., 2013; Imanaga *et al*., 2014). There is evidence that the commercially available hormones LHRHa, hCG and Ovaprim® can have a major role in egg maturation and spawning (Haddy and Pankhurst, 2000; Krol *et al*., 2009). Therefore, this current study will utilise these hormones in an attempt to induce artificial spawning of *L. bergylta*. This research will be the first to study the artificial spawning induction of *L. bergylta*, including manipulation of both wild and captive fish. The strategy of comparing wild and captive populations has led to increased understanding in the past (e.g. Haddy and Pankhurst, 2000) and it may help explain why natural spawning of captive *L. bergylta* is successful in Norway but failed elsewhere. The study will measure reproductive steroid levels in the blood before and after each experimental treatment (relative to sham); also, a full experimental set-up will be employed to examine any agonist (stimulatory) and antagonist (inhibitory) interactions among three artificial spawning treatments in the captive individuals. Finally, evaluations of egg viability, fertilisation success and larval survival rate until 7 days post-hatch (dph) will be made, as this data is important in evaluating the success of the induction protocol for *L. bergylta*.

3.2 Materials and Methods

3.2.1 Broodstock sampling and management

The study of spawning induction of *L. bergylta* was conducted in April 2011 at Carna Research Station of National University of Ireland Galway, Republic of Ireland. Broodstock were captured by trammel nets at Carna Bay, Co. Galway, Ireland. The hormonal induction of spawning was carried out in 2011 in several trials that distinguished between 'wild' *L. bergylta* broodstock sampled just prior to experimentation and 'captive' *L. bergylta* that had been sampled up to one year prior to experimentation. Broodstock sampling occurred in either April - September 2010 for a 'captive trial' or in March 2011, just two weeks prior to the experimental treatment for 'wild trial 1' and 'wild trial 2'.

Broodstock were fed to satiation three times a week on defrosted mussel meats and chopped squid tubes. All experimental animals were tagged on their left shoulder using passive integrated transponders (PIT tags, AVID, UK) (Figure 3.2a). They were maintained in round plastic tanks (5000 liters, 1.5 meter depth and 4.30 m^2) at a density of 5 to 6 kgm^{-3} and exposed to natural photoperiod and water temperatures was ranged from ~13 to 15°C (Figure 3.2b). The tank sides and base were blacked out to reduce stress and contained negatively buoyant shredded black plastic as artificial cover. All tanks were on continuous flow-through of full strength mechanically filtered (50 micron) natural seawater at an exchange rate of 400% total volume/d^{-1}. Dissolved oxygen (DO), temperature and salinity levels were recorded daily (Oxy-Guard Handy Series of Portable DO Meters) and maintained respectively at 6.5±0.3 ppm (8.3 mg/l saturation), 13 to 15°C and 34‰. Tanks were covered in 3 cm square mesh net to prevent fish from jumping out of the tanks.

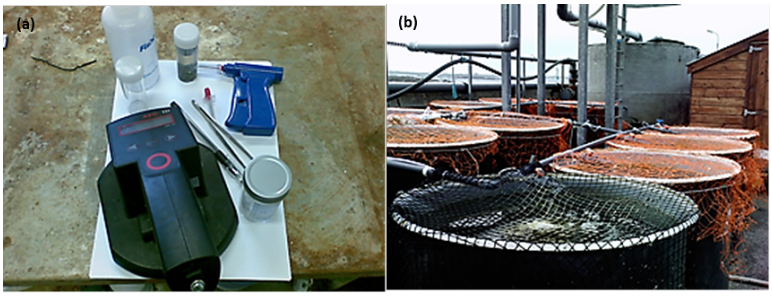


Figure 3.2: (a) PIT Tag (2x12mm bio glass transponder), PIT Tag reader, needle (40 mm length) and implanter (b) open air experimental fish tanks.

Ninety-six individual *L. bergylta* from the 'captive trial' broodstock were distributed in 12 spawning tanks (eight individuals per tank) prior to artificial spawning induction. Within each tank, individuals ranged in size from 400 g to 1200 g total body weight (-see below for more details on experimental set-up). Similarly, for the 'wild trial(s)' broodstock, 64 individuals ranging from 400 g to 1200 g total body weight were distributed within eight spawning tanks (eight individuals per tank). The wild experimental trial was further divided into trial 1 and trial 2 (four tanks each), as shall be explained below.

It was often not possible to determine sex in experimental individuals, so *L. bergylta* in experimental tanks were graded: each tank contained three small [500 to 700 g body weight], two medium [700 g to 900 g] and three large [>900 g]) individuals, giving a total of eight individual fish per tank. The grouping was designed to reflect the varying body sizes found within wild harem structures in an attempt to mimic normal breeding behaviour interactions within experimental units. In addition to the set-up described above, 15 mature and productive males were identified in early Spring 2011 based on the presence of expressible milt upon gentle abdominal pressure (for egg fertilisation). In order to be sure of a supply of males for the experiment, these individuals were quarantined in a separate tank. To minimize stress, blood samples and body weight/length were taken only prior to hormonal injection (see below).

3.2.2 Hormonal induction – Handling protocol

Experimental treatments for all trials (wild and captive) were initiated during the natural spawning period for this species starting on April 20th and finishing on May 15th 2011. Prior to blood sampling or hormonal injection, individuals were netted and sedated by immersion in 6 litres of aerated saltwater with 100mg/L concentration of tricane methanesulfonate (MS222, Sigma) to reduce tissue injury and minimize handling stress. Blood samples were taken twice from each individual fish for evaluating changes of plasma steroids pre- and post-treatment. The first blood samples were collected before the first injection ('before' sample) and the second blood samples were collected after the third induction injection ('after' sample); sometimes the 'after' sample took place after spawning had occurred. During blood sampling, 2 ml of blood was taken from a

puncture of caudal vein (venepuncture) with blood and plasma sampling following the protocol described in Chapter two (2.2.6 Blood and tissue sampling). Blood plasma was stored at -80°C prior to enzyme-linked immuno-sorbent assay (ELISA) evaluations for estradiol, testosterone, progesterone and cortisol levels. ELISA has been previously used to indicate these steroids in blood serum (Barry *et al*., 1993) and plasma (Chapter two; Velasco and Cruz, 2007; Muncaster *et al*., 2010).

To perform a hormonal injection, the broodstock were first anaesthetised with MS-222 before a needle was inserted beneath a scale at a 45 degree angle into the musculature (IM) of the upper left shoulder until the tip is fully inserted before the compound injected (Figure 3.3). The needle was left within the musculature for several seconds after delivery of the hormone before withdrawing to prevent back-seepage. All equipment was sterile to limit infection potential. This procedure follows the "blind puncture" technique to inject substances into *in-situ* tissue (Ardashev and Kiseleva, 2000).

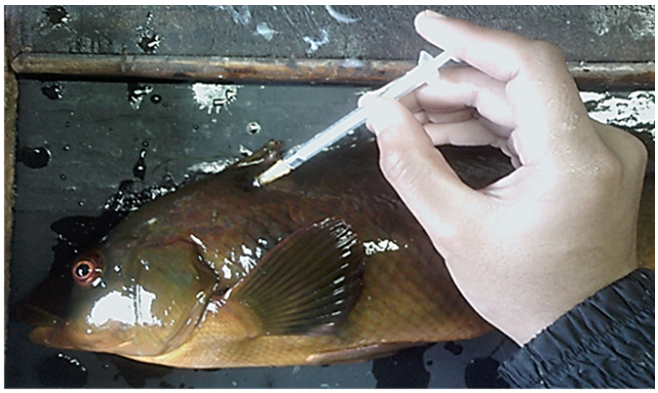


Figure 3.3: Intramuscular injection in spawning induction protocol on the left shoulder of anaesthetized *Labrus bergylta.*

3.2.3 Hormonal treatments and experimental design

The hormonal induction protocol was a modification of methodology from Billard *et al*. (1984) and Shibatta *et al*. (2011). This protocol involved three consecutive injections with various combinations of placebo/sham, LHRHa, hCG and Ovaprim®. These treatments were chosen because of their anticipated activity at various points in the hypothalamo-pituitary-gonad axis (Figure 3.1). The time interval between each injection was 24 hours. Designated experimental treatment groups for captive and wild experimental trials are shown in Table 3.1 and 3.2, respectively (Appendix 3).

Table 3.1: Schematic design of hormonal treatment groups used for captive fish, n=8 in each tank were graded into small (n=3), medium (n=2) and large (n=3) sizes.

TANK 1 **Control** 3x shams *replicate 1*	TANK 2 **Control** 3x shams *replicate 2*	TANK 3 **LHRHa** Treatment + 2x shams *replicate 1*	TANK 4 **LHRHa** Treatment + 2x shams *replicate 2*
TANK 5 **LHRHa + hCG** 2 Treatments + sham *replicate 1*	TANK 6 **LHRHa + hCG** 2 Treatments + sham *replicate 2*	TANK 7 **hCG** Treatment + 2x shams *replicate 1*	TANK 8 **hCG** Treatment + 2x shams *replicate 2*
TANK 9 **LHRHa + hCG + Ovaprim®** 3 Treatments *replicate 1*	TANK 10 **LHRHa + hCG + Ovaprim®** 3 Treatments *replicate 2*	TANK 11 **hCG + Ovaprim®** 2 Treatments + sham *replicate 1*	TANK 12 **hCG + Ovaprim®** 2 Treatment + sham *replicate 2*

Table 3.2: Schematic design of hormonal treatment groups used for wild fish -please note that this design was carried out once for 'wild trial 1' and repeated for 'wild trial 2'.

TANK 1 (Trial 1/Trial 2) **LHRHa + hCG + Ovaprim®** 3 Treatments *replicate 1*	TANK 2 (Trial 1/Trial 2) **LHRHa + hCG + Ovaprim®** 3 Treatments *replicate 2*
TANK 3 (Trial 1/Trial 2) **Control** 3x shams *replicate 1*	TANK 4 (Trial 1/Trial 2) **Control** 3x shams *replicate 2*

Agonistic and antagonistic interactions between hormonal treatments on blood steroids levels and spawning was evaluated in captive fish as follows: captive individuals were divided into six treatment groups, represented by different colours in Table 3.1, with two replicate tanks for each treatment. Group (1) (Tank 1 and 2), fish were treated with three sham injections of saline (0.5% NaCl) as control. Group (2)

(Tank 3 and 4), fish received one LHRHa and two sham injections. Group (3) (Tank 5 and 6), fish were treated with one each LHRHa, hCG and sham injection. Group (4) (Tank 7 and 8), fish were treated with one hCG and two sham injections. In Group (5) (Tank 9 and 10), fish were treated with one injection each of LHRHa, hCG and Ovaprim®. Finally, Group (6) (Tank 11 and 12), fish were treated with one injection each of hCG, Ovaprim® and a sham.

In wild trials of *L. bergylta*, the broodstock were obtained only two weeks prior to their natural spawning period for experimental induction. These individuals had been conditioned in the wild and therefore represented a useful contrast with animals that had been held in captivity for several months. There were only two treatment groups in the wild trial, as illustrated in Table 3.2. This simplified experimental design comprised two replicate tanks in which fish were treated sequentially with LHRHa + hCG + Ovaprim® injections; and two further replicate tanks in which fish were treated with three sham injections of saline (0.5% NaCl) as control. 'Wild trial 2' was identical to 'wild trial 1' in design, with a single difference, which was evaluating the same individual fish for ability to carry out repeat spawning.

3.2.4 Preparation of hormone dosage

To calculate the correct dosage, the recommended dose, approximate weight of the brood fish and desired volume of the injection must be determined. For example, the amount of hormone to administer for Ovaprim® (Syndel Laboratory Company), is based on a standard fish hormonal induction protocol: [weight of fish (g) x hormone dosage (ml/kg) = volume of Ovaprim® for injection].

Ovaprim® (Syndel Laboratories Ltd, Canada), consisting of Salmon Gonadotropin Releasing Hormone Analog (20µg/ml) and Domperidone (10mg/ml), was administered at the recommended dose of 0.5ml/kg body weight (BW). 1mg (1,000 µg) of powdered LHRHa (Syndel Laboratories Ltd, Canada) was dissolved in a calculated amount of distilled water based on the fish body weight (BW) and administered at a dosage of 0.06mg/kg (60µg/kg BW). 10,000 International Unit (I.U.) hCG (Argent Chemical Laboratories, Redmond, WA, USA) was dissolved in 4 ml of 0.7% physiological saline (NaCl) to obtain a final concentration of 500I.U./kg BW hCG

(Omeljaniuk, Shih and Peter, 1987). A standard calculation protocol for preparing each LHRHa, hCG and Ovaprim® dosages are summarised in Appendix 2. The volume of distilled water was adjusted for each of the three size classes of *L. bergylta* to get the same dosage per size class. The volume of the saline sham was identical to the hormonal treatment in each case. Fishing handling during routine husbandry and experimentation was carried out according to national and institutional regulations and the current European Union legislation on handling experimental animals.

3.2.5 Egg collection, fertilisation and incubation

To establish the presence of eggs, fish were manually checked by exerting gentle abdominal pressure 24 hours post-treatment (with each individual injection or control). Female fish were anaesthetised using MS222 and the genital area were cleaned with fresh water and dried. Eggs were removed by gentle hand stripping into dry clean 100 ml glass beaker (Figure 3.4a). Unspawned females were checked again for ovulation 12 hours later.

Stripped eggs were kept suspended in ovarian fluid and isolated from mixing with sea water before fertilisation was initiated. The reason was to avoid premature activation of stickiness and clumping of the gelatinous layer of the eggs. Eggs were kept cool for up to 15 minutes to avoid drying. The total egg volume was recorded and weighed in grams. A fecundity estimate was carried out by sub-sampling the fluid in a 0.5 ml tube (see section 3.2.6). Suspended eggs were promptly mixed with 0.05 ml fresh milt per batch of eggs from randomly selected productive males which had been hand stripped using a dry teaspoon to collect the milt. The mixture was gently stirred and 1ml of clean sea water was added then slowly mixed for 5 minutes to activate the sperm and rehydrate the eggs for optimal fertilisation.

Batches of eggs were then placed on a corrugated perspex plate which acted as an artificial substrate for egg attachment and easy handling during incubation in the hatchery. The incubation plate was horizontally immersed in 4 cm of seawater in order to spread the eggs evenly for good aeration during the incubation period (Figure 3.4b). Fertilized eggs were spread on the incubation plate using a teaspoon with eggs being dropped on the water surface and allowed to disperse for even attachment (Figure 3.4c).

The sticky eggs that had directly attached to the perspex plate (Figure 3.4d) were then ready for transportation and incubation in a cone (Figure 3.4e). The batches of eggs were maintained in separate cone incubators (Figure 3.4f) until hatching to determine egg quality. After a period of two hours post fertilisation, sub-samples of eggs were evaluated for fertilisation (see section 3.2.6).

Egg incubation temperature was recorded by Tiny Tag® remote temperature sensor device attached in the water column. Egg batches were incubated at an ambient temperature of either 12.80±0.37°C (wild trial 1) or 11.38±0.27°C (wild trial 2) in 70 L cones with upwelling. Water was gently aerated from a bottom central point to create an upwelling current. Dissolved oxygen concentration in the cones was recorded daily at a dissolved oxygen concentration of 6.0 to 8.0mg/L for all batches and at a low flow rate of 200 to 500ml/min to achieve a daily water exchange of 50 to 70% seawater and ensure the required oxygen concentration in each cone was always above 5mg/L or 80%. A 24-hour light photoperiod was maintained throughout the incubation phase. The incubated eggs were not disinfected as the outer sticky egg layer protected against contamination e.g. fungus or bacteria (Paxton and Willoughby, 2000) and no egg contamination was observed during the incubation period. The incubated eggs were checked twice a day for hatching and survival rates (see section 3.2.6). The developmental stage of the eggs in each incubator was determined daily according to D'arcy *et al*. (2012).

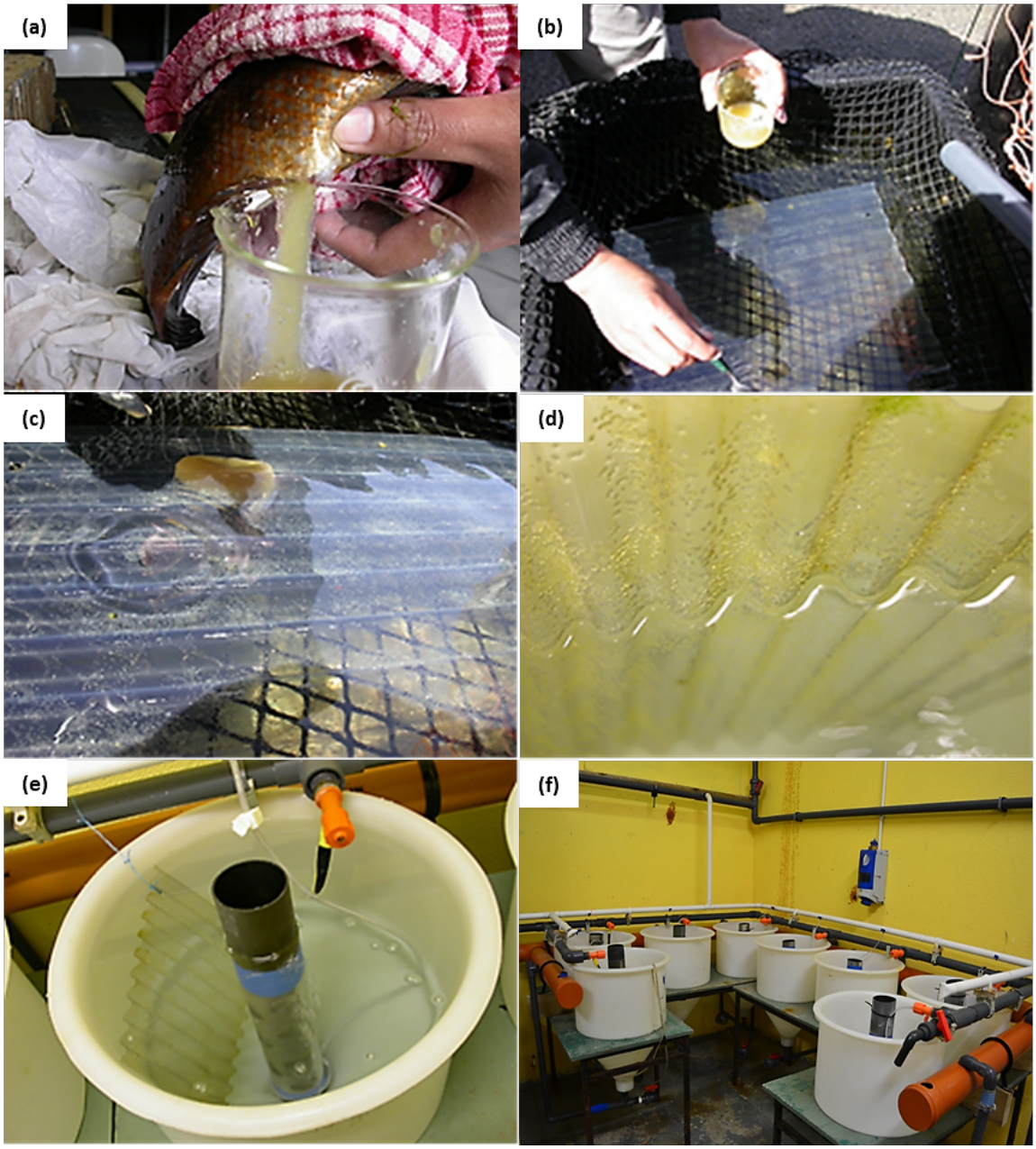


Figure 3.4: Sequence of an artificial fertilisation and incubation protocol of *Labrus bergylta* eggs; (a) hand stripping to express viable eggs; (b) attachment of fertilized eggs onto immersed corrugated perspex plate; (c) egg dispersed by dropping on the water surface; (d) attached eggs on a perspex plate; (e) incubated eggs in a cone with upwelling; (f) batches of incubated eggs in separate cones.

3.2.6 Determination of spawning performance and egg quality

Batch fecundity was determined volumetrically and then converted to the number of eggs per ml based on estimates showing that 0.1 ml volume of stripped eggs in ovarian fluid contains approximately 100±10 eggs. Each egg batch was placed inside a floating incubator made of plankton netting (500 µm) for optimal water flow and aeration (Figure 3.5) and was observed daily for egg development from 0 until 7 days post hatch (dph). Observation of eggs was carried out via dissecting microscope by removing 100 to 300 eggs from each batch and allowing these to attach to a 4 cm diameter transparent petri-dish which was placed in a submerged container.

Fertilisation success was determined and recorded by observing egg batches under dissecting microscope and digital camera to determine the gastrulation stage. The hatching rate was determined by calculating the number of hatched larvae divided by the number of incubated eggs. Larval survival was determined by counting larval numbers at 7 dph divided by larval numbers at 1 dph. All data was expressed as percentage. The new larvae were maintained in room temperature on a succession of live feed with algae (green water) and rotifers for the first week of a larval weaning trial (which will be discussed in Chapter four). Monitoring of water temperature, water exchange and aeration was conducted hourly over this entire period.

Figure 3.5: Floating incubator to subsample eggs for observing egg development, fertilisation, hatching and survival rates of the larvae.

3.2.7 Statistical analysis

Separate statistical analyses were conducted for each plasma steroid in all cases, below. A one-way ANOVA was carried out to evaluate the effect of 'Groups' i.e. captive *L. bergylta* versus wild *L. bergylta* from two sampling years 2010 and 2011. This analysis examined whether plasma steroids differed between groups before any hormonal treatments were given. The main analysis to evaluate variation in plasma steroids within different treatments and time points was carried out with a three-way ANOVA examining the effects of 'Treatment' (hormone or sham), 'Time' (before or after treatment), 'Size' (small, medium or large fish) and their interactions on the concentration of steroids. This statistical design was used to evaluate both experiments involving captive and wild *L. bergylta*, with separate statistical analyses being used in each case. Finally, although 'wild trial 2' was a repeat of 'wild trial 1' to evaluate repeat spawning, the statistical differences between both trials was evaluated using a four-way ANOVA, this time adding the factor 'Trial' (1 or 2) to the factors 'Treatment', 'Time' and 'Size', as before. For significant effects, post-hoc multiple comparisons were performed. A $\log_{10}$ transformation of the raw data was used when normality and homoscedasticity assumptions were not met.

The null hypothesis is that there is no effect of treatment (hormones/shams) on the outcome of spawning in *L. bergylta* was tested using Fisher's Exact Probability Test, which is a non-parametric version of Pearson's Chi Squared test of independence. This decision was made because the experiment was relatively small: 50% of the expected frequencies were <5 in wild trial 1 and 100% were <5 in wild trial 2. Even with continuity correction, the assumption that at least 80% of the cells must have an expected frequency of 5 or greater was not met. Fishers Exact Probability for count data was calculated using the 'stats' package in R Studio v 0.97.551 for Windows (R Core Team, 2013). This was carried out for a 4 x 2 contingency table using the *fisher.test* command. As almost no spawning occurred in the captive experiment, the latter procedure was only carried out in wild trials 1 and 2.

3.3 Results

3.3.1 Spawning induction - Overview

With the exception of a two individual spawners in the captive *L. bergylta* experiment, spawning only took place in wild *L. bergylta*. Spawning was significantly associated with hormonal treatments over sham (see below) and *L. bergylta* which spawned were between 30 and 40 cm in total length (700 – 900 g body weight). When spawning occurred, this mostly took place after the second injection or 24 hours post-hCG injection especially in wild trial 1. During wild trial 2 (i.e. repeat-spawning trial), the third treatment, Ovaprim®, produced eggs which were slightly lower in quality (maturity) and quantity. In the latter trial, some of the female *L. bergylta* had already spawned (post-hCG) and very few viable eggs were expressed post-treatment with Ovaprim® (Appendix 4).

3.3.2 Comparison of reproductive steroid concentrations in wild and captive *Labrus bergylta*

Table 3.3 shows the mean squares of estradiol, testosterone, progesterone and cortisol concentrations among three 'Groups' of fish which were captive *L. bergylta* (n=96) from 2010, wild *L. bergylta* from 2010 (n=21) and wild *L. bergylta* from 2011 (n=64). These steroid concentrations were obtained from the first blood samples ('before' sample) prior to the hormonal injection during their respective spawning periods each year (April-May). 'Before' treatment estradiol, progesterone and cortisol were significantly higher in wild versus captive *L. bergylta*; although in progesterone, this occurred only in 2011, possibly because of small sample size in 2010. Meanwhile testosterone was of similar concentration in wild and captive individuals (Table 3.4).

Estradiol concentrations were almost four-fold higher in wild 2010 samples and six-fold higher in wild 2011 samples by comparison with captive *L. bergylta* (Table 3.4). Cortisol was almost three-fold higher in wild samples (both years). The results suggest that estradiol, progesterone and cortisol concentrations of captive *L. bergylta* fell below the natural circulating steroids during the spawning period. Reproductive steroids showed quite a bit of variability; based on homogeneity of variances and levene statistics, estradiol recorded the highest variance (30.92) followed by progesterone

(14.25), cortisol (11.90) and testosterone (1.87). The variances were statistically significant among groups for estradiol, progesterone and cortisol ($p<0.01$) but not for testosterone ($p>0.05$).

Table 3.3: Mean squares from ANOVA for the effect of 'Groups' (i.e. captive, wild 2010 and wild 2011) in *Labrus bergylta* 'before' treatment steroids during the spawning period. Estradiol, testosterone, progesterone and cortisol concentrations were analysed separately by ANOVA.

		Means squares			
Source of variation	d.f	Estradiol	Testosterone	Progesterone	Cortisol
Groups	2	68.08**	5.33 n.s	7714.34**	17576.45**
Error	178	3.88	1.30	1363.63	1332.82
Total	180				

**($p<0.01$) highly significant
(n.s) not significant

Table 3.4: Least-square means of steroids and their standard errors in 'Groups' (i.e. captive, wild 2010 and wild 2011) of *Labrus bergylta* 'before' treatment during the spawning period. Estradiol, testosterone, progesterone and cortisol concentrations are given (ng/ml).

Groups	Estradiol	Testosterone	Progesterone	Cortisol
Captive (n=96)	0.40±0.03[a]	0.84±0.12[a]	16.85±2.97[a]	14.17±2.35[a]
Wild 2010 (n=21)	1.50±0.24[b]	0.84±0.09[a]	20.10±5.55[a]	40.99±9.02[b]
Wild 2011 (n=64)	2.27±0.40[b]	1.35±0.15[a]	36.63±6.09[b]	42.44±6.16[b]

[a, b]Means within a column within a group with different superscripts are significantly different at $p<0.05$.

3.3.3 Spawning induction and plasma steroids in captive *Labrus bergylta*

No captive *L. bergylta* spawned except for one fish out of eight in each of Tanks 7 and Tank 11 (i.e. n=2/96 spawners). Both cases of spawning were similar occurring post-hCG treatment i.e. prior to Ovaprim® in Tank 11 (Table 3.5). The results demonstrate that hormonal induction does not produce spawning relative to sham in the vast majority of cases of captive *L. bergylta*. The results show that hCG can induce spawning in a minority of fish.

Table 3.5: Different treatment groups of hormonal injections in captive *Labrus bergylta* with the respective spawning results.

TANK 1 **Control** 3x shams **(0/8 spawned)**	TANK 2 **Control** 3x shams **(0/8 spawned)**	TANK 3 **LHRHa** + 2x shams **(0/8 spawned)**	TANK 4 **LHRHa** + 2x shams **(0/8 spawned)**
TANK 5 **LHRHa + hCG**+ sham **(0/8 spawned)**	TANK 6 **LHRHa + hCG** + sham **(0/8 spawned)**	TANK 7 **hCG**+ 2x shams **(1/8 spawned post-hCG)**	TANK 8 **hCG**+2x shams **(0/8 spawned)**
TANK 9 **LHRHa + hCG + Ovaprim®** **(0/8 spawned)**	TANK 10 **LHRHa + hCG + Ovaprim®** **(0/8 spawned)**	TANK 11 **hCG + Ovaprim®** + sham **(1/8 spawned post-hCG)**	TANK 12 **hCG + Ovaprim®** + sham **(0/8 spawned)**

Statistical examination of the plasma steroid concentrations in captive *L. bergylta* showed a significant effect 'Treatment' x 'Time' interaction, indicating a significant effect of hormonal treatment over sham for three of the steroids: estradiol, progesterone and cortisol (Table 3.6). Estradiol and progesterone were significantly higher before treatment than afterwards, whereas cortisol was significantly lower before treatment than afterwards. There was no difference in testosterone levels before versus after treatment (Table 3.7). The non-significant 'Treatment' x 'Time' x 'Size' interaction indicated that the size of individual fish had no significant effect on whether the hormonal treatment altered steroid concentrations in any of the four cases of steroids.

Table 3.6: Mean squares from ANOVA for the effect of treatment (various hormonal combinations –see Table 3.5), time ('before' versus 'after'), fish size and the interactions of these factors on estradiol, testosterone, progesterone and cortisol concentrations in captive *Labrus bergylta.* The term of most interest is indicated in bold.

		Means squares			
Source of variation	d.f	Estradiol	Testosterone	Progesterone	Cortisol
Treatment	5	0.04^{**}	0.06^{**}	1.30^{**}	2.68^{**}
Time	1	0.15^{**}	0.67^{**}	4.83^{**}	2.89^{**}
Size	2	0.02^{*}	$0.02^{n.s}$	$0.13^{n.s}$	$0.21^{n.s}$
Treatment*Time	**5**	$\mathbf{0.02^{*}}$	$\mathbf{0.01^{n.s}}$	$\mathbf{2.40^{**}}$	$\mathbf{0.63^{**}}$
Treatment*Size	10	0.02^{**}	0.03^{*}	0.39^{*}	$0.23^{n.s}$
Time*Size	2	$0.00^{n.s}$	$0.03^{n.s}$	$0.02^{n.s}$	$0.28^{n.s}$
Treatment*Time*Size	10	$0.00^{n.s}$	$0.01^{n.s}$	$0.27^{n.s}$	$0.11^{n.s}$
Error	156	0.01	0.01	0.21	0.17
Corrected total	191				

**($p<0.01$) highly significant
*($0.01<p<0.05$) significant
$^{(n.s)}$ not significant

If we consider the treatment groups overall (i.e. 'before' and 'after' combined), the LHRHa + hCG had the highest mean concentration of estradiol (0.65±0.27ng/ml), but this was not significantly different from the sham (perhaps as there was high variability in the LHRHa + hCG treatment). However significantly lower estradiol was observed in hCG (0.23±0.03ng/ml) or LHRHa+hCG+Ovaprim® (0.16±0.02ng/ml) treatments relative to sham (0.38±0.05ng/ml; Table 3.7). Interestingly, the same two treatments showed significant differences for testosterone which had a lower concentration relative to sham, at concentrations of 0.29±0.04ng/ml (hCG) and 0.38±0.12ng/ml (LHRHa+hCG+Ovaprim®). Meanwhile the hCG + Ovaprim® treatment group (0.80±0.21ng/ml) had the highest mean level of testosterone but this was not different from the sham. For progesterone too, the LHRHa + hCG + Ovaprim® group (2.98±0.72ng/ml) had the lowest level of this steroid and was the only treatment which was significantly different from the sham (14.35±3.85ng/ml). The treatment with the highest progesterone level was LHRHa (25.84±6.63ng/ml). Cortisol concentrations were highest in hCG + Ovaprim® (61.17±15.91ng/ml) and sham (38.38±7.34ng/ml) groups, both of which were significantly higher than the other treatment groups.

Table 3.7: Least-square means and their standard errors for the effects of treatment (various hormonal combinations –see Table 3.5), time ('before versus 'after') and fish size on concentrations of estradiol, testosterone, progesterone and cortisol concentrations (ng/ml) in captive *Labrus bergylta.*

Factors	Subclass	Estradiol	Testosterone	Progesterone	Cortisol
Treatment	(1) Control	0.38±0.05^{cd}	0.76±0.30^{b}	14.35±3.85^{bc}	38.38±7.34^{d}
	(2) LHRHa	0.32±0.04^{bcd}	0.58±0.07^{b}	25.84±6.63^{c}	16.05±2.92^{c}
	(3) LHRHa+hCG	0.65±0.27^{d}	0.58±0.08^{b}	7.38±1.76^{ab}	17.31±3.44^{c}
	(4) hCG	0.23±0.03^{ab}	0.29±0.04^{a}	18.00±8.08^{ab}	9.20±2.45^{b}
	(5)LHRHa+hCG+Ovaprim	**0.16±0.02^{a}**	**0.38±0.12^{a}**	**2.98±0.72^{a}**	**7.05±2.73^{a}**
	(6) hCG+Ovaprim	0.30±0.04^{bc}	0.80±0.21^{b}	22.60±6.30^{c}	61.17±15.91^{d}
Time	**(1) Before**	**0.40±0.03^{a}**	**0.84±0.12^{a}**	**16.85±2.97^{a}**	**14.17±2.35^{a}**
	(2) After	**0.28±0.09^{b}**	**0.29±0.03^{b}**	**13.53±3.27^{b}**	**35.55±6.07^{b}**
Size	(1) Small	0.27±0.03^{a}	0.56±0.13^{a}	14.22±3.40^{a}	21.40±4.26^{a}
	(2) Medium	0.52±0.18^{b}	0.43±0.05^{a}	15.09±4.10^{a}	21.45±5.02^{a}
	(3) Large	0.29±0.02^{a}	0.66±0.11^{a}	16.23±3.99^{a}	30.60±7.06^{a}

$^{a, b, c}$Means within a column within a group with different superscripts are significantly different at $p<0.05$.
*Size of fish: 1 = <700 grams, 2 = 700 to 900 grams and 3 = >900 grams

Concentration of steroids before and after the different treatments is illustrated in Figures 3.6, 3.7 and 3.8. Figure 3.6 (a & b) shows that steroid concentrations decreased in the 'after' samples, irrespective of whether *L. bergylta* were treated with a sham or LHRHa. This decreasing trend in steroids post-treatment occurred in estradiol, progesterone and testosterone. By contrast, cortisol concentrations increased after injection with either sham or LHRHa.

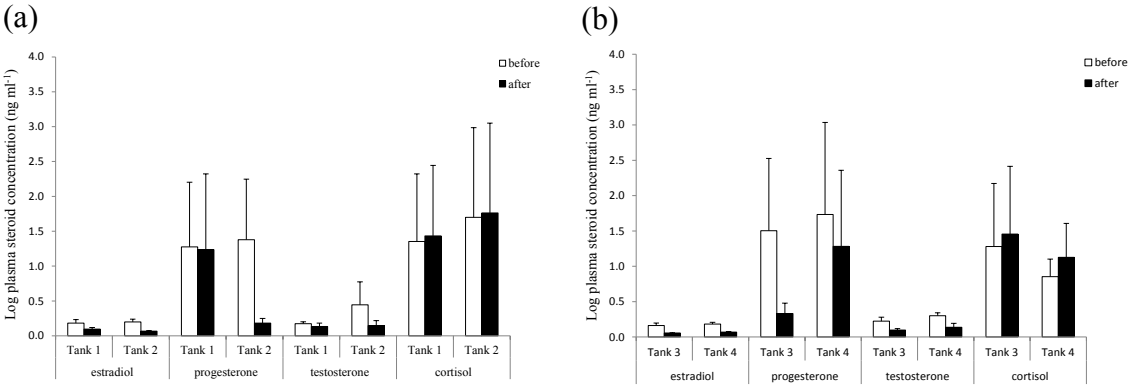


Figure 3.6: Log concentrations (ng/ml) of estradiol, progesterone, testosterone and cortisol before and after (a) sham and (b) LHRHa injections in captive *Labrus bergylta.*

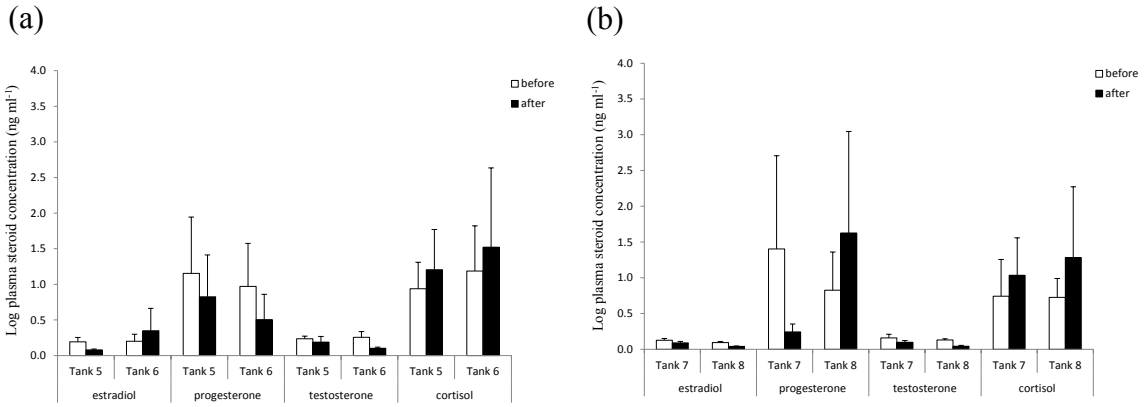


Figure 3.7: Log concentrations (ng/ml) of estradiol, progesterone, testosterone and cortisol before and after (a) LHRHa + hCG and (b) hCG injections in captive *Labrus bergylta.*

Figure 3.7 shows that results were similar, but a bit less conclusive for LHRHa + hCG or hCG treatments. Concentrations of estradiol in one replicate (Tank 6) and progesterone in another (Tank 8) increased after injection with LHRHa + hCG and hCG, respectively. However replicates of the respective treatments (Tank 5 and Tank 7) showed the opposite effect, with these steroids decreasing after the injections. The other results were as before; testosterone concentrations decreased in all replicates after treatments; whereas cortisol concentrations increased after both treatments, as before.

When LHRHa + hCG + Ovaprim® were administered to broodstocks, all steroid concentrations decreased afterwards, including cortisol in one of the replicate tanks (Tank 10). Albeit in Tank 9, the usual trend of cortisol increasing post-treatment was

observed (Figure 3.8 (a)). The only treatment showing a completely different trend was observed after injection with hCG + Ovaprim® (Figure 3.8 (b)), because this time, three steroids were elevated after treatment: estradiol, progesterone and cortisol (both replicate tanks). Only testosterone was lower post treatment with hCG + Ovaprim®. Overall the results suggest an antagonistic effect when LHRHa + hCG + Ovaprim® are administered in rapid succession whereas a synergistic effect in elevating reproductive steroids appears to be observed using hCG + Ovaprim® in combination. The reason this effect can be termed 'synergistic', is because hCG and Ovaprim® used in isolation did not have the effect of elevating the steroids.

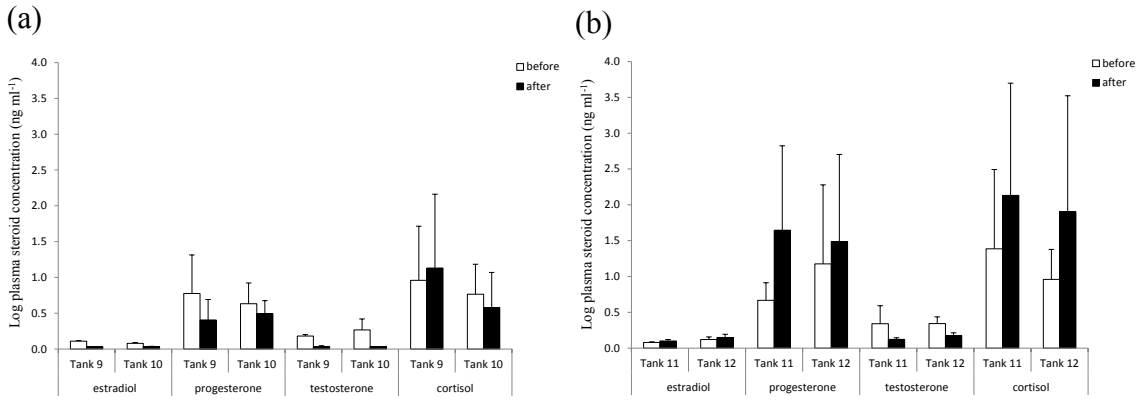


Figure 3.8: Log concentrations (ng/ml) of estradiol, progesterone, testosterone and cortisol before and after (a) LHRHa + hCG + Ovaprim® and (b) hCG + Ovaprim® injections in captive *Labrus bergylta.*

Only estradiol showed a pattern according to body size, as overall this steroid (i.e. before and after combined) was significantly higher in medium-sized *L. bergylta* ($p<0.05$); the estradiol concentration in the medium size class was 0.52±0.18ng/ml, compared with 0.27±0.03 and 0.29±0.02ng/ml respectively in the small and large size classes (Table 3.7). In general, the steroid concentrations of various sizes of captive *L. bergylta* showed similar responses to hormonal intervention. Comparing 'before' and 'after' samples demonstrated that treatments mostly had the effect of decreasing estradiol, testosterone and progesterone concentrations, irrespective of body size in captive broodstocks. However, there were slight deviations from this: more equivalent before and after levels were observed in estradiol (medium-sized individuals) and progesterone (small and large individuals) (Figure 3.9). There was a general increase in the concentration of cortisol after hormone treatment in all size classes of broodstock.

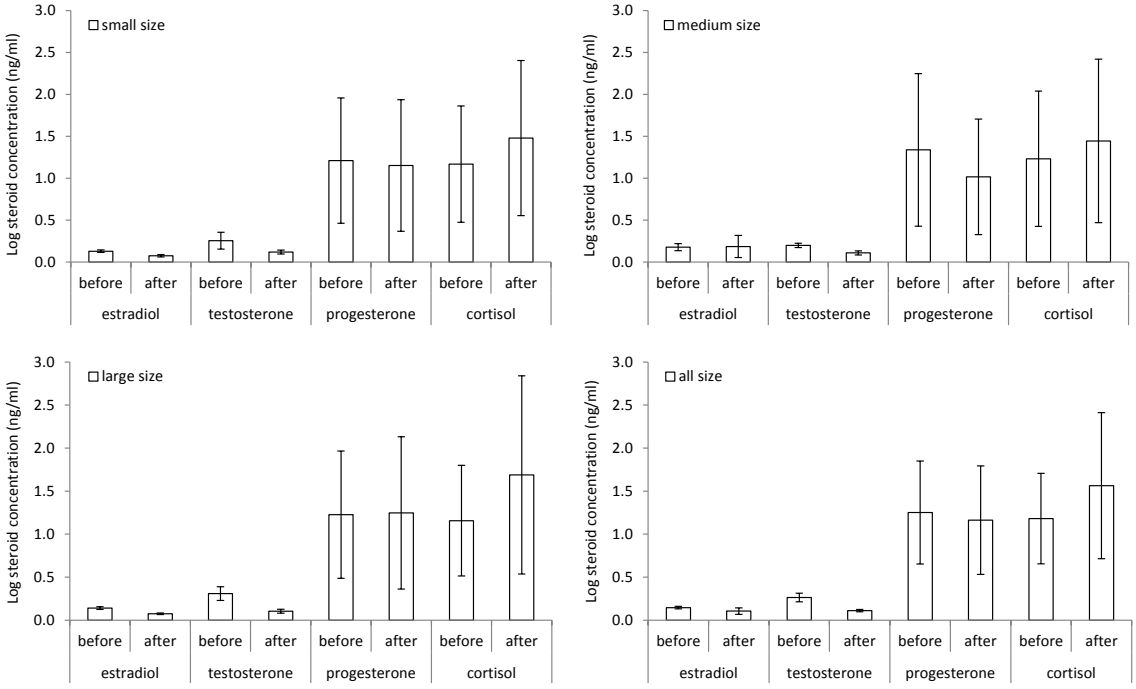


Figure 3.9: Effect of body size (small, medium, large and overall) in captive *Labrus bergylta* on log concentrations (ng/ml) of estradiol, testosterone, progesterone and cortisol before and after hormonal injections.

3.3.4 Spawning induction and plasma steroids in wild *Labrus bergylta*

Wild *L. bergylta* were also broken into three different body sizes, but the experimental design was simpler, containing a single hormonal treatment which consisted of LHRHa + hCG + Ovaprim® and shams (two replicate tanks in each case). The experiment was also divided into two consecutive trials named 'wild trial 1' and 'wild trial 2', the second of which determined capability for repeat spawning.

3.3.4.1 Wild trial 1

Hormonal treatments induced spawning in 6 / 8 *L. bergylta* in one replicate (Tank 1) and 4 / 8 *L. bergylta* in the second replicate (Tank 2). Fewer *L. bergylta* spawned in control tanks (1 / 8 each in Tanks 3 and 4). Spawning mostly took place post-hCG (10 individuals) and less commonly post-Ovaprim (2 individuals). The observed and expected frequencies of spawning are given in Table 3.8. Fishers Exact Probability Test returned a two sided p-value = 0.0294. Therefore, the null hypothesis was rejected and the alternative hypothesis was accepted: there was a significant effect of hormone treatment on the outcome of spawning in *L. bergylta*. The spawning fish were of small (5), medium (4) or large (3) body sizes.

Table 3.8: Observed and expected frequencies of spawning in 4 x 2 contingency table for wild trial 1 in *Labrus bergylta*.

Observed values	Spawned	Unspawned	Expected values	Spawned	Unspawned
LHRHa + hCG + Ovaprim®	4	4	LHRHa + hCG + Ovaprim®	3	5
LHRHa + hCG + Ovaprim®	6	2	LHRHa + hCG + Ovaprim®	3	5
Sham 1	1	7	Sham 1	3	5
Sham 1	1	7	Sham 1	3	5

Regarding steroids concentrations, there was a significant effect of the interaction of 'Treatment' x 'Time' for estradiol and testosterone on wild *L. bergylta*, as the hormone induction was associated with significantly decreased levels of these steroids relative to sham. This may be attributed to the fact that many of the *L. bergylta*

spawned in treatment tanks and the respective 'after' samples were relatively depleted in steroids. The interaction of 'Treatment' x 'Time' had no effect on progesterone and cortisol (Table 3.9). As before, 'Time' (before versus after hormone treatment) significantly affected the concentrations of all steroids. This time there was a different trend in cortisol, as this decreased post-treatment (whereas cortisol generally increased post-treatment in the captive *L. bergylta*).

Table 3.9: Mean squares from ANOVA for the effect of treatment (LHRHa + hCG + Ovaprim® versus sham), time ('before' versus 'after'), fish size and the interactions of these factors on estradiol, testosterone, progesterone and cortisol concentrations in wild trial 1 in *Labrus bergylta.* The term of most interest is indicated in bold.

		Mean squares			
Source of variation	d.f	Estradiol	Testosterone	Progesterone	Cortisol
Treatment	1	$0.01^{n.s}$	$0.00^{n.s}$	$0.17^{n.s}$	$0.02^{n.s}$
Time	1	0.94^{**}	0.60^{**}	19.58^{**}	2.27^{**}
Size	2	$0.00^{n.s}$	$0.03^{n.s}$	$0.16^{n.s}$	$0.08^{n.s}$
Treatment*Time	**1**	$\mathbf{0.31}^{**}$	$\mathbf{0.26}^{**}$	$\mathbf{0.0}^{n.s}$	$\mathbf{0.02}^{n.s}$
Treatment*Size	2	$0.07^{n.s}$	$0.01^{n.s}$	$0.07^{n.s}$	$0.54^{n.s}$
Time*Size	2	$0.01^{n.s}$	$0.01^{n.s}$	$0.14^{n.s}$	$0.32^{n.s}$
Treatment*Time*Size	2	$0.03^{n.s}$	$0.00^{n.s}$	$0.08^{n.s}$	$0.08^{n.s}$
Error	52	0.05	0.01	0.21	0.12
Total (corrected)	63				

**($p<0.01$) highly significant
*($0.01<p<0.05$) significant
$^{(n.s)}$ not significant

Table 3.10: Least-square means and their standard errors for the effects of treatment (LHRHa + hCG + Ovaprim® versus sham), time ('before' versus 'after') and fish size on estradiol, testosterone, progesterone and cortisol concentrations (ng/ml) in wild trial 1 of *Labrus bergylta.*

Factors	Subclass	Estradiol	Testosterone	Progesterone	Cortisol
Treatment	(1) LHRHa+hCG[§]	1.46±0.45[a]	1.10±0.23[a]	26.93±9.42[a]	28.89±5.84[a]
	(2) Control	1.20±0.32[a]	0.87±0.08[a]	24.98±6.87[a]	32.00±5.75[a]
Time	(1) Before	2.18±0.50[a]	1.46±0.20[a]	50.52±9.84[a]	44.01±6.04[a]
	(2) After	0.48±0.11[b]	0.51±0.07[b]	1.40±0.47[b]	16.88±4.36[b]
Size	(1) Small	1.41±0.55[a]	0.76±0.10[a]	22.23±8.30[a]	33.54±7.38[a]
	(2) Medium	1.22±0.33[a]	1.26±0.35[b]	37.12±14.67[a]	30.41±7.79[a]
	(3) Large	1.31±0.45[a]	1.02±0.19[ab]	22.24±8.79[a]	27.38±6.27[a]

[a, b]Means within a column within a group with different superscripts are significantly different at $p<0.05$.
*Size of fish: 1 = <700 grams, 2 = 700 to 900 grams and 3 = >900 gram
[§]Most individuals spawned 24h post-hCG and blood samples were taken right after egg stripping. Ovaprim® was administered after that so is not relevant to steroid concentration in this case.

Table 3.10 shows the least-square means and their standard errors for the main effects of hormone treatment, time ('before' versus 'after') and fish size on steroid concentrations of wild *L. bergylta.* Significantly lower concentrations of reproductive steroids were generally observed after injections (sham and treatment combined). These differences were very pronounced compared with captive *L. bergylta* -in the case of progesterone, there was almost a 50-fold decrease in concentration after injection in wild fish. This was followed by cortisol (three-fold), estradiol (two-fold) and testosterone (one-fold). Also, generally speaking, steroid concentrations were generally much higher in wild than in captive *L. bergylta*.

Figure 3.10 shows the overall log concentrations (ng/ml) of all steroids decreased after the injections, irrespective of whether *L. bergylta* were treated with (a) LHRHa + hCG + Ovaprim® or (b) sham. Progesterone concentration was the most affected, however estradiol and testosterone also fell markedly. The relative decrease in the latter three steroids tended to be especially pronounced in hormonal treatment groups relative to the sham groups. Wild *L. bergylta* showed higher variance and magnitude of steroid concentrations before and after the injections compared with captive fish.

Fish body size did not have an effect on steroid concentrations ($p>0.05$; Table 3.9). Figure 3.11 illustrates that small, medium and large fish had more or less the same concentrations in both treatments for estradiol, testosterone, progesterone and cortisol.

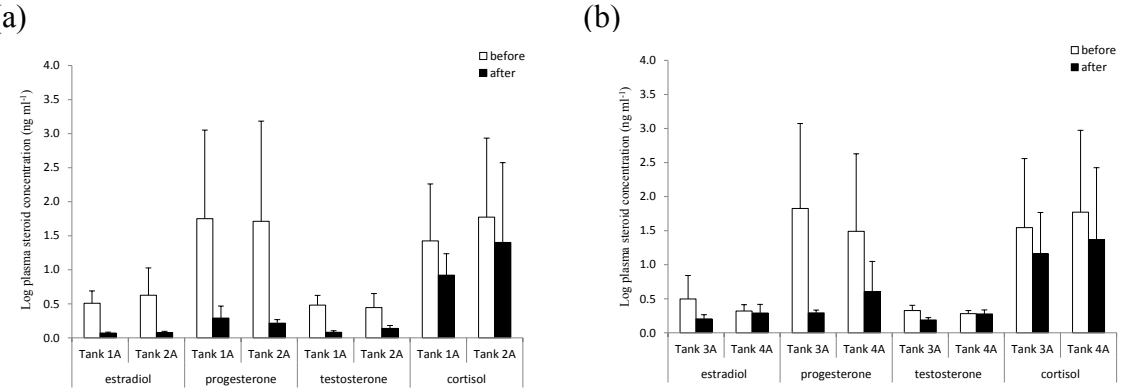


Figure 3.10: Log concentrations (ng/ml) of estradiol, progesterone, testosterone and cortisol before and after (a) LHRHa + hCG + Ovaprim®* and (b) sham injections in wild trial 1 *Labrus bergylta*. *Most individuals spawned 24h post-hCG and blood samples were taken right after egg stripping. Ovaprim® was administered after that so is not relevant to steroid concentration in this case.

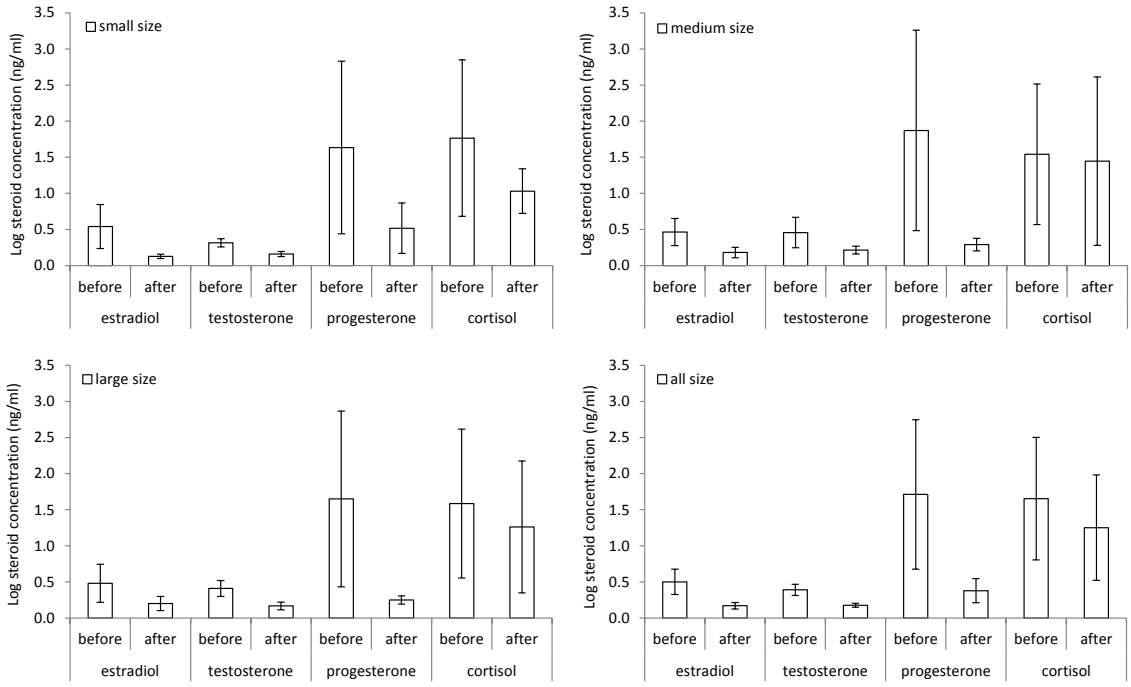


Figure 3.11: Effect of body size (small, medium, large and overall) in wild trial 1 of *Labrus bergylta* on log concentrations (ng/ml) of estradiol, testosterone, progesterone and cortisol before and after hormonal injections.

There was an interesting pattern in the concentration of steroids, particularly estradiol and progesterone, between spawned and unspawned fish in the hormone treatment (LHRHa + hCG + Ovaprim®, Figure 3.12). Overall, the *L. bergylta* which spawned showed higher concentrations in estradiol and progesterone *before* injection, relative to the unspawned fish. This illustrates that the wild *L. bergylta* which could be induced to spawn already had high estradiol and progesterone coming into the experiment. Notwithstanding this difference before hormonal treatment, the concentration of all steroids, in both spawned and unspawned fish, decreased after the hormonal injections, indicating a possible handling effect in addition to the spawning effect (on decreasing steroids).

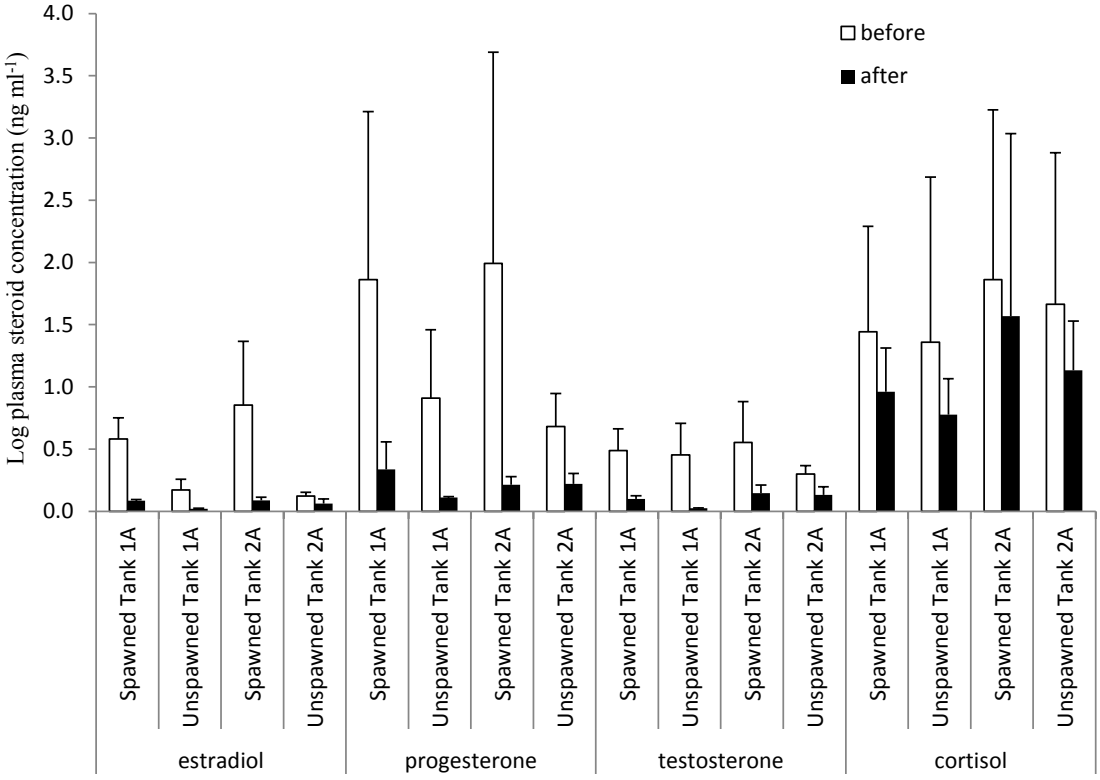


Figure 3.12: Log concentrations (ng/ml) of estradiol, progesterone, testosterone and cortisol in LHRHa + hCG + Ovaprim® treatments, organised by spawned and unspawned *Labrus bergylta* in wild trial 1; steroid concentrations before and after hormonal treatments are shown.

3.3.4.2 Wild trial 2 – Evaluation of repeat spawning

Hormonal treatment induced spawning in 5 / 8 wild *L. bergylta* in one replicate (Tank 1) and 8 / 8 *L. bergylta* in the second replicate (Tank 2; Table 3.11). The spawners were mostly medium-size fish which had spawned either post-hCG (8 individuals) or post-Ovaprim® (5 individuals). No fish spawned in the control tanks after receiving sham injections. The observed and expected frequencies of spawning are given in Table 3.11. Fishers Exact Probability Test returned a two sided p-value = 0.000. Therefore the null hypothesis was rejected and the alternative hypothesis was accepted: there was a significant effect of hormone treatment on the outcome of spawning in *L. bergylta*.

Table 3.11: Hormonal and sham injections in wild trial 2 in *Labrus bergylta* with the respective spawning results and contingency table.

Observed values	Spawned	Unspawned	Expected values	Spawned	Unspawned
LHRHa + hCG + Ovaprim®	5	3	LHRHa + hCG + Ovaprim®	3.25	4.75
LHRHa + hCG + Ovaprim®	8	0	LHRHa + hCG + Ovaprim®	3.25	4.75
Sham 1	0	8	Sham 1	3.25	4.75
Sham 1	0	8	Sham 1	3.25	4.75

In wild trial 2, there was a significant 'Treatment' x 'Time' effect ($p<0.05$) for two steroids, testosterone and progesterone. Both of these steroids were highly variable, although they decreased after hormone treatments with LHRHa + hCG + Ovaprim®, they either decreased or increased after shams (Table 3.12; Figure 3.13). The fact that spawning occurred only in treatments (and not in shams) would have tended to act to decrease reproductive steroids in 'after' samples from fish receiving hormonal treatments relative to shams. In addition, and as usual, there was a trend of decreasing steroid concentrations post injection, irrespective of whether treatment was hormone or sham, in most cases (Table 3.13). This included cortisol concentrations, which mostly decreased after hormones (both replicate tanks) and sham treatments (one replicate tank, Figure 3.13). Overall, the steroids concentrations in wild trial 2 agree somewhat with wild trial 1, apart from the three cases where there was a post-sham rise in steroids (i.e. one case each for progesterone, testosterone and cortisol).

Table 3.12: Mean squares from ANOVA for the effect of treatment (LHRHa + hCG + Ovaprim® versus sham), time ('before' versus 'after'), fish size and their interactions on estradiol, testosterone, progesterone and cortisol concentrations in wild trial 2 of *Labrus bergylta.* The term of most interest is indicated in bold.

		Mean squares			
Source of variation	d.f	Estradiol	Testosterone	Progesterone	Cortisol
Treatment	1	$0.15^{n.s}$	0.20^{**}	$0.003^{n.s}$	$0.65^{n.s}$
Time	1	0.62^{**}	$0.06^{n.s}$	2.505^{**}	2.01^{**}
Size	2	0.53^{**}	0.06^{*}	$0.006^{n.s}$	0.71^{*}
Treatment*Time	**1**	$\mathbf{0.04^{n.s}}$	$\mathbf{0.09^{*}}$	$\mathbf{0.987^{*}}$	$\mathbf{0.63^{n.s}}$
Treatment*Size	2	$0.04^{n.s}$	$0.03^{n.s}$	$0.280^{n.s}$	$0.12^{n.s}$
Time*Size	2	$0.07^{n.s}$	$0.04^{n.s}$	$0.342^{n.s}$	$0.07^{n.s}$
Treatment*Time*Size	2	$0.01^{n.s}$	$0.02^{n.s}$	$0.386^{n.s}$	$0.10^{n.s}$
Error	52	0.06	0.02	0.231	0.21
Total (corrected)	63				

**($p<0.01$) highly significant
*($0.01<p<0.05$) significant
$^{(n.s)}$ not significant

Table 3.13: Least-square means and their standard errors for the effects of treatment, time and size of hormonal injections on estradiol, testosterone, progesterone and cortisol concentrations (ng/ml) in Trial 2 of wild *Labrus bergylta.*

Factors	Subclass	Estradiol	Testosterone	Progesterone	Cortisol
Treatment	(1) LHRHa+hCG[§]	2.31 ± 0.80^{a}	1.38 ± 0.22^{a}	18.81 ± 6.48^{a}	29.87 ± 10.56^{a}
	(2) Control	1.05 ± 0.35^{a}	0.64 ± 0.09^{b}	13.51 ± 5.39^{a}	24.30 ± 4.99^{a}
Time	(1) Before	2.36 ± 0.64^{a}	1.23 ± 0.22^{a}	22.75 ± 6.44^{a}	40.86 ± 10.84^{a}
	(2) After	0.99 ± 0.59^{b}	0.79 ± 0.12^{a}	9.57 ± 5.22^{b}	13.31 ± 2.70^{b}
Size	(1) Small	1.01 ± 0.35^{a}	1.00 ± 0.24^{a}	19.07 ± 6.88^{a}	39.84 ± 11.22^{b}
	(2) Medium	3.87 ± 1.35^{b}	1.31 ± 0.20^{b}	17.81 ± 9.10^{a}	12.98 ± 2.93^{a}
	(3) Large	0.46 ± 0.11^{a}	0.71 ± 0.08^{a}	8.83 ± 3.68^{a}	19.04 ± 7.69^{a}

$^{a, b}$Means within a column within a group with different superscripts are significantly different at $p<0.05$.
*Size of fish: 1 = <700 grams, 2 = 700 to 900 grams and 3 = >900 grams
[§]Most individuals spawned 24h post-hCG and blood samples were taken right after egg stripping. Ovaprim® was administered after that so is not relevant to steroid concentration in this case.

Steroids decreased overall after treatment (irrespective of sham or hormone) in wild trial 2; with differences generally between two- and three-fold, apart from testosterone which decreased to less of an extent (Table 3.13). This trend was the same but less pronounced than in wild trial 1, where three- to four-fold decreases were generally seen (and even more than this in progesterone).

Figure 3.13 shows the overall log concentrations (ng/ml) of steroids before (white bar) and after (black bar) injection. Most of the steroid concentrations decreased after the injections irrespective of whether *L. bergylta* were treated with LHRHa + hCG + Ovaprim® (Figure 3.13a) or sham (Figure 3.13b). However, as mentioned above, the concentrations of progesterone, testosterone and cortisol in Tank 4 increased slightly in sham tanks. Again, this may have arisen due to the relatively low number (zero) of spawners in the sham tanks.

An analysis of body size revealed a non-significant difference among small and large *L. bergylta* in concentrations of estradiol, testosterone and progesterone, with all steroids having similar patterns before and after injections (Table 3.13; Figure 3.14). Either handling stress or spawning events post-treatment may have contributed to reduction in steroid concentrations after injections (of all types, either treatment or sham). Medium body sized *L. bergylta* maintained high testosterone and progesterone concentrations before and after injections, they also had higher estradiol concentrations relative to the other size classes (Figure 3.14).

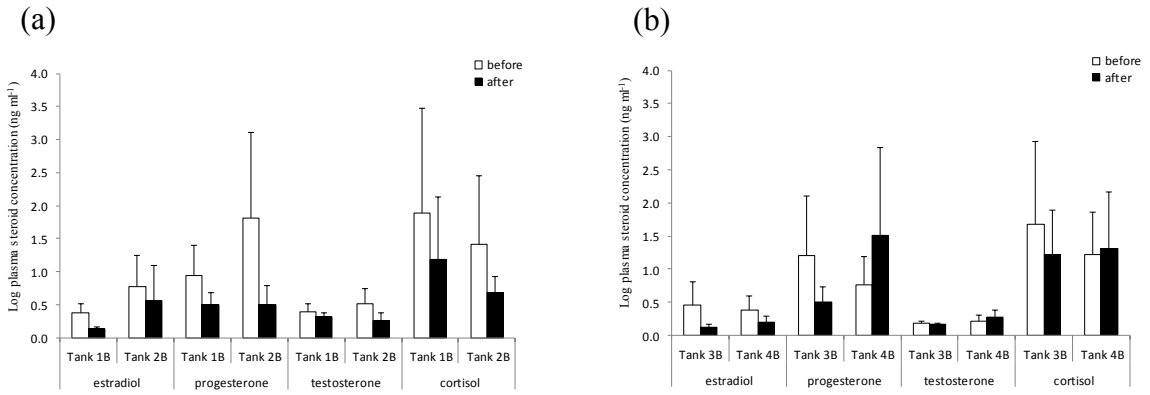


Figure 3.13: Log concentrations (ng/ml) of estradiol, progesterone, testosterone and cortisol before and after (a) LHRHa + hCG + Ovaprim®* and (b) sham injections in wild trial 2 for *Labrus bergylta*. *Most individuals spawned 24h post-hCG and blood samples were taken right after egg stripping. Ovaprim® was administered after that so is not relevant to steroid concentration in this case.

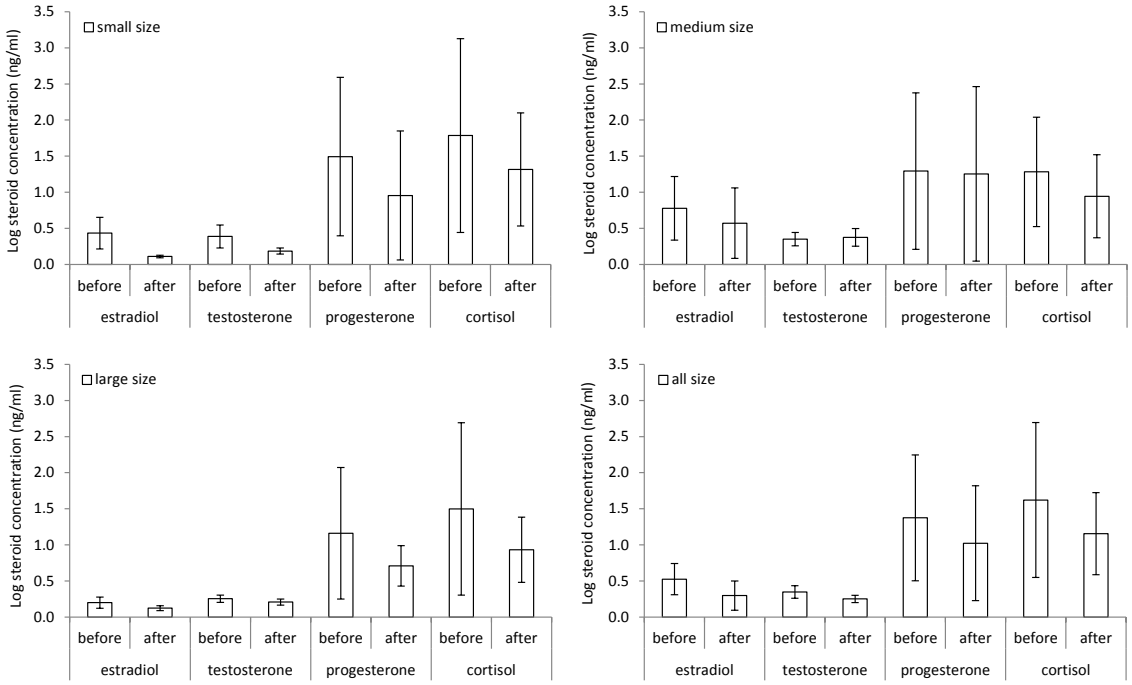


Figure 3.14: Effect of body size (small, medium, large and overall) in wild trial 2 of *Labrus bergylta* on log concentrations (ng/ml) of estradiol, testosterone, progesterone and cortisol before and after hormonal injections.

3.3.4.3 Comparison between wild trial 1 and wild trial 2

Mean squares from analysis of variance and least squares means respectively are shown in Table 3.14 and 3.15. There was no significant effect of 'Trial' for most steroids (apart from cortisol), even though wild trial 2 was a repeat spawning or re-run using the same individuals. The most striking result was a highly significant effect of Time i.e. the steroids generally showed significantly lower concentrations after the injections, irrespective of whether these were LHRHa + hCG + Ovaprim®, or, sham (Table 3.14). The exception was testosterone. It can be seen in Table 3.14 that this was the only steroid in which there was a significant interaction of Treatment and Time. In other words, there was a significant association between hormonal treatment versus sham and a reduction in testosterone.

In other results, there was a significant effect of body size on estradiol and testosterone concentrations. In this case, medium sized *L. bergylta* had significantly higher levels of estradiol and testosterone than either small or large individuals. Cortisol was elevated in the smallest sized individuals, though not significantly so. There were no significant differences between the trials in any of the steroids but except cortisol, which was higher in trial 1. The remaining steroids were not significantly different between the treatments, as steroid concentrations were decreased after the injections in both treatments and sham.

Table 3.14: Mean squares from ANOVA for the effect of treatment (LHRHa + hCG + Ovaprim® versus sham), time ('before' versus 'after'), fish size, trial ('wild trial 1' versus 'wild trial 2'), and their interactions on estradiol, testosterone, progesterone and cortisol concentrations in *Labrus bergylta.* The term of primary interest is highlighted in bold.

		Mean squares			
Source of variation	d.f	Estradiol	Testosterone	Progesterone	Cortisol
Treatment	1	$0.05^{n.s}$	0.12^{**}	$0.06^{n.s}$	$0.46^{n.s}$
Time	1	1.55^{**}	0.50^{**}	17.86^{**}	4.27^{**}
Size	2	0.31^{**}	0.07^{**}	$0.09^{n.s}$	0.51^{*}
Trial	1	$0.01^{n.s}$	$0.00^{n.s}$	$0.16^{n.s}$	1.32^{**}
Treatment*Time	**1**	$\mathbf{0.29^{n.s}}$	$\mathbf{0.32^{**}}$	$\mathbf{0.70^{n.s}}$	$\mathbf{0.44^{n.s}}$
Treatment*Size	2	$0.03^{n.s}$	$0.02^{n.s}$	$0.16^{n.s}$	$0.48^{n.s}$
Treatment*Trial	1	$0.11^{n.s}$	0.09^{*}	$0.11^{n.s}$	$0.22^{n.s}$
Time*Size	2	$0.06^{n.s}$	$0.01^{n.s}$	$0.13^{n.s}$	$0.34^{n.s}$
Time*Trial	1	$0.01^{n.s}$	0.14^{*}	3.86^{**}	$0.00^{n.s}$
Size*Trial	2	0.23^{*}	$0.02^{n.s}$	$0.06^{n.s}$	$0.25^{n.s}$
Treatment*Time*Size	2	$0.03^{n.s}$	$0.00^{n.s}$	$0.37^{n.s}$	$0.18^{n.s}$
Treatment*Time*Trial	1	$0.06^{n.s}$	$0.02^{n.s}$	$0.34^{n.s}$	$0.22^{n.s}$
Treatment*Size*Trial	2	$0.08^{n.s}$	$0.02^{n.s}$	$0.20^{n.s}$	$0.17^{n.s}$
Time*Size*Trial	2	$0.02^{n.s}$	0.04^{*}	$0.35^{n.s}$	$0.07^{n.s}$
Treatment*Time*Size*Trial	2	$0.01^{n.s}$	$0.02^{n.s}$	$0.14^{n.s}$	$0.01^{n.s}$
Error	104	0.05	0.02	0.22	0.16
Total (corrected)	127				

**($p<0.01$) highly significant; *($0.01<p<0.05$) significant; $^{(n.s)}$ not significant

Table 3.15: Least-square means and their standard errors for the effects of treatment (LHRHa + hCG + Ovaprim® versus sham), time ('before' versus 'after'), fish size and trial ('wild trial 1' versus 'wild trial 2') on estradiol, testosterone, progesterone and cortisol concentrations (ng/ml) in *Labrus bergylta.*

Factors	Subclass	Estradiol	Testosterone	Progesterone	Cortisol
Treatment	(1) LHRHa+hCG	1.88±0.46[a]	1.24±0.16[a]	22.87±5.70[a]	29.38±5.99[a]
	(2) Control	1.12±0.24[a]	0.75±0.06[b]	19.25±4.39[a]	28.15±3.81[a]
Time	(1) Before	2.27±0.40[a]	1.35±0.15[a]	36.63±6.09[a]	42.44±6.16[a]
	(2) After	0.74±0.30[b]	0.65±0.07[b]	5.48±2.65[b]	15.10±2.55[b]
Size	(1) Small	1.19±0.31[a]	0.89±0.14[a]	20.48±5.27[a]	37.04±7.00[a]
	(2) Medium	2.63±0.76[b]	1.29±0.19[b]	26.90±8.45[a]	21.18±4.20[a]
	(3) Large	0.97±0.28[a]	0.90±0.12[a]	16.87±5.53[a]	24.04±4.84[a]
Trial	(1) 1	1.33±0.27[a]	0.98±0.12[a]	25.96±5.79[a]	30.45±4.07[a]
	(2) 2	1.68±0.44[a]	1.01±0.13[a]	16.16±4.19[a]	27.08±5.81[b]

[a, b]Means within a column within a group with different superscripts are significantly different at $p<0.05$.
*Size of fish: 1 = <700 grams, 2 = 700 to 900 grams and 3 = >900 grams

3.3.5 Egg quality, fertilisation and development of larvae in hormonal induction

Eggs in all incubation cones were healthy in appearance. The average diameter of newly spawned eggs from 150 random individual eggs sampled from all treatments was 1.01±0.03mm. Fertilisation rates were 76.3±9.5% in *L. bergylta* wild trial 1 and 68.4±11.9% in *L. bergylta* wild trial 2. Both trials showed good hatching rates at 79.6±9.1% and 67.2±16.9%, respectively. There were no significant differences in the fertilisation ($F = 2.26$; d.f = 1; $p = 0.15$ ($p>0.05$)) and hatching ($F = 4.02$; d.f = 1; $p = 0.06$ ($p>0.05$)) rates between the trials. Newly hatched larvae for both batches measured 3.57±0.03mm and 3.58±0.02mm in standard length, respectively. Overall, hatching started approximately 6 days after fertilisation (DAF) or 137 hours post-fertilisation equivalent to 91.26 degree days at a mean temperature of 13.72±0.51°C for *L. bergylta* wild trial 1 larvae and 80.54 degree days at a mean temperature of 11.44±0.74°C for *L. bergylta* wild trial 2 larvae. Over the incubation period, salinity and pH values ranged from 33.0 to 33.5 ppt and 7.8 to 8.1, respectively. The average standard length and myotome height of newly hatched larvae (0dph) in *L. bergylta* wild trial 1 was 3.57±0.03mm and 0.22±0.01mm, respectively, meanwhile in *L. bergylta* wild trial 2 was

3.58±0.02mm and 0.21±0.01mm, respectively. Table 3.16 summarises the results on egg quality and larval development for both trials.

Absorption of the yolk-sac was completed after the 5th day when larvae reached 5.87±0.28mm on average standard length in both wild *L. bergylta* trials. During early feeding, larvae were fed with the rotifers *Brachionus plicatilis* and green algae. Figure 3.15 describes the embryonic development of *L. bergylta* produced by hormonal injections from fertilisation through hatching. Larval mortality rates by 7 dph were 10.5±6.8% and 18.4±15.5%, respectively in both trials. There was no relationship between the percentage fertilisation, hatching and survival rates of larvae and body weight of the spawner broodstock ($F = 1.91$; $d.f = 4$; $p = 0.15$ ($p>0.05$)).

Table 3.16: Mean and standard deviations (mean ± s.d.) for reproductive parameters between wild trial 1 and wild trial 2 of spawned *Labrus bergylta*.

Batches of larvae	**Wild trial 1**	**Wild trial 2**
Lengths of spawner (cm)	35.86±2.27	33.25±3.48
Weights of spawner (g)	818.57±173.14	652.18±194.78
Condition factor (K)	1.75±0.13	1.73±0.09
Egg volume (ml)	13.50±6.64	13.82±14.00
Approximate number of ovulated eggs (1000±100ml^{-1})	14850±7306	15200±15399
Egg diameter (mm)	1.00±0.01	1.00±0.01
Milt volume (ml/egg batch)	0.05	0.05
Fertilization date	30th April 2011 (120 day-of-year)	18th May 2011 (138 day-of-year)
Fertilization rate (%)	76.29±9.51	68.36±11.91
Incubation period (hours)	137.00	137.00
Hatching date	6th May 2011 (126 day-of-year)	24th May 2011 (144 day-of-year)
Cumulative degree days of incubation (CDD)	91.26	80.54
Temperature at hatch (°C)	13.72±0.51	11.44±0.74
Hatching rate (%)	79.58±9.11	67.16±16.86
Larval mortality after 7 days of hatching (%)	10.47±6.81	18.40±15.50
Standard length at 0 dph (mm)	3.57±0.03	3.58±0.02
Myotome height at 0 dph (mm)	0.22±0.01	0.21±0.01

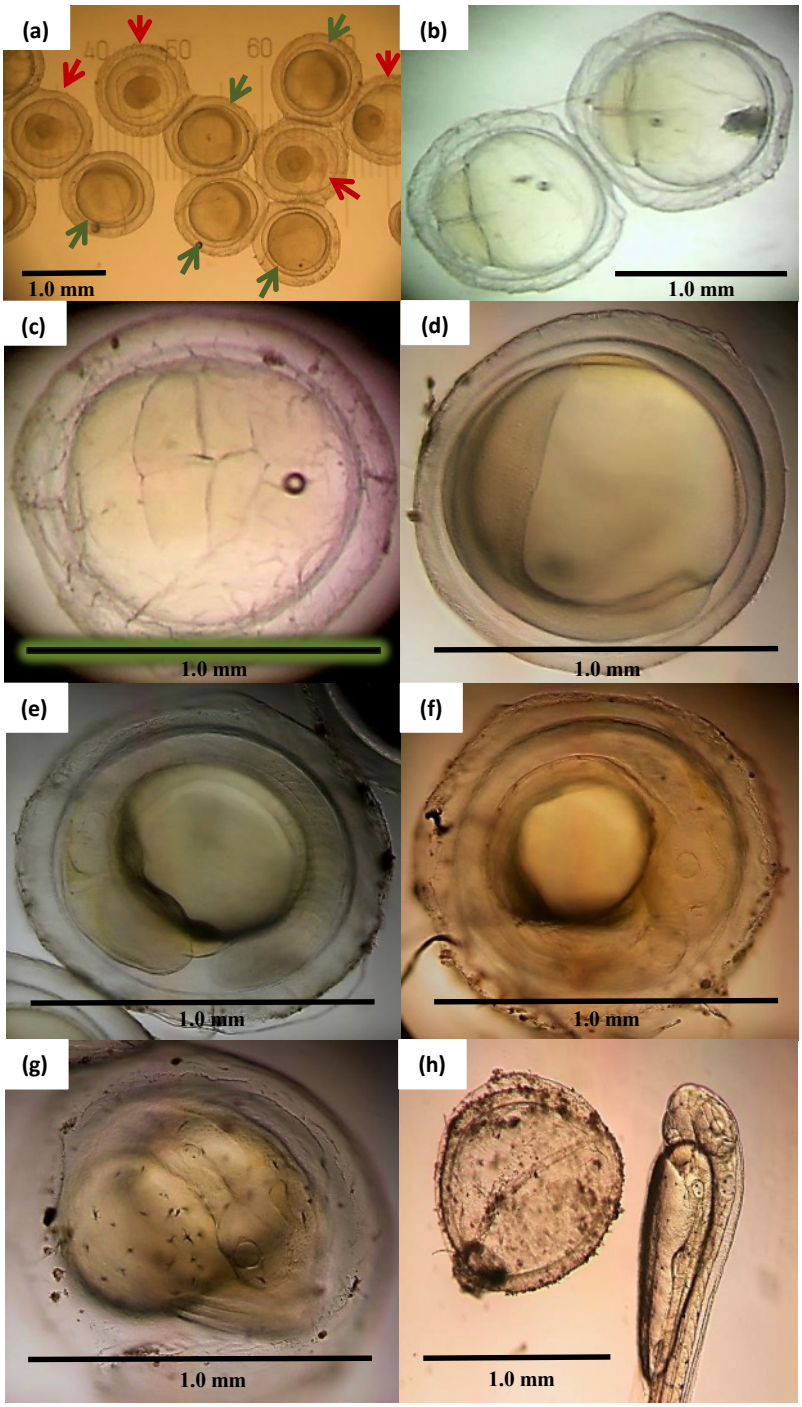


Figure 3.15: Development of *Labrus bergylta* eggs produced by hormonal induction: (a) Fertilisation (green arrows) is indicated via development of the blastula including formation of a clearly visible blastodisc; unfertilized eggs are indicated with red arrows (b) Cleavage: two cells (c) Cleavage: eight cells (d) Gastrula: embryonic pole (3 days post fertilisation) (e) Early segmentation (f) Late segmentation (g) Complete embryo (h) Hatched with new larva (0 day post hatch). Scale bar = 1.0 mm.

3.4 Discussion

The use of exogenous hormones to induce ovary maturation, ovulation, and spawning has become commonplace in the aquaculture of many species, including salmonids (Donaldson *et al*., 1981; Mylonas *et al*., 1992; Vikingstad *et al*., 2008), flounders, *Paralichthys dentatus* (Berlinsky *et al*., 1997; Harmin and Crim, 1992), black sea bass, *Centropristis striata* (Watanabe *et al*., 2003), European sea bass, *Dicentrarchus labrax*, (Fornies *et al*., 2001), sea breams, *Sparus aurata* (Barbaro *et al*., 1997), wild black bream, *Acanthopagrus butcheri* (Haddy and Pankhurst, 2000) and ornamental fishes (Hill *et al*., 2009). The benefits of well-developed artificial fertilisation protocols are that they can simplify egg development and fertilisation under systematic handling procedures, reduce the risk of egg contamination, as well as simplifying and optimizing larval development.

This is the first study of hormonal-induced spawning and artificial fertilisation of *L. bergylta* with evaluation of subsequent egg and larval success. It is also the first empirical dataset specifying reproductive steroid concentrations throughout that process, including evaluation of wild and captive broodstocks for spawning success. The results showed that spawning in *L. bergylta* could be induced with significant success over placebo, but generally this was only possible in wild broodstock captured immediately prior to the natural spawning season (i.e. 'wild trial 1' and 'wild trial 2'). Repeat spawning was possible ('wild trial 2') and blood steroids did not differ significantly on repeat spawning (apart from cortisol which decreased slightly). Broodstock that had been captive for approximately one year ('captive' trial), almost completely failed to spawn with induction. Spawning in wild *L. bergylta* generally tended to occur in medium sized *L. bergylta* ~30-40 cm standard length and 700 to 900 grams, even though an association between size class and plasma concentration of steroids was not always evident.

A combination of hormones was successful in spawning wild *L. bergylta*; the three hormones were administered 24h apart, with stripping occurring 24h after each individual treatment. In both wild trials, spawning mostly occurred after LHRHa + hCG (18 individuals), but a few also spawned after the third treatment with Ovaprim® (seven

individuals). Spawning after LHRHa + hCG + Ovaprim® mostly occurred in wild trial 2 (5/7 individuals). Spawning success and larval development parameters were slightly better in wild trial 1 than in wild trial 2. This may either have been because wild trial 2 involved repeat spawning, or due a higher involvement of post-Ovaprim® spawning in this trial. Specific fertilisation rates were 76.3±9.5% and 68.4±11.9% respectively in wild trial 1 and 2; hatching rates were 79.6±9.1% and 67.2±16.9% respectively; and mortality rates at 7 days post hatch were 10.5±6.8% and 18.4±15.5% respectively).

In the present study, the incubation period of hormonally-induced eggs of *L. bergylta* was a minimum of 137 hours post-fertilisation, equivalent to 80.54 - 91.26 degree days at mean temperatures of 11.44±0.74°C and 13.72±0.51°C respectively. A previous study within this temperature range reported slightly shorter incubation time, where hatching occurred 123 hours post-fertilisation at 12.2±1.10°C (D'arcy *et al.*, 2012). In another study, hatching in *Labrus viridis* from natural spawning began at 127 hours post-fertilisation when the incubation temperature was 14.4°C which was almost similar to *L. bergylta* (Kožul *et al.,* 2011). In a comparison of *L. bergylta* egg diameters, the sizes reported ranged from 0.7-0.8 mm to 1.0 mm (Dulcic *et al*., 1999) which suggests that egg diameters reported in the present study (1.01±0.03mm) are at the larger end of the typical range. Some literature is available on *L. bergylta* embryonic development (D'arcy *et al.,* 2012; Dunaevskaya *et al*., 2012; Artuz, 2005). While Artuz (2005) notes a length at hatch of 2.7±0.2mm, the other studies indicate a much larger size where the larvae emerging from the yolk sac were 3.72±0.13mm (in Ireland), 3.64±0.1mm (in Norway) and 4.1±0.1 - 4.3±0.3mm (in Scotland) (D'arcy *et al*., 2012). In the present study, the average standard length and myotome height of newly hatched larvae was found to be very consistent: 3.57±0.03mm and 0.22±0.01mm respectively in wild trial 1, or 3.58±0.02mm and 0.21±0.01mm, respectively in wild trial 2. It is likely that the observed variations are largely due to the initial size of eggs, as has been found with many marine fish species (Marteinsdottir and Steinarsson, 1998).

This research provided some information on the complicated question of how estradiol, progesterone and cortisol concentrations are associated with the natural spawning period in wild *L. bergylta*. In the present study, plasma concentrations of

estradiol and progesterone appeared most critical for spawning. This was demonstrated by the comparison of these steroids in spawned versus unspawned *L. bergylta*. The wrasse which spawned had much higher levels of these steroids even *before treatment*, as well as a bigger differential between pre- and post- treatment levels. Testosterone is also interesting because this may be converted into estrogen to regulate the supply of both estradiol and testosterone in the bodies of both female and male individuals (Yaron, 1995; Castillo *et al*., 2013). Indeed, the synthesis and metabolism of sex steroids is very labile and subject to endocrine disruption from chemical agents in the environment (Thibaut and Porte, 2004; Rempel and Schlenk, 2008; Munshi *et al*., 2012). However, in the current study, relatively low testosterone concentrations were observed in most females during the spawning period and testosterone did not vary a lot between spawned and unspawned individuals. This agrees with a previous study of *L. bergylta* females, where relatively low concentrations of testosterone were observed during spawning events and there was a high association of estradiol with the events leading up to egg maturation (Muncaster *et al*., 2010; Muncaster *et al*., 2013).

In addition, it appears from the present results that the reason captive broodstocks did not spawn was because the circulating reproductive steroid concentrations of estradiol and progesterone fell well below those of wild populations; for example, estradiol concentrations prior to any treatments were four- to five-fold higher in wild compared to captive fish. Captivity may suppress reproductive steroids, for example Imanaga *et al*. (2014) found that captive-rearing stress may negatively influence the transcription of *gnrh1* in the brain and gonadotropin (GtH) synthesis in the pituitary and thereby impair vitellogenesis of jack mackerel (*Trachurus japonicus*). An alternative explanation is condition: a study on Atlantic cod (*Gadus morhua*) revealed that low condition of individuals (e.g. due to captivity) can lead to almost total reproductive failure for the population (Scott *et al*., 2006). In the present study, captive *L. bergylta* broodstock may have had poorer quality dietary conditions (e.g. El-Sayed and Kawanna, 2008) and therefore insufficient energy reserves relative to wild broodstocks to complete their reproductive efforts.

Overall, it appears from the present results that estradiol and progesterone have a major role for egg development and spawning in *L. bergylta*, an observation that has been made previously in many other fish species (Yaron, 1995; Zohar and Mylonas, 2001; Guzman *et al*., 2009; Ahmadi *et al*., 2011). The role of cortisol within spawning is more complex and will be discussed at length –see below.

In captive *L. bergylta*, many of the plasma steroid concentrations (apart from cortisol) after injection were significantly lower than those before injection in all treatments, including the sham. This indicated that, although they did not spawn, the fish responded to injection. We could term this a 'handling effect' whereby steroids were reduced due to handling stress. (As the captive individuals mostly did not spawn, this complicating factor is removed.) In agreement, was the observation that cortisol was higher in these captive *L. bergylta* after handling. In wild trials too, plasma steroids were often reduced after treatments. But this time, the same explanation is not entirely relevant. The reason is because in both wild trials, cortisol (with one exception) reduced after handling from treatments. We can explain the reduction in cortisol post-treatment as being associated with spawning because the reduction in mean steroid concentrations (including cortisol) was most pronounced in fish which spawned relative to those which didn't. This is almost certainly not a handling effect since all of these fish (spawned/unspawned) had been handled to the same extent. Cortisol may have a role in energy (protein, glucose) metabolism during spawning, including mobilisation of energy reserves to the egg yolk (Sundararaj *et al*., 1982; Ding *et al*., 1994; Berg *et al*., 2003; Berg *et al*., 2004; Phartyal *et al*., 2008). Specifically, cortisol may have a role in stimulating the size and number of cells in the yolk-sac membrane of the ovary (Ayson *et al*., 1995). If handling stress in *L. bergylta* is analogous to salmonids, then stress response mechanisms may be suppressed during spawning. In fact, in natural reproductive cycles of salmonids, cortisol concentrations increased progressively from maturation, to spawning and senescence (Baker and Vynne, 2014). The phenomenon means that, as they complete sexual maturation, individuals' responsiveness to external stressors diminishes due to an attenuated corticosteroid stress response and increased metabolic clearance of cortisol (Shrimpton and Randall, 1994; Baker and Vynne, 2014). In artificial spawning also, treatments involving hormones (GnRHa) have raised plasma

cortisol concentrations (Joy *et al*., 1998). Elevated plasma cortisol concentrations in the periovulatory phase has been observed in numerous other species and this may be explained by the fact that progesterone in many teleost fish can be converted to forms such as 17alpha,20beta-dihydroxyprogesterone (17α20β-P), and this molecule may be a physiological regulator of cortisol (Barry *et al*., 1995). However other possible stimulants of adrenocorticotrophin (ACTH) pathways and hence cortisol concentrations are also linked to reproductive hormones (Singh and Yadav, 1975; Joy *et al.*, 1998; Van Lier *et al*., 1999). A contrasting view is that cortisol, is present as part of the stress response (Bonga *et al*., 1995) and this suppresses reproductive steroids e.g. in pink salmon (*Oncorhynchus gorbuscha*) (McConnachie *et al*. 2012). We believe the evidence in the present study points towards the former proposition, as cortisol was depressed in the captive *L. bergylta* relative to wild broodstock in the present study. Also, in the wild *L. bergylta,* the varying spawner versus non-spawner cortisol levels in present study are consistent with a role of this steroid in spawning of *L. bergylta*.

Reproductive steroids tend to be naturally variable, which makes analysis difficult. For example, extremely high variances were associated with these steroids, particularly progesterone and cortisol. Notwithstanding great variability, some patterns were evident in the present study; steroids were generally decreased post-treatment relative to before, irrespective of whether that treatment was a hormone or sham injection. Handling may reduce reproductive steroid concentrations (as discussed above). In addition, Figure 3.16 describes the likely pattern of an example steroid (e.g. estradiol) in the context of spawning in *L. bergylta* –the plasma concentration is higher prior to spawning than afterwards, with the peak concentration in between these time points occurring just before spawning. Steroid levels are reduced immediately after spawning and egg stripping. These patterns are evident in other studies: spawning of silver perch (*Bidyanus bidyanus*) occurred 40 hours after injection with GnRH and highest plasma 17β oestradiol (E_2) levels were recorded 24 hours after the injection, but levels were very low after spawning. Analogous patterns were seen in the levels of 17α20β-P (≈ progesterone) in *B. bidyanus*; levels gradually increased after injection and peaked 12 hours before spawning, but levels were low during spawning itself (Levavi-Sivan *et al*., 2004).

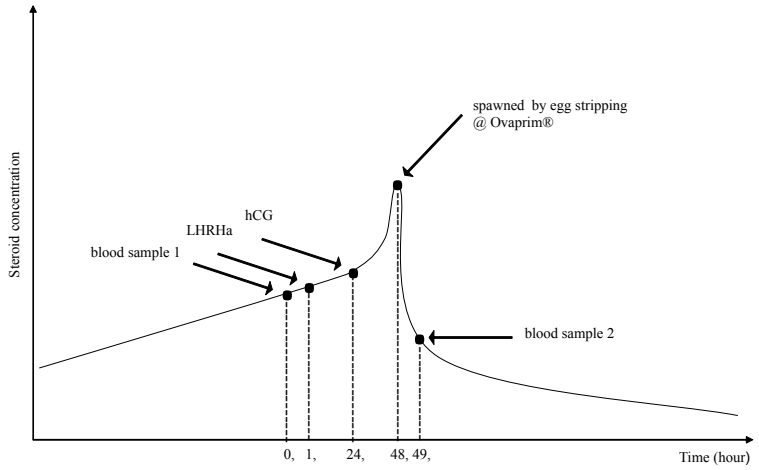


Figure 3.16: Schematic diagram representing plasma steroid concentration of estradiol in *L. bergylta* with respect to artificial spawning injections using LHRHa + hCG and subsequent spawning by egg stripping. Blood samples from 'before' (blood sample 1) and 'after' (blood sample 2) the process are indicated, as is a lower plasma estradiol concentration 'after' relative to 'before'. The same process is suggested for all steroids.

Inhibitory (antagonistic) or stimulatory (agonistic) effects on steroids by hormonal intervention was evaluated by examining single treatments and combinations. This was nicely illustrated in captive fish, even though most of them did not spawn. A good example was seen in estradiol; there was no effect on this steroid when either LHRHa or hCG were used on their own. However, an agonistic (positive) effect was observed when hCG was used in combination with either LHRHa or Ovaprim®. Interestingly, an antagonistic (negative) effect on estradiol was shown when all three LHRHa + hCG + Ovaprim® were used. Something similar was seen with progesterone concentrations, but this time the combination of hCG + Ovaprim® had the best effect in elevating this steroid, whereas again, all three hormonal treatments depressed it. We can hypothesise that LHRHa plus Ovaprim® in the same treatment has an antagonistic effect on estradiol, progesterone and also cortisol.

Antagonistic effects of Ovaprim® on other interventions may be due to negative feedback. The synthesis and secretion of estradiol is stimulated by follicle-stimulating hormone (FSH), which is in turn controlled by the hypothalamic gonadotropin releasing hormone (GnRH), equivalent in this case to either LHRHa or Ovaprim® –the former is

a synthetic analogue of the naturally occurring GnRH hormone and the latter is a synthetic GnRHa with dopamine antagonist. Essentially, LHRHa and Ovaprim® do the same job, using them both together presumably caused overstimulation of estradiol and resulted in negative feedback/antagonistic effect. Progesterone production is stimulated by luteinizing hormone (LH), which is also stimulated by GnRH. Therefore elevated levels of progesterone may be controlled by the same negative-feedback mechanism. Again, a suppression of the steroids occurred when LHRHa + hCG + Ovaprim® were used in combination. In wild trials, a reduced experimental set-up was used and, although the individual effects of hormonal treatments were not evaluated, it was clear that LHRHa + hCG was an efficient method of inducing spawning in *L. bergylta*. Ovaprim® was intended to be included with the other two hormones in our treatments, but as discussed above, most spawning actually occurred prior to the Ovaprim® injection, and the 'after' blood samples were taken at the point of spawning so the steroid concentrations reflect LHRHa + hCG rather than LHRHa + hCG+ Ovaprim®. The high spawning occurrence and the steroid profiles typical of spawning (i.e. conforming to Figure 3.16) in these LHRHa + hCG treatments are also suggestive that this combination had an agonistic effect on spawning in wild broodstocks (see also Malison *et al*., 1998).

The discovery of a successful artificial spawning and fertilisation protocol in *L. bergylta* in the present study will be helpful from a management perspective. The ability to control spawning in captivity allows for the management of captive broodstocks in aquaculture, reducing dependence on collection of wild fish from their spawning grounds. This knowledge allows the industry to guarantee and plan production and produce a known quantity and quality of eggs. Spawning may be synchronised on predetermined dates that do mature and improve production efficiency in relation to demand. Hormonal treatment of fish may also compress the spawning season. A compressed spawning season represents considerable economic advantage from labour perspective as it decreases the amount of time crews must attend the captive adult fish. Batch spawning of fish also permits batch ponding and first feeding of fry as exemplified by Chapter four. This can permit uniform growth of fish within the population by decreasing the duration of the first feeding phase. Uniformity of fish is

desirable because husbandry practices are reduced if fish are of uniform size. It is also reasonable to assume that *L. bergylta* of a uniform size would be needed for use in salmon cages. Additional efficiencies in the control of spawning in future might focus on developing rapid biochemical assays (e.g. involving reproductive hormones) for monitoring reproductive milestones as well as a better understanding of the influence of captivity on the neuroendocrine system.

References

Abe, H., & Oka, Y. (2011). Mechanisms of Neuromodulation by a Nonhypophysiotropic GnRH System Controlling Motivation of Reproductive Behavior in the Teleost Brain. *Journal of Reproduction and Development, 57*(6), 665-674.

Ahmadi, M., Amiri, B. M., Abdoli, A., Fakharzade, S. M. E., & Hoseinifar, S. H. (2011). Sex steroids, gonadal histology and biological indices of fall and spring Caspian lamprey (*Caspiomyzon wagneri*) spawning migrants in the Shirud River, Southern Caspian Sea. *Environmental Biology of Fishes, 92*(2), 229-235.

Ardashev, A. A., & Kiseleva, M. I. (2000). Hormonal-metabolic rearrangements in the dog salmon *Oncorhynchus keta* at high population density and hydrocortisone injection. *Journal of Evolutionary Biochemistry and Physiology, 36*(5), 525-530.

Artuz, M. L. (2005). Embryonic and larval development of the ballan wrasse *Labrus bergylta* Ascanius, 1767. *Hidrobiologica*, 10, 98-101.

Ayson, F. G., Kaneko, T., Hasegawa, S., & Hirano, T. (1995). Cortisol Stimulates the Size and Number of Mitochondrion-Rich Cells in the Yolk-Sac Membrane of Embryos and Larvae of Tilapia (*Oreochromis mossambicus*) in-Vitro and in-Vivo. *Journal of Experimental Zoology, 272*(6), 419-425.

Baker, M. R., & Vynne, C. H. (2014). Cortisol profiles in sockeye salmon: Sample bias and baseline values at migration, maturation, spawning, and senescence. *Fisheries Research, 154*, 38-43.

Barbaro, A., Francescon, A., Bozzato, G., Merlin, A., Belvedere, P., & Colombo, L. (1997). Induction of spawning in gilthead seabream, *Sparus aurata* L., by a long-acting GnRH agonist and its effects on egg quality and daily timing of spawning. *Aquaculture, 154*(3-4), 349-359.

Barry, T. P., Lapp, A. F., Kayes, T. B., & Malison, J. A. (1993). Validation of a Microtitre Plate Elisa for Measuring Cortisol in Fish and Comparison of Stress Responses of Rainbow-Trout (*Oncorhynchus mykiss*) and Lake Trout (*Salvelinus namaycush*). *Aquaculture, 117*(3-4), 351-363.

Barry, T. P., Malison, J. A., Held, J. A., & Parrish, J. J. (1995). Ontogeny of the Cortisol Stress-Response in Larval Rainbow-Trout. *Gen Comp Endocrinol, 97*(1), 57-65.

Baumann, L., Holbech, H., Keiter, S., Kinnberg, K. L., Knorr, S., Nagel, T., & Braunbeck, T. (2013). The maturity index as a tool to facilitate the interpretation of changes in vitellogenin production and sex ratio in the Fish Sexual Development Test. *Aquat Toxicol, 128-129*, 34-42.

Beresford, N., Brian, J. V., Runnalls, T. J., Sumpter, J. P., & Jobling, S. (2011). Estrogenic activity of tropical fish food can alter baseline vitellogenin concentrations in male fathead minnow (*Pimephales promelas*). *Environ Toxicol Chem, 30*(5), 1139-1145.

Berg, A. H., Westerlund, L., & Olsson, P. E. (2004). Regulation of Arctic char (*Salvelinus alpinus*) egg shell proteins and vitellogenin during reproduction and in response to 17 beta-estradiol and cortisol. *Gen Comp Endocrinol, 135*(3), 276-285.

Berg, H. A., Westerlund, L., & Olsson, P. E. (2003). Differences in cortisol effects on vitellogenin and vitelline envelope protein production in the teleost, arctic char (*Saluelinus alpinus*). *Biology of Reproduction, 68*, 348-349.

Berlinsky, D. L., King, W., & Smith, T. I. J. (2005). The use of luteinizing hormone releasing hormone analogue for ovulation induction in black sea bass (*Centropristis striata*). *Aquaculture, 250*(3-4), 813-822.

Berlinsky, D. L., William, K., Hodson, R. G., & Sullivan, C. V. (1997). Hormone induced spawning of summer flounder *Paralichthys dentatus*. *Journal of the World Aquaculture Society, 28*(1), 79-86.

Billard, R., Reinaud, P., Hollebecq, M. G., & Breton, B. (1984). Advancement and Synchronization of Spawning in Salmo-Gairdneri and Salmo-Trutta Following Administration of Lrh-a Combined or Not with Pimozide. *Aquaculture, 43*(1-3), 57-66.

Black, B. J., & Black, M. (2013). Efficacy of two exogenous hormones (GnRHa and hCG) for induction of spontaneous spawning in captive yellowfin bream, *Acanthopagrus australis* (Sparidae) and influence of sex ratio on spawning success. *Aquaculture, 416*, 105-110.

Bonga, S. E. W., Balm, P. H. M., & Lamers, A. E. (1995). The Involvement of Acth and Msh in the Stress-Response in Teleost Fish. *Netherlands Journal of Zoology, 45*(1-2), 103-106.

Castillo-Briceno, P., Aguila-Martinez, S., Liarte, S., Alcazar, A. G., Meseguer, J., Mulero, V., & Garcia-Ayala, A. (2013). In situ forming microparticle implants for delivery of sex steroids in fish: Modulation of the immune response of gilthead seabream by testosterone. *Steroids, 78*(1), 26-33.

Chatakondi, N. G. (2014). Suspending Mammalian LHRHa-injected Channel Catfish, *Ictalurus punctatus*, in Individual Soft Mesh Bags Reduces Stress and Improves Reproductive Performance. *Journal of the World Aquaculture Society, 45*(5), 604-612.

Chaube, R., Singh, R. K., & Joy, K. P. (2014). Effects of ovaprim, a commercial spawning inducer, on vasotocin and steroid hormone profiles in the catfish *Heteropneustes fossilis*: In vivo and in vitro studies. *Gen Comp Endocrinol, 195*, 190-200.

Chaves-Pozo, E., Arjona, F. J., Garcia-Lopez, A., Garcia-Alcazar, A., Meseguer, J., & Garcia-Ayala, A. (2008). Sex steroids and metabolic parameter levels in a seasonal breeding fish (*Sparus aurata* L.). *Gen Comp Endocrinol, 156*(3), 531-536.

Cook, K. V., McConnachie, S. H., Gilmour, K. M., Hinch, S. G., & Cooke, S. J. (2011). Fitness and behavioral correlates of pre-stress and stress-induced plasma cortisol titers in pink salmon (*Oncorhynchus gorbuscha*) upon arrival at spawning grounds. *Horm Behav, 60*(5), 489-497.

D'Arcy, J., Dunaevskaya, E., Treasurer, J. W., Ottesen, O., Maguire, J., Zhuravleva, N., Karlsen, A., Rebours, C., & FitzGerald, R. D. (2012). Embryonic development in ballan wrasse *Labrus bergylta*. *J Fish Biol, 81*(3), 1101-1110.

de Lapeyre, B. A., Muller-Belecke, A., & Horstgen-Schwark, G. (2009). Control of spawning activity in female Nile tilapia (*Oreochromis niloticus*) (L.) by temperature manipulation. *Aquaculture Research, 40*(9), 1031-1036.

DiMaggio, M. A., Broach, J. S., & Ohs, C. L. (2014). Evaluation of Ovaprim and Human Chorionic Gonadotropin Doses on Spawning Induction and Egg and Larval Quality of Pigfish, *Orthopristis chrysoptera. Journal of the World Aquaculture Society, 45*(3), 243-257.

Ding, J. L., Lim, E. H., & Lam, T. J. (1994). Cortisol-Induced Hepatic Vitellogenin Messenger-Rna in *Oreochromis aureus* (Steindachner). *Gen Comp Endocrinol, 96*(2), 276-287.

Donaldson, E. M., & Hunter, G. A. (1983). Induced Final Maturation, Ovulation, and Spermiation in Cultured Fish. *Fish Physiology, 9*, 351-403.

Donaldson, E. M., Hunter, G. A., & Dye, H. M. (1981). Induced Ovulation in Coho Salmon (*Oncorhynchus kisutch*) .2. Preliminary-Study of the Use of Lh-Rh and 2 High Potency Lh-Rh Analogs. *Aquaculture, 26*(1-2), 129-141.

Dulcic, J., Kozul, V., Kraljevic, M., Skaramuca, B., Glamuzina, B., & Re, P. (1999). Embryonic and larval development of the brown wrasse *Labrus merula* (Pisces : Labridae). *Journal of the Marine Biological Association of the United Kingdom, 79*(2), 327-332.

Dunaevskaya, E., Amin, A. B., Ottesen, O. H. (2012). Organogenesis of Ballan Wrasse *Labrus bergylta* (Ascanius 1767) Larvae. *J Aquacult Res Dev*, 3, 142.

El-Sayed, A. F. M., & Kawanna, M. (2008). Effects of dietary protein and energy levels on spawning performance of Nile tilapia (*Oreochromis niloticus*) broodstock in a recycling system. *Aquaculture, 280*(1-4), 179-184.

Espmark, A. M., Eriksen, M. S., Salte, R., Braastad, B. O., & Bakken, M. (2008). A note on pre-spawning maternal cortisol exposure in farmed Atlantic salmon and its impact on the behaviour of offspring in response to a novel environment. *Applied Animal Behaviour Science, 110*(3-4), 404-409.

Fornies, M. A., Mananos, E., Carrillo, M., Rocha, A., Laureau, S., Mylonas, C. C., Zohar, Y., & Zanuy, S. (2001). Spawning induction of individual European sea bass females (*Dicentrarchus labrax*) using different GnRHa-delivery systems. *Aquaculture, 202*(3-4), 221-234.

Ganesh, C. B. (2014). Follicular development status and profile of 17 beta estradiol and cortisol levels during spawning cycle in *Oreochromis mossambicus* (Peters). *Indian Journal of Fisheries, 61*(2), 45-51.

Gomes, C. C., Costa, F. G., & Borella, M. I. (2013). Distribution of GnRH in the brain of the freshwater teleost *Astyanax altiparanae* (Garutti & Britski, 2000). *Micron, 52-53*, 33-38.

Guzman, J. M., Cal, R., Garcia-Lopez, A., Chereguini, O., Kight, K., Olmedo, M., Sarasquete, C., Mylonas, C. C., Peleteiro, J. B., Zohar, Y., & Mananos, E. L. (2011). Effects of in vivo treatment with the dopamine antagonist pimozide and gonadotropin-releasing hormone agonist (GnRHa) on the reproductive axis of Senegalese sole (*Solea senegalensis*). *Comp Biochem Physiol A Mol Integr Physiol, 158*(2), 235-245.

Guzman, J. M., Ramos, J., Mylonas, C. C., & Mananos, E. L. (2009). Spawning performance and plasma levels of GnRHa and sex steroids in cultured female Senegalese sole (*Solea senegalensis*) treated with different GnRHa-delivery systems. *Aquaculture, 291*(3-4), 200-209.

Haddy, J. A., & Pankhurst, N. W. (2000). The efficacy of exogenous hormones in stimulating changes in plasma steroids and ovulation in wild black bream *Acanthopagrus butcheri* is improved by treatment at capture. *Aquaculture, 191*(4), 351-366.

Hamaguchi, Y., Sakai, Y., Takasu, F., & Shigesada, N. (2002). Modeling spawning strategy for sex change under social control in haremic angel fishes. *Behavioral Ecology, 13*(1), 75-82.

Haniffa, M. A., Marimuthu, K., Nagarajan, M., Arokiaraj, A. J., & Kumar, D. (2004). Breeding behaviour and parental care of the induced bred spotted murrel *Channa punctatus* under captivity. *Current Science, 86*(10), 1375-1376.

Haniffa, M. A., & Sridhar, S. (2002). Induced spawning of spotted murrel (*Channa punctatus*) and catfish (*Heteropneustes fossilis*) using human chorionic gonadotropin and synthetic hormone (Ovaprim). *Vet. Archiv*, 72, 51-56.

Harmin, S. A., & Crim, L. W. (1992). Gonadotropic Hormone-Releasing Hormone Analog (Gnrh-a) Induced Ovulation and Spawning in Female Winter Flounder, *Pseudopleuronectes americanus* (Walbaum). *Aquaculture, 104*(3-4), 375-390.

Hassin, S., Gothilf, Y., Blaise, O., & Zohar, Y. (1998). Gonadotropin-I and -II subunit gene expression of male striped bass (*Morone saxatilis*) after gonadotropin-releasing hormone analogue injection: Quantitation using an optimized ribonuclease protection assay. *Biology of Reproduction, 58*(5), 1233-1240.

Hill, J. E., Baldwin, J. D., Graves, J. S., Leonard, R., Powell, J. F. F., & Watson, C. A. (2005). Preliminary observations of topical gill application of reproductive hormones for induced spawning of a tropical ornamental fish. *North American Journal of Aquaculture, 67*(1), 7-9.

Hill, J. E., Kilgore, K. H., Pouder, D. B., Powell, J. F. F., Watson, C. A., & Yanong, R. P. E. (2009). Survey of Ovaprim Use as a Spawning Aid in Ornamental Fishes in the United States as Administered through the University of Florida Tropical Aquaculture Laboratory. *North American Journal of Aquaculture, 71*(3), 206-209.

Hoogenboom, M. O., Metcalfe, N. B., Groothuis, T. G. G., de Vries, B., & Costantini, D. (2012). Relationship between oxidative stress and circulating testosterone and cortisol in pre-spawning female brown trout. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology, 163*(3-4), 379-387.

Imanaga, Y., Nyuji, M., Amano, M., Takahashi, A., Kitano, H., Yamaguchi, A., & Matsuyama, M. (2014). Characterization of gonadotropin-releasing hormone and gonadotropin in jack mackerel (*Trachurus japonicus*): Comparative gene expression analysis with respect to reproductive dysfunction in captive and wild fish. *Aquaculture, 428*, 226-235.

Ingram, B., Sungan, S., Tinggi, D., Sim, S. Y., & De Silva, S. S. (2007). Breeding performance of Malaysian mahseer, *Tor tambroides* and *Tor douronensis* broodfish in captivity. *Aquaculture Research, 38*(8), 809-818.

Joy, K. P., Senthilkumaran, B., & Sudhakumari, C. C. (1998). Periovulatory changes in hypothalamic and pituitary monoamines following GnRH analogue treatment in the catfish *Heteropneustes fossilis*: a study correlating changes in plasma hormone profiles. *Journal of Endocrinology, 156*(2), 365-372.

Kelley, J. L., Magurran, A. E., & Garcia, C. M. (2006). Captive breeding promotes aggression in an endangered Mexican fish. *Biological Conservation, 133*(2), 169-177.

Kime, D. E. (1993). Classical and Nonclassical Reproductive Steroids in Fish. *Reviews in Fish Biology and Fisheries, 3*(2), 160-180.

Koulish, S., & Kramer, C. R. (1989). Human Chorionic-Gonadotropin (hCG) Induces Gonad Reversal in a Protogynous Fish, the Bluehead Wrasse, *Thalassoma bifasciatum* (Teleostei, Labridae). *Journal of Experimental Zoology, 252*(2), 156-168.

Kozul, V., Glavic, N., Tutman, P., Bolotin, J., & Onofri, V. (2011). The spawning, embryonic and early larval development of the green wrasse *Labrus viridis* (Linnaeus, 1758) (Labridae) in controlled conditions. *Anim Reprod Sci, 125*(1-4), 196-203.

Krol, J., Kowalski, R. K., Hliwa, P., Dietrich, G. J., Stabinski, R., & Ciereszko, A. (2009). The effects of commercial preparations containing two different GnRH analogues and dopamine antagonists on spermiation and sperm characteristics in the European smelt *Osmerus eperlanus* (L.). *Aquaculture, 286*(3-4), 328-331.

Lee, W. K., & Yang, S. W. (2002). Relationship between ovarian development and serum levels of gonadal steroid hormones, and induction of oocyte maturation and ovulation in the cultured female Korean spotted sea bass *Lateolabrax maculatus* (Jeom-nong-eo). *Aquaculture, 207*(1-2), 169-183.

Lee, W.K., Yang, S.W., & Kwak, E.J. (2000). Relationship between ovarian development and plasma levels of steroid hormones, and induction of oocyte maturation and ovulation in the cultured female Korean sea bass, *Lateolabrax japonicus. Dev. Reprod*. 4 (2), 187–193.

Legendre, M., Slembrouck, J., Subagja, J., & Kristanto, A. H. (2000). Ovulation rate, latency period and ova viability after GnRH or hCG-induced breeding in the Asian catfish *Pangasius hypophthalmus* (Siluriformes, Pangasiidae). *Aquatic Living Resources, 13*(3), 145-151.

Lethimonier, C., Madigou, T., Munoz-Cueto, J. A., Lareyre, J. J., & Kah, O. (2004). Evolutionary aspects of GnRHs, GnRH neuronal systems and GnRH receptors in teleost fish. *Gen Comp Endocrinol, 135*(1), 1-16.

Levavi-Sivan, B., Vaiman, R., Sachs, O., & Tzchori, I. (2004). Spawning induction and hormonal levels during final oocyte maturation in the silver perch (*Bidyanus bidyanus*). *Aquaculture, 229*(1-4), 419-431.

Li, N., Takeyama, T., Jordan, L. A., & Kohda, M. (2015). Female control of paternity by spawning site choice in a cooperatively polyandrous cichlid. *Behaviour, 152*(2), 231-245.

Malison, J. A., Procarione, L. S., Kayes, T. B., Hansen, J. F., & Held, J. A. (1998). Induction of out-of-season spawning in walleye (*Stizostedion vitreum*). *Aquaculture, 163*(1-2), 151-161.

Marteinsdottir, G., & Steinarsson, A. (1998). Maternal influence on the size and viability of Iceland cod *Gadus morhua* eggs and larvae. *J Fish Biol, 52*(6), 1241-1258.

Martins, R. S. T., Pinto, P. I. S., Guerreiro, P. M., Zanuy, S., Carrillo, M., & Canario, A. V. M. (2014). Novel galanin receptors in teleost fish: Identification, expression and regulation by sex steroids. *Gen Comp Endocrinol, 205*, 109-120.

McConnachie, S. H., Cook, K. V., Patterson, D. A., Gilmour, K. M., Hinch, S. G., Farrell, A. P., & Cooke, S. J. (2012). Consequences of acute stress and cortisol manipulation on the physiology, behavior, and reproductive outcome of female Pacific salmon on spawning grounds. *Horm Behav, 62*(1), 67-76.

Moore, J. W., Schindler, D. E., Carter, J. L., Fox, J., Griffiths, J., & Holtgrieve, G. W. (2007). Biotic control of stream fluxes: Spawning salmon drive nutrient and matter export. *Ecology, 88*(5), 1278-1291.

Morehead, D. T., Pankhurst, N. W., & Ritar, A. J. (1998). Effect of treatment with LHRH analogue on oocyte maturation, plasma sex steroid levels and egg production in female striped trumpeter *Latris lineata* (Latrididae). *Aquaculture, 169*(3-4), 315-331.

Morrey, C., Nagahama, Y., & Grau, E. G. (1995). Hcg Differentially Affects in-Vitro Steroidogenesis in Gonadal Fragments of a Protogynous Hermaphroditic Wrasse. *Biology of Reproduction, 52*, 125-125.

Muncaster, S., Andersson, E., Kjesbu, O. S., Taranger, G. L., Skiftesvik, A. B., & Norberg, B. (2010). The reproductive cycle of female Ballan wrasse *Labrus bergylta* in high latitude, temperate waters. *J Fish Biol, 77*(3), 494-511.

Muncaster, S., Andersson, E., Skiftesvik, A. B., Opstad, I., Taranger, G. L., & Norberg, B. (2008). Seasonal reproductive cycle of Ballan wrasse (*Labrus bergylta*) in Norway. *Cybium, 32*(2), 199-199.

Muncaster, S., Norberg, B., & Andersson, E. (2013). Natural sex change in the temperate protogynous Ballan wrasse *Labrus bergylta*. *J Fish Biol, 82*(6), 1858-1870.

Munshi, A. B., Boardman, G. D., Flick, G. J., Cobb, J., Begum, S., & Lane, R. B. (2012). Impact of Endocrine Disrupting Chemicals (EDCs) on Estrone and Estradiol Content in Fish. *Journal of the Chemical Society of Pakistan, 34*(2), 396-403.

Mylonas, C. C., Hinshaw, J. M., & Sullivan, C. V. (1992). Gnrha-Induced Ovulation of Brown Trout (*Salmo trutta*) and Its Effects on Egg Quality. *Aquaculture, 106*(3-4), 379-392.

Mylonas, C. C., Scott, A. P., & Zohar, Y. (1997). Plasma gonadotropin II, sex steroids, and thyroid hormones in wild striped bass (*Morone saxatilis*) during spermiation and final oocyte maturation. *Gen Comp Endocrinol, 108*(2), 223-236.

Mylonas, C. C., & Zohar, Y. (2001a). Endocrine regulation and artificial induction of oocyte maturation and spermiation in basses of the genus Morone. *Aquaculture, 202*(3-4), 205-220.

Mylonas, C. C., & Zohar, Y. (2001b). Use of GnRHa-delivery systems for the control of reproduction in fish. *Reviews in Fish Biology and Fisheries, 10*(4), 463-491.

Noori, A., Amiri, B. M., Mirvaghefi, A., & Baker, D. W. (2010). LHRHa-induced ovulation of the endangered-Caspian brown trout (*Salmo trutta caspius*) and its effect on egg quality and two sex steroids: testosterone and 17 alpha-hydroxyprogestrone. *Aquaculture Research, 41*(6), 871-877.

Nyina-Wamwiza, L., Defreyne, P. S., Ngendahayo, L., Milla, S., Mandiki, S. N. M., & Kestemont, P. (2012). Effects of partial or total fish meal replacement by agricultural by-product diets on gonad maturation, sex steroids and vitellogenin dynamics of African catfish (*Clarias gariepinus*). *Fish Physiology and Biochemistry, 38*(5), 1287-1298.

Oguz, A. R., Kaptaner, B., & Unal, G. (2015). Vitellogenin in the male Lake Van fish (*Chalcalburnus tarichi* Pallas, 1811). *Bull Environ Contam Toxicol, 94*(2), 188-192.

Omeljaniuk, R. J., Shih, S. H., & Peter, R. E. (1987). Invivo Evaluation of Dopamine Receptor-Mediated Inhibition of Gonadotropin-Secretion from the Pituitary-Gland of the Goldfish, *Carassius auratus*. *Journal of Endocrinology*, 114(3), 449-458.

Pankhurst, N. W., Vanderkraak, G., Peter, R. E., & Breton, B. (1986). Effects of (D-Ala6,Pro9n Ethylamide) - Lhrh on Plasma-Levels of Gonadotropin, 17-Alpha,20-Beta-Dihydroxy-4-Pregnen-3-One and Testosterone in Male Goldeye (*Hiodon alosoides* Rafineque). *Fish Physiology and Biochemistry, 1*(3), 163-170.

Patino, R., & Sullivan, C. V. (2002). Ovarian follicle growth, maturation, and ovulation in teleost fish. *Fish Physiology and Biochemistry, 26*(1), 57-70.

Paxton, C. G. M., & Willoughby, L. G. (2000). Resistance of perch eggs to attack by aquatic fungi. *J Fish Biol, 57*(3), 562-570.

Perini, V. D., Paschoalini, A. L., da Cruz, C. K. F., de Rocha, R. D. G. A., Senhorini, J. A., Ribeiro, D. M., Formagio, P. S., Bazzoli, N., & Rizzo, E. (2013). Profiles of sex steroids, fecundity and spawning of a migratory characiform fish from the Paraguay-Parana basin: a comparative study in a three-river system. *Fish Physiology and Biochemistry, 39*(6), 1473-1484.

Peter, R. E., Lin, H. R., & Vanderkraak, G. (1988). Induced Ovulation and Spawning of Cultured Fresh-Water Fish in China - Advances in Application of Gnrh Analogs and Dopamine Antagonists. *Aquaculture, 74*(1-2), 1-10.

Phartyal, R., Goswami, S. V., & Sehgal, N. (2008). Effect of cortisol on vitellogenin synthesis in the primary hepatocyte culture of African catfish, *Clarias gariepinus*. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology, 151*(1), S4-S5.

Phelps, R. P., Papanikos, N., Bourque, B. D., Bueno, F. T., Hastey, R. P., Maus, D. L., Ferry, A., & Davis, D. A. (2009). Spawning of Red Snapper (*Lutjanus campechanus*) in Response Hormonal Induction or Environmental Control in a Hatchery Setting. *Reviews in Fisheries Science, 17*(2), 149-155.

Planas, J. V., Athos, J., Goetz, F. W., & Swanson, P. (2000). Regulation of ovarian steroidogenesis in vitro by follicle-stimulating hormone and luteinizing hormone during sexual maturation in salmonid fish. *Biology of Reproduction, 62*(5), 1262-1269.

Podhorec, P., & Kouril, J. (2009). Induction of final oocyte maturation in Cyprinidae fish by hypothalamic factors: a review. *Veterinarni Medicina, 54*(3), 97-110.

Prat, F., Zanuy, S., & Carrillo, M. (2001). Effect of gonadotropin-releasing hormone analogue (GnRHa) and pimozide on plasma levels of sex steroids and ovarian development in sea bass (*Dicentrarchus labrax* L.). *Aquaculture, 198*(3-4), 325-338.

Raizada, S., Lal, K. K., Sarkar, U. K., Varshney, P. K., Sahu, V., Yadav, K. C., Agnihotri, P., Awasthi, A., & Jena, J. K. (2013). Captive Breeding and Embryonic Development of Butter Catfish (*Ompok bimaculatus*, Bloch 1794), a Threatened Fish of Indian Sub-continent in Northern India. *Proceedings of the National Academy of Sciences India Section B-Biological Sciences, 83*(3), 333-339.

Rawat, V. S., Rani, K. V., Phartyal, R., & Sehgal, N. (2013). Vitellogenin genes in fish: differential expression on exposure to estradiol. *Fish Physiology and Biochemistry, 39*(1), 39-46.

Rempel, M. A., & Schlenk, D. (2008). Effects of environmental estrogens and antiandrogens on endocrine function, gene regulation, and health in fish. *International Review of Cell and Molecular Biology, Vol 267, 267*, 207-252.

Rosenfeld, H., Mylonas, C. C., Bridges, C. R., Heinisch, G., Corriero, A., Vassallo-Aguis, R., Medina, A., Belmonte, A., Garcia, A., De la Gandara, F., Fauvel, C., De Metrio, G., Meiri-Ashkenazi, I., Gordin, H., & Zohar, Y. (2012). GnRHa-mediated stimulation of the reproductive endocrine axis in captive Atlantic bluefin tuna, *Thunnus thynnus*. *Gen Comp Endocrinol, 175*(1), 55-64.

Sahoo, S. K., Giri, S. S., Chandra, S., & Sahu, A. K. (2007). Spawning performance and egg quality of Asian catfish *Clarias batrachus* (Linn.) at various doses of human chorionic gonadotropin (HCG) injection and latency periods during spawning induction. *Aquaculture, 266*(1-4), 289-292.

Sahoo, S. K., Giri, S. S., & Sahu, A. K. (2010). Evaluation of human chorionic gonadotropin (HCG) dose and latency period combinations on the weight of stripped eggs during induced spawning of *Clarias batrachus* (Linn.). *Indian Journal of Fisheries, 57*(3), 83-85.

Sarkar, U. K., Deepak, P. K., Negi, R. S., Singh, S., & Kapoor, D. (2006). Captive breeding of endangered fish *Chitala chitala* (Hamilton-Buchanan) for species conservation and sustainable utilization. *Biodiversity and Conservation, 15*(11), 3579-3589.

Scott, B. E., Marteinsdottir, G., Begg, G. A., Wright, P. J., & Kjesbu, O. S. (2006). Effects of population size/age structure, condition and temporal dynamics of spawning on reproductive output in Atlantic cod (*Gadus morhua*). *Ecological Modelling, 191*(3-4), 383-415.

Shibatta, O. A., Novelli, J. L., Dias, J. H. P., Britto, S. G. D., & Caetano, M. (2011). Reproduction of duckbill catfish *Sorubim lima* in captivity (Siluriformes, Pimelodidae) by means of hormonal induction. *Semina-Ciencias Agrarias, 32*(1), 363-372.

Shrimpton, J. M., & Randall, D. J. (1994). Down-Regulation of Corticosteroid Receptors in Gills of Coho Salmon Due to Stress and Cortisol Treatment. *American Journal of Physiology, 267*(2), R432-R438.

Silverstein, J. T., Bosworth, B. G., & Wolters, W. R. (1999). Evaluation of dual injection of LHRHa and the dopamine receptor antagonist pimozide in cage spawning of channel catfish *Ictalurus punctatus*. *Journal of the World Aquaculture Society, 30*(2), 263-268.

Singh, B. R., & Yadav, B. N. (1975). Effect of ACTH and bilateral gonadectomy on the adrenocortical tissue of an air-breathing fish *Heteropneustes fossilis* (Bloch). *Z Mikrosk Anat Forsch, 89*(3), 486-494.

Stuartkregor, P. A. C., Sumpter, J. P., & Dodd, J. M. (1981). The Involvement of Gonadotropin and Sex Steroids in the Control of Reproduction in the Parr and Adults of Atlantic Salmon, *Salmo-Salar* L. J Fish Biol, 18(1), 59-72.

Su, B. F., Perera, D. A., Zohar, Y., Abraham, E., Stubblefield, J., Fobes, M., Beam, R., Argue, B., Ligeon, C., Padi, J., Waters, P., Umali-Maceina, G., Chatakondi, N., Kristanto, A., Hutson, A., Templeton, C., Ballenger, J., Chaimongkol, A., Gima, A., Gima, M., Zuberi, A., Lambert, D. M., Kim, S., Mandour, M., & Dunham, R. A. (2013). Relative effectiveness of carp pituitary extract, luteininzing hormone releasing hormone analog (LHRHa) injections and LHRHa implants for producing hybrid catfish fry. *Aquaculture, 372*, 133-136.

Sundararaj, B. I., Goswami, S. V., & Lamba, V. J. (1982). Role of Testosterone, Estradiol-17-Beta, and Cortisol during Vitellogenin Synthesis in the Catfish, *Heteropneustes fossilis* (Bloch). *Gen Comp Endocrinol, 48*(3), 390-397.

Tacon, P., Baroiller, J. F., Le Bail, P. Y., Prunet, P., & Jalabert, B. (2000). Effect of egg deprivation on sex steroids, gonadotropin, prolactin, and growth hormone profiles during the reproductive cycle of the mouthbrooding cichlid fish *Oreochromis niloticus*. *Gen Comp Endocrinol, 117*(1), 54-65.

Thibaut, R., & Porte, C. (2004). Effects of endocrine disrupters on sex steroid synthesis and metabolism pathways in fish. *Journal of Steroid Biochemistry and Molecular Biology, 92*(5), 485-494.

Treasurer, J. W. (1994). The Distribution, Age and Growth of Wrasse (Labridae) in Inshore Waters of West Scotland. *J Fish Biol, 44*(5), 905-918.

Van Lier, E., Regueiro, M., Perez-Clariget, R., Andersson, H., Kindahl, H., & Forsberg, M. (1999). Effects of adrenocorticotrophin (ACTH) and progesterone on luteinising hormone (LH) secretion in recently castrated rams. *Anim Reprod Sci, 55*(2), 115-126.

Vazirzadeh, A., Hajimoradloo, A., Esmaeili, H. R., & Akhlaghi, M. (2008). Effects of emulsified versus saline administration of GnRHa on induction of ovulation in rainbow trout, *Oncorhynchus mykiss*. *Aquaculture, 280*(1-4), 267-269.

Velasco-Santamaria, Y., & Cruz-Casallas, P. (2007). Methodology for determination of plasma cortisol in fish using competitive enzyme-linked immunosorbent assay (ELISA). *Revista Mvz Cordoba, 12*(1), 869-877.

Vikingstad, E., Andersson, E., Norberg, B., Mayer, I., Klenke, U., Zohar, Y., Stefansson, S. O., & Taranger, G. L. (2008). The combined effects of temperature and GnRHa treatment on the final stages of sexual maturation in Atlantic salmon (*Salmo salar* L.) females. *Fish Physiology and Biochemistry, 34*(3), 289-298.

Viveiros, A. T., Goncalves, A. C., Di Chiacchio, I. M., Nascimento, A. F., Romagosa, E., & Leal, M. C. (2013). Gamete quality of streaked prochilod *Prochilodus lineatus* (Characiformes) after GnRHa and dopamine antagonist treatment. *Zygote*, 1-10.

Wang, S. H., Yuen, S. S. F., Randall, D. J., Hung, C. Y., Tsui, T. K. N., Poon, W. L., Lai, J. C. C., Zhang, Y., & Lin, H. R. (2008). Hypoxia inhibits fish spawning via LH-dependent final oocyte maturation. *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology, 148*(4), 363-369.

Watanabe, W. O., Ellis, S. C., Ellis, E. P., Head, W. D., Kelley, C. D., Moriwake, A., Lee, Cheng-Sheng., & Bienfang, P. K. (1995). Progress in controlled breeding of Nassau grouper (*Epinephelus striatus*) broodstock by hormone induction. *Aquaculture, 138*(1–4), 205-219.

Watanabe, W. O., Smith, T. I. J., Berlinsky, D. L., Woolridge, C. A., Stuart, K. R., Copeland, K. A., & Denson, M. R. (2003). Volitional spawning of black sea bass *Centropristis striata* induced with pelleted luteinizing hormone releasing hormone-analogue. *Journal of the World Aquaculture Society, 34*(3), 319-331.

Westring, C. G., Ando, H., Kitahashi, T., Bhandari, R. K., Ueda, H., Urano, A., Dores, R. M., Sher, A. A., & Danielson, P. B. (2008). Seasonal changes in CRF-I and urotensin I transcript levels in masu salmon: Correlation with cortisol secretion during spawning. *Gen Comp Endocrinol, 155*(1), 126-140.

Wootton, R. J., & Smith, C. (2014). Reproductive Biology of Teleost Fishes. *In*: Endocrinology of reproduction. Wiley-Blackwell, pp. 81-125.

Yaron, Z. (1995). Endocrine Control of Gametogenesis and Spawning Induction in the Carp. *Aquaculture, 129*(1-4), 49-73.

Zairin, M., Asahina, K., Furukawa, K., & Aida, K. (1992). Plasma Steroid-Hormone Profiles during Hcg Induced Ovulation in Female Walking Catfish *Clarias batrachus*. *Zoological Science, 9*(3), 607-617.

Zairin, M., Furukawa, K., & Aida, K. (1992a). Changes in Ovarian Maturity in the Tropical Walking Catfish, *Clarias batrachus* Reared under 23-25-Degrees-C. *Nippon Suisan Gakkaishi, 58*(11), 2033-2037.

Zairin, M., Furukawa, K., & Aida, K. (1992b). Induction of Ovulation by Hcg Injection in the Tropical Walking Catfish *Clarias batrachus* Reared under 23-25-Degrees-C. *Nippon Suisan Gakkaishi, 58*(9), 1681-1685.

Zanuy, S., Carrillo, M., & Ruiz, F. (1986). Delayed Gametogenesis and Spawning of Sea Bass (*Dicentrarchus labrax* L) Kept under Different Photoperiod and Temperature Regimes. *Fish Physiology and Biochemistry, 2*(1-4), 53-63.

Zohar, Y., & Mylonas, C. C. (2001). Endocrine manipulations of spawning in cultured fish: from hormones to genes. *Aquaculture, 197*(1-4), 99-136.

Chapter four

Larval Weaning and Growth in Hormonal Induced *Labrus bergylta* Larvae

4.1 Introduction

4.1.1 Early growth and development of fish larvae

At the time of hatching, many marine fish larvae have immature organs and nutrition is provided endogenously through the yolk sac (Osse *et al*., 1997; Jaworski and Kamler, 2002; Yufera and Darias, 2007). The main organ systems differentiate during the period leading up to metamorphosis and become functional by the time of first feeding (Falk-Petersen, 2005; Yufera and Darias, 2007). During this period, the available resources are directed into developing the organs most needed to enhance further growth and survival, and organ differentiation has to take place over a relatively short period of time (Osse and van den Boogaart, 2004). Overall, the ontogenesis of fish larvae may generally be divided into different stages: yolk sac, preflexion, flexion, postflexion stage and metamorphosis (Tytler and Blaxter, 1988; Cahu *et al*., 2009).

In *L. bergylta* larvae, preliminary studies have shown that pre-flexion larvae are partly pigmented on the body, while there are no pigments in the caudal region (Dunaevskaya *et al*, 2012). The flexion stage is characterized by the existence of the interspinous membrane of the caudal fin at a larval size of 5 mm, and at 7 mm the urochord is fully bent upwards. When the larval length is around 8 mm the anal and dorsal fins develop and melanophores appear anteriorly on the anal fin (Darcy *et al*., 2012). The swim bladder, which acts as a hydrostatic organ, is filled in many species soon after hatching. *E.g*., in cod it is developed at the age of 10 days post-hatch (dph) (Grotmol *et al*., 2005), whereas in other species this may take several weeks or months (Falk-Petersen, 2005). A successful inflation of the swim bladder during early development is vital for larval manoeuvring ability and thus larvae feeding success. This event occurs within certain intervals during early development, often associated with the transition from endogenous to exogenous feeding in larvae (Trotter, Pankhurst and Battaglene, 2005). The chronological age of the larva does not necessarily indicate its physiological age, which depends mainly on water temperature (Meekan *et al*., 2003; Green and Fisher, 2004; Johnston and Hall, 2004).

Thus, staging the larval ontogenesis based on external morphological features should be related to larval age given as days and as degree-days.

After opening of the mouth, a quick growth and differentiation of the digestive organs is necessary to enable digestion and nutrient absorption (Yufera and Darias, 2007). In turbot (*Psetta maxima*) and common dentex (*Dentex dentex*), the digestive organs increased in volume at a relatively faster rate than the other organs before a switch occurred to exclusive exogenous feeding (Sala *et al*., 2005). This priority of growth / unequal growth of the different systems is a common feature in larval development (Osse and van den Boogaart, 2004; Sala *et al*., 2005; Wiff and Roa-Ureta, 2008). Outer structures also grow unevenly during larval development, for example, a higher growth rate of the head and tail region, as opposed to the trunk, is a common feature (Fuiman, 1983; Osse *et al*., 1997; vanSnik *et al*., 1997; Peña and Dumas, 2009). This results in an increased ability to capture prey, in addition to being beneficial when escaping predators and reducing the energy expenditure during locomotion (Osse *et al*., 1997).

Uneven growth rates in early life, or 'allometry', representing sharp changes during the larval period is common during development before more isometric growth is observed in older fish (Nakayama and Fuiman, 2010). Information about the growth of various structures may help in understanding critical points in the early development of fish larvae (Sala *et al*., 2005). Nevertheless, such growth and development studies are still limited in *L. bergylta*, even though these provide the basis for designing and optimising hatchery protocols for this new species. In particular, nutrition is one of the most important extrinsic factors regulating growth (Osse and van den Boogaart, 2004), and different nutritional requirements may occur during different periods of larval development.

4.1.2 Nutritional requirements of marine fish larvae

The first feeding period is considered a major bottleneck in fish larval growth and development. At this stage, larvae are very small and require feed of specific size and nutritional composition. Proteins are important for developing fish larvae as growth primarily is an increase in body muscle mass by protein synthesis and accretion, and this group makes up between 60 and 80% of the larval dry weight (Galloway *et al*., 1999; Kjørsvik *et al*., 2004). Specific growth rates during the larval

phase can be high, and a daily rate close to 30% has been measured for Atlantic cod (*Gadus morhua*) reared at 14°C (Otterlei *et al*., 1999). Amino acids are the building blocks of proteins, and they are also considered a major energy source during larval development (Fyhn, 1989; Finn *et al*., 1995; Rønnestad *et al*., 1999; Rønnestad *et al*., 2003). They are provided through the diet incorporated in proteins and as free amino acids (FAA), with FAAs in particular being rapidly and efficiently absorbed by fish larvae (Rust *et al*., 1993; Rønnestad *et al*., 2000a; Applebaum and Rønnestad, 2004). The protein and FAA content varies according to type of live feed organism, life stage and rearing conditions of the live feed (Helland *et al*., 2003; Pedersen *et al*., 2004; van der Meeren *et al*., 2008). Although proteins are the most important dietary component in terms of quantity, the right amount and composition of lipids is equally important in the larval diet (Rainuzzo *et al*., 1997; Sargent *et al*., 1999; Giraldo *et al*., 2013).

Lipids are grouped as either neutral or polar, with triacylglycerides (TAG) and wax esters (WE) constituting neutral lipids and functioning as a major source of energy. Phospholipids (PL) are polar lipids, and besides serving as a source of energy, are important structural and functional components of cell membranes, gonads, brain and eye tissue (Navarro *et al*., 1993; Watanabe and Kiron, 1994; Furuita *et al*., 1998; Murzina *et al*., 2008). PL biosynthesis does not take place at a sufficient rate to meet the PL requirement in the fast growing larvae (Geurden *et al*., 1995; Tocher *et al*., 2008). A sufficient supply of PL through the diet is therefore important, as larval stages are sensitive towards PL deficiency and require higher levels of dietary PL than juveniles (Geurden *et al*., 1995; Coutteau *et al*., 1997). Compared to TAG, PLs are more easily digested (Olsen *et al*., 1991; Tocher *et al*., 2008) and their presence may enhance digestion of other lipids; in addition, PLs are a key component of the lipoproteins transporting nutrients after uptake by the intestinal absorptive cells (enterocytes) (Coutteau *et al*., 1997; Tocher *et al*., 2008).

The PLs (polar) and neutral lipids are composed of fatty acids, *e.g.*, *n-3* polyunsaturated fatty acids (PUFAs), some of which are essential to ensure optimal larval growth and development (Watanabe and Kiron, 1994; Furuita *et al*., 1998; Izquierdo *et al*., 2000; Evjemo *et al*., 2003). Since these essential fatty acids (EFAs) cannot be synthesized by the fish larvae *de novo*, they need to be provided through the diet (Bell *et al*., 2003). The PUFAs docosahexaenoic acid (22:6*n-3*; DHA),

eicosapentaenoic acid (20:5*n-3*; EPA) and arachidonic acid (20:4*n-6*; ARA) are considered to be the most important EFAs for marine fish larvae (Sargent *et al*., 1999; Bell *et al.*, 2003). They can be supplied by either the PL or the neutral lipid fraction, but these EFAs are more beneficial and readily digested when incorporated in the PL fraction (Izquierdo *et al*., 2000; Gisbert *et al*., 2005; Kjørsvik *et al*., 2009; Wold *et al*., 2009). Studies on Atlantic cod found that incorporation of the EFA in the PL fraction led to better larval growth, more developed digestive organs, earlier ossification and increased levels of DHA in the tissue (Kjørsvik *et al*., 2009 ; Wold *et al*., 2009). The amount and ratio of the EFAs provided is also greatly important, with a varying optimum from species to species (Sargent *et al*., 1999). In general, the demand is greater during the larval stages compared to juveniles and adults (Coutteau *et al*., 1997).

There is little information on protein and lipid requirements of *L. bergylta* larvae. The digestive system has an altricial development (Balon, 1979), meaning "requiring nourishment", refers to a pattern of growth and development in organisms which are incapable of moving around on their own soon after hatching with the intestine being a straight, undifferentiated tube and lacking a stomach at the time of first feeding (Dunaevskaya *et al*., 2012). This is a common feature among marine fish larvae, *e.g*., Atlantic cod (Kjørsvik *et al*., 1991), Atlantic halibut (*Hippoglossus hippoglossus*) (Luizi *et al*., 1999), turbot (*Scophthalmus maximus*) (Segner *et al*., 1994), Senegalese sole (*Solea senegalensis*) (Ribeiro *et al*., 1999), common dentex (*Dentex dentex*) (Santamaria *et al*., 2004). The need for similar nutrition to other marine fish larvae with altricial digestive development is likely, with a need for proteins and lipids that are easily digestible at time of first feeding. Recent studies have actually found that *L. bergylta* lacks a stomach as an adult (Hamre and Saele, 2011), a factor which emphasises the above requirements when developing formulated feeds.

4.1.3 First feeding from live into dry food for *Labrus bergylta* larvae

Live feed is commonly used in first feeding of marine fish larvae (Conceicao *et al*., 2010), and this is the preferred food of larvae when presented in combination with inert diets (Person-Le Ruyet *et al*., 1993; Fernández-Díaz *et al*., 1994; Conceicao *et al*., 2010). The movement of the live feed keeps it suspended in the water column

and may help to stimulate feeding behaviour. Also, the high water content (normally above 80%) and availability of varying sizes make live feed ideal in the early rearing stages of marine fish larvae (Turingan *et al*., 2005; Conceicao *et al*., 2010). The most commonly used live feed is rotifer (*Brachionus* sp.) and brine shrimp (*Artemia* sp.), due to the existence of standardized cost-effective protocols for their mass production (Lubzens, 1987; Sorgeloos *et al*., 2001; Conceicao *et al*., 2010; Boglino *et al*., 2012). These species have also been used as first feed in the cultivation of *L. bergylta* (Skiftesvik *et al*., 2011) during exogenous feeding which occurs from 4 dph. *L. bergylta* larvae are fed rotifers up to approximately 30 dph, followed by *Artemia* until weaning on formulated dry feed is successful (Treasurer, 1994; Dunaevskaya *et al*., 2012). Rotifers and *Artemia* do not fulfil the fish larval requirements for EFAs and these are therefore enriched to ensure nutritional quality (Lubzens, 1987; Evjemo and Olsen, 1997; Oie and Olsen, 1997; Sorgeloos *et al*., 2001; Conceicao *et al*., 2010). During enrichment, the EFAs are incorporated into the neutral lipid fraction (Rainuzzo *et al*., 1994), where they are less available to the fish larvae (Izquierdo *et al*., 2000; Gisbert *et al*., 2005; Kjørsvik *et al*., 2009; Wold *et al*., 2009). Skeletal deformities and low survival rates were observed in *L. bergylta* larvae first feeding on rotifers and *Artemia* (Grøntvedt, 2010; Helland *et al*., 2012), suggesting that the current first feeding practice is suboptimal and may be improved.

Commercial dry diets are more convenient than live food and do not require labour-intensive facilities for live food production (Rottmann *et al*., 1991). Mixed diets of live and dry food can be chosen to match the nutritional requirements of larvae and provide a back-up food supply in case of production failure; they can also be used to enhance weaning of juvenile fish onto artificial diets (Rottmann *et al*., 1991; Rosenlund *et al*., 1997; Abdel-Warith *et al*., 2001). For example, the difficulty of getting cultured cyprinids to accept dry starter diets can often be overcome by feeding a mixed live and dry food diet initially and then weaning the fish onto dry food after one to two weeks (Opuszynski and Shireman, 1993). Good growth and high survival of goldfish reared under intensive conditions have been achieved with a mixed diet of *Artemia* and dry food (Abiayad and Kestemont, 1994; Kestemont, 1995).

The best growth and development of larvae may be obtained through early rearing with mixed live and dry diets (Rottmann *et al*. 1991). Kestemont (1995)

achieved the best growth rate of larvae at 28ºC ranging from 27% to 34% day^{-1} in intensively-reared goldfish on a mixed diet of *Artemia* and dry food, although it was not known if the fish fed selectively on the *Artemia* component of the diet. One of the problems associated with feeding dry diets is the relatively fast deterioration of water quality and tank cleanliness, and the rapid proliferation of micro-organisms on uneaten food (Charlon and Bergot, 1984; Brock *et al*., 1992; Sales, 2011). This may affect the growth and survival of larvae (Rottmann *et al*., 1991; Sharma and Chakrabarti, 1999; Cavalin and Weirich, 2009). The problem of water quality degradation due to feed type is not limited to the dry food component of a mixed diet because uneaten *Artemia* may also reduce water quality. Hygiene considerations are probably less serious for *L. bergylta* that has a capability of cleaning their surroundings (Treasurer, 1994).

Polychaete is a natural food source for many fish and very good fishing bait for many marine fish species. Polychaetes are naturally rich in both DHA and EPA incorporated into their PLs (Beneliahu and Golani, 1990; Whitfield *et al*., 1999; Drazen *et al*., 2008), which should ensure that these molecules easily accessible for marine fish larvae with an immature digestive system. They have also been found to have a higher protein and FAA content than rotifers and *Artemia* (Costa *et al*., 2000; Cardinaletti *et al*., 2009). The digestive system of fish larvae initially has a high assimilation capacity towards FAA and a low protein digestibility, but the latter increases as the proteolytic capacity matures (Rønnestad *et al*., 2000b; Cahu and Infante, 2001; Rønnestad *et al*., 2003; Applebaum and Rønnestad, 2004). Polychaete has never been tested in the early feeding of *L. bergylta* larvae, for example, as a transitional feed between live and artificial feeding. Formulated dry food from polychaetes is available as a freeze dried product for early feeding. There are two types of marine polychaetes used in the aquaculture and aquarium trade since the 90's: king ragworm (*Nereis virens*) and lugworm (*Arenicola marina*). Powdered freeze dried worm that is certified disease-free product is available with the commercial name of *Lyo-p* from © Shoreline Polychaete Farms LLP 2010 in U.K.

The aim of this study was to examine the early growth and weaning of *L. bergylta* larvae from hatch to 112 dph. Also, to determine the effect of two types of dry diets, namely, micropellet and micropellet + polychaete in two spawning batches of *L. bergylta* larvae. Both batches of larvae received co-feeding regimens i.e. they

received live feed (rotifers and *Artemia*) along with the different formulations of dry diets between 49 to 112 days post hatch (dph). The hypothesis tested was whether dried polychaete + micropellet would promote better growth in *L. bergylta* larvae compared to micropellet alone.

4.2 Materials and Methods

4.2.1 Larval maintenance in feeding trials

L. bergylta larvae were produced in the hatching facility at Carna Research Station from artificial spawning using hormonal treatments between April and May 2011 (Chapter three). Spawning took place in two batches and fertilized eggs from these batches were incubated in separate cones. The age of larvae in both spawning batches was separated by three weeks as spawning batch I was fertilized on 30/4/2011 and hatched on 6/5/2011, whereas spawning batch II was fertilized on 18/5/2011 and hatched on 23/5/2011. Reproductive parameters such as egg diameter and fertilization / hatching / survival rates were recorded for every spawner and spawning batch. Egg diameters were calculated just prior to hatch using measurements from two perpendicular axes. Hatched larvae were distributed at an estimated density of 10,000 larvae per 1000L fibreglass tank. Four rearing tanks were used in the co-feeding trials: two tanks each were used for spawning batch I and II, with each tank getting a separate diet 'A' or 'B' (-see section 4.2.3).

The larvae were maintained in a flow-through system in which water-flow remained constant at approximately 600ml/min throughout the experiment. Inflowing sea water (34 ppt and ambient temperature) was treated with a sand filter and filtered through a 10 μm mesh. Any dead fish or debris was removed by siphoning the bottom every second or third day from the beginning of dry feeding at 49 days post-hatch (dph). Each tank was aerated at the bottom and attached with a sieve tube in the middle of the tank where the water outlet was situated (Figure 4.1). Mesh size on the sieves was increased from 200μm at the beginning of the experiment (from 0 to 49 dph) to 750μm at the end of the experiment (from 49 to 112 dph). Temperature and oxygen (O_2) concentrations were measured daily using Traceable® VWR® Digital Thermometer, (VWR, USA), pH/mV-meter, (WTW pH 315i, Germany) and care was taken to ensure that the O_2 level was above 80% or 8.3 mg/l throughout the experiment. Cumulative Degree Days (CDD) were calculated according to dph x mean daily temperature for each tank. All the larval tanks were maintained in diurnal photoperiod with 16L:8D with illumination measuring 1500 lux (daylight fluorescent tubes, Philips MASTER TL-D 90 Graphica, 18W/965).

Spawning batch I Diet A	Spawning batch I Diet B
Spawning batch II Diet A	Spawning batch II Diet B

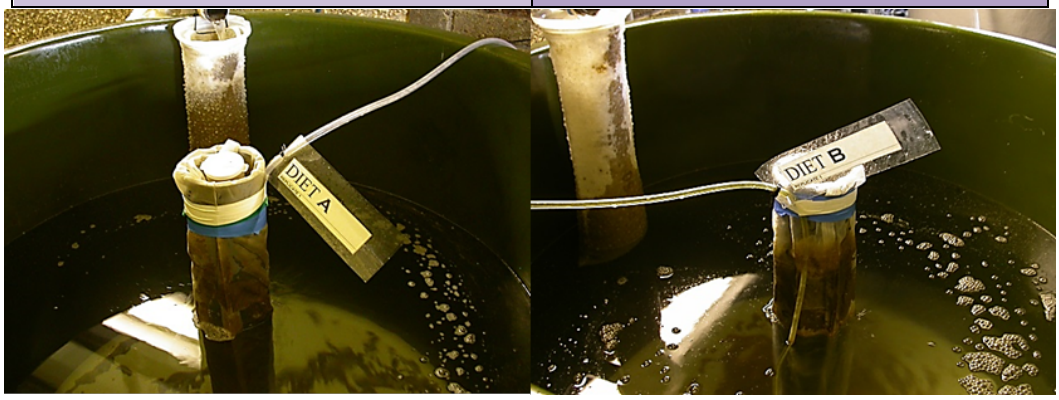


Figure 4.1: Experimental tanks for spawning batch I and II *L. bergylta* larvae with Diets A and B.

4.2.2 Formulated dry feeds

Growth was compared in two dry feed regimens for both batches of larvae (Figure 4.2). Diet A comprised pre-prepared micropellets and Diet B contained micropellet + freeze-dried polychaete. In Diet A, a standard micropellet was purchased from the O.range larval diet range from INVE Technologies nv Norway (website: www.inve.com). Micropellets named O.range Start, O.range Wean and O.range Grow have particles sized from 100 to 200 µm, 300 to 500 µm and 600 to 800 µm, respectively. The larvae were fed particles according to the size of their mouth or based on their age. As reported from the manufacturer, the overall nutrient content of this micropellet was 56% crude protein, 12% crude oils and fats, 12% crude ash, 4% insoluble ash in hydrochloric acid, 1% crude fiber, 1.2% phosphorus, Docosahexaenoic acid (DHA), Eicosapentaenoic acid (EPA) and 40mg/g dwt Sum (n-3) highly unsaturated fatty acids (HUFA). In Diet B, freeze-dried polychaetes were added to micropellets as an extra component. The typical nutritional profile of polychaete meal is: 55% crude protein, 17% crude oils and fats, 7.5% crude ash, 4% insoluble ash in hydrochloric acid, 1% crude fiber, 1.2% phosphorus, DHA, EPA and 40mg/g dwt Sum (n-3) HUFA.

Figure 4.2: Ready prepared Diet A (micropellet from O.range larval diet range) and Diet B (micropellet + freeze-dried polychaete) in bottles.

4.2.3 Start feeding and co-feeding

Each feeding regime had two replicates, one from each of the spawning batches, in a total of four trial tanks (Table 4.1; Figure 4.1). The treatments started off with identical feeding regimens: larvae in all rearing tanks were given live feed from day one onwards, when they were fed exclusively on green algae (*Nannochloropsis* sp.) and rotifers (*Brachionus* sp.). Live cultures of *Nannochloropsis* sp. were added to the tank twice daily. At 3 dph, the larvae were fed once daily with cultured rotifers, *Brachionus* sp. (final density: approximately 10 rotifers/ml) enriched with Selco®. Rotifers were reared on INVE Culture Selco Plus and enriched for 6 hours on DHA Selco (Docosahexaenoic Acid Self-Emulsified Liquid Concentrate). Enriched rotifers were then treated with Pyceze (supplied by Atlantic Veterinary Services Ltd.) for 1 – 3 hours. The number of feeds was increased gradually to two feeds at 7 dph and three feeds at 9 dph until 28 dph. The amount of feed was also gradually increased from 20 to 60 litres mixed live feed per day to each tank by 28 dph (Table 4.1).

From approximately 14 dph, larvae were weaned onto *Artemia* sp.. During the weaning period the numbers of rotifers were gradually reduced until fish larvae were feeding primarily on *Artemia* sp. (at 35 – 42 dph). *Artemia* sp. were enriched with GadiSelco® in 250 L cylindro-conical cones for 24 hours. *Artemia* sp. cysts were hydrated for one hour, decapsulated in sodium hypochlorite for 1 – 3 minutes, rinsed and set up in 250 L hatching vessels. *Artemia* sp. were separated from unhatched cysts and cuticular membranes using oxygen flocculation and then these *Artemia* sp. were treated with Pyceze (50mg/L water) for 1 – 2 hours to control

fungal and bacterial infections. Enriched *Artemia* sp. was introduced to the larvae at 14 dph until 84 dph (final density: approximately 1 individual/ml).

Fish larvae were weaned onto inert commercial (INVE) diets starting between 49 and 112 dph. During this weaning period, the amount of *Artemia* sp. was reduced until fish larvae were feeding primarily on dry feed (~91 – 112 dph). Co-feeding consisting of *Artemia* sp. plus either Diet A or Diet B commenced after 42 dph. This co-feeding trial remained in place between 49 dph and 84 dph, and is the period that was compared during statistical analysis (-see 4.2.5 Data analysis). The doses of each diet were adjusted according to the appetite of the fish larvae, keeping the food density at 3000/L. Micropellets of 100 to 200 μm in size were used between 49 to 63 dph. This was increased to 300 to 500 μm between 70 to 84 dph and to 600 to 800 μm between 91 to 112 dph. Thereafter, the fish larvae were fed manually three times a day at about 10 g per tank/day, normally at 9.00 am, 12.00 noon and 16.00 pm. The formulated food was distributed equally in excess over the water surface in the rearing tanks. Weaning, i.e. exclusive feeding with dry feed, commenced from 91 dph to 112 dph (see Table 4.1 below) and the experiment was terminated at 112 dph.

Table 4.1: Experimental setup of co-feeding regimens of microalgae, rotifers, *Artemia*, micropellet (Diet A) or micropellet + polychaete meal (Diet B) fed to the Spawning batches I and II *L. bergylta* larvae. Sampling points for various parameters at different days post hatch (dph) are also indicated.

Days post hatch (Dph)	1	2	3	4	5	6	7	14	21	28	35	42	49	56	63	70	77	84	91	98	105	112
Microalgae (Nannochloropsis sp.)																						
Tank 1 (Spawning batch I) Diet A																						
Tank 2 (Spawning batch I) Diet B																						
Tank 3 (Spawning batch II) Diet A																						
Tank 4 (Spawning batch II) Diet B																						
Rotifers (Brachionus sp.)																						
Tank 1 (Spawning batch I) Diet A																						
Tank 2 (Spawning batch I) Diet B																						
Tank 3 (Spawning batch II) Diet A																						
Tank 4 (Spawning batch II) Diet B																						
Artemia (Artemia sp.)																						
Tank 1 (Spawning batch I) Diet A																						
Tank 2 (Spawning batch I) Diet B																						
Tank 3 (Spawning batch II) Diet A																						
Tank 4 (Spawning batch II) Diet B																						
Micropellet																						
Tank 1 (Spawning batch I) Diet A																						
Tank 3 (Spawning batch II) Diet A																						
Micropellet + Polychaeta																						
Tank 2 (Spawning batch I) Diet B																						
Tank 4 (Spawning batch II) Diet B																						
Sampling																						
Standard length (mm)	•		•			•	•	•	•	•	•	•	•	•	•	•		•		•		•
Myotome height (mm)	•		•			•	•	•	•	•	•	•	•	•	•	•		•		•		•
Dry weight (g)							•	•	•	•	•	•	•	•	•	•		•		•		•
Wet weight (g)													•	•	•	•		•		•		•
Outflow mesh size (µm)	200													750								

4.2.4 Larval sampling

In the first days after hatching (1 dph, 3 dph and 6 dph), larvae were randomly sampled and measured for various parameters (Table 4.1). This sampling was done on a weekly basis from day 7 – 112 dph for standard length (±0.01mm), myotome height (±0.01mm), wet weight (±0.001g) and dry weight (±0.001g). In each sampling, 10 to 15 larvae were examined and measured from each tank. Sampled larvae were anaesthetized in a solution of tricaine methane sulfonate (0.05mg/l MS-222, Sigma) before rinsing them in distilled water. Each larva was gently placed (by pipette) on a 1 ml welled slide, etched with a 1 mm^2 grid. The measurements were performed on photographs of the fish larvae taken using a compound microscope with 10mm eye lens (Olympus CX21, Germany; Nikon digital sight DS-SM, Nikon Corporation, Japan). A graticule or grid paper (0.1mm / 10mm) was used as scale for accurate measurements in larger fish larvae. Standard length (SL) was measured from the tip of the upper lip to the end of the vertebra pre-flexion and to the peduncle (root of the caudal fin) post-flexion. Body depth, or myotome height (MH) provided the perpendicular depth of the trunk relative to the axial skeleton; this measure was taken right behind the anus from the ventral to the dorsal margin (Figure 4.3). Samples for wet weight (WW) and dry weight (DW) were measured with a milligram balance (UMX2, Mettler-Toledo, U.S.). For DW, larvae were transferred into an oven and dried at 60^0C for a minimum of 24 hours prior to being weighed (Figure 4.4).

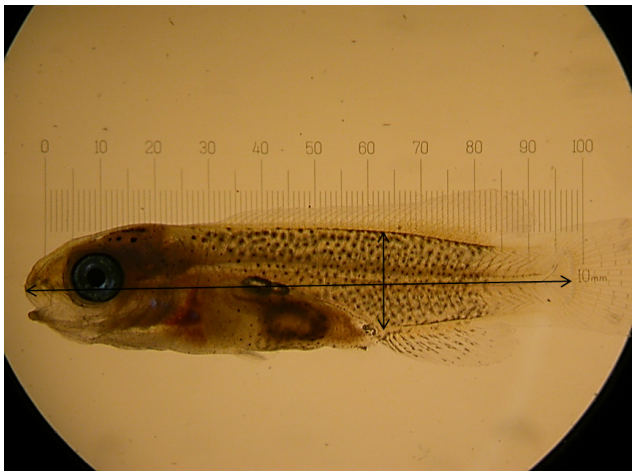


Figure 4.3: Standard length is measured from the tip of the upper lip to the end of the vertebra (horizontal line), while myotome height is perpendicular to the axial skeleton right behind the anus (vertical line) of *L. bergylta* larvae.

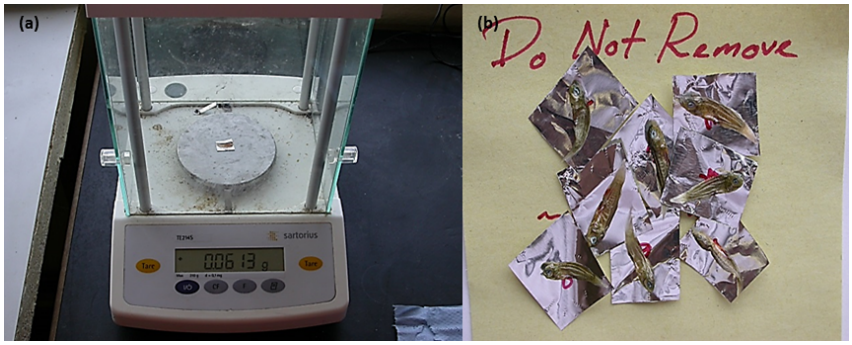


Figure 4.4: Weighing scales (a) and samples for dry weight (b).

Specific growth rate (SGR) was calculated using equations according to Ricker (1958). Specific growth rates of myotome height (SGRMH) for the whole period of development were calculated for both batches/diets using the following formula:

$$SGRMH = [(LnMHf - LnMHi))/t(dph)] \times 100$$

LnMHf = the natural logarithm of the final myotome height; LnMHi = the natural logarithm of the initial myotome height, and t(dph) = time (days post hatch) between LnMHf and LnMHi. The SGRMH was calculated for Diet A and Diet B, respectively. The formula was adapted to also calculate specific growth rates for standard length (SGRSL), dry weight (SGRDW) and wet weight (SGRWW). Specific growth rate (SGR) on a weekly basis was expressed as a percentage of the geometric mean (geomean) for all the growth parameters of the larvae which was calculated using the measurements at the beginning and the end of each sampling (dph).

4.2.5 Data analysis

To safeguard against tendencies for the data to break assumptions of homogeneity of variance, all response variables of standard length, myotome height, wet weight and dry weight were $\log_{10}$ transformed to normalise them against two different predictor variables; Cumulative Degree Days (CDD) and age, expressed as days post-hatch (dph). The difference in means for different diet types was tested via two-way ANOVA ($p=0.05$), with any differences between spawning batches also being tested. Statistical analyses were performed with SPSS Version 20.0 (SPSS Inc., USA, 2011) for PC.

4.3 Results

4.3.1 Reproductive background for spawning batch I and II *Labrus bergylta* larvae

Labrus bergylta broodstock were in good condition and the overall body weight-length relationship of the broodstock with a quadratic relationship was described by $y = 1.55x^2 - 44.11x + 399.32$; $F = 125.51$; $R^2 = 0.92$, $P<0.01$ (Figure 4.5).

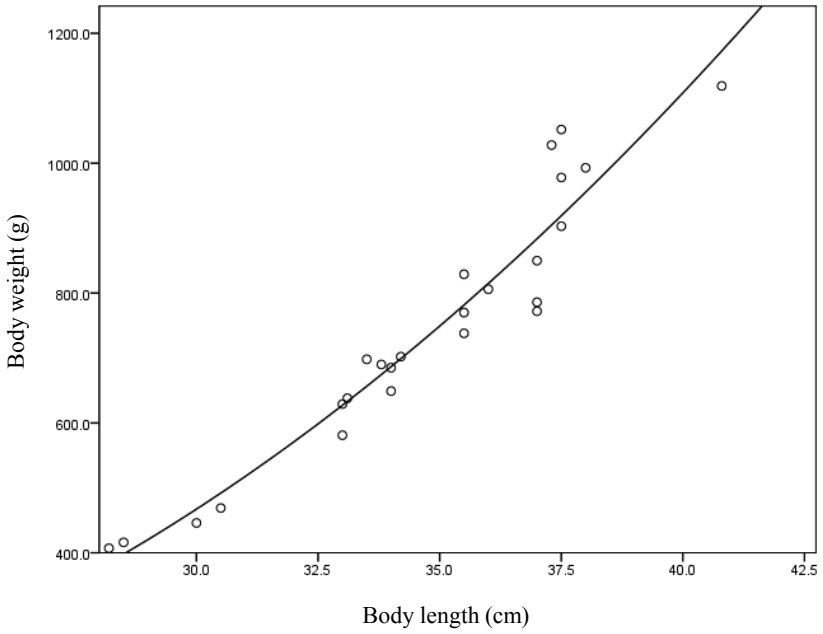


Figure 4.5: Relationship between body weight (g) and body length (cm) of spawned *L. bergylta* broodstock (n = 25) for spawning batch I and II larvae.

Table 4.2 shows the various reproductive and environmental measures from the point of spawning to when hatching took place for spawning batches I and II. Eggs of both spawning batches were healthy in appearance and hatched normally. Hatching for both spawning batch I and II occurred approximately 6 days post fertilization. Newly hatched larvae for both batches measured 3.57±0.03mm and 3.58±0.02mm in standard length, respectively.

Table 4.2: Overall mean and standard deviations (mean ± s.d.) of reproductive measures for spawning batch I and II *L. bergylta* larvae. dph: days post hatch.

Batches of larvae	Spawning batch I	Spawning batch II
Lengths of spawner (cm)	35.86±2.27	33.25±3.48
Weights of spawner (g)	818.57±173.14	652.18±194.78
Condition factor (K)	1.75±0.13	1.73±0.09
Egg volume (ml)	13.50±6.64	13.82±14.00
Approximate number of ovulated eggs (1000±100ml^{-1})	14850±7306	15200±15399
Egg diameter (mm)	1.00±0.01	1.00±0.01
Milt volume (ml/egg batch)	0.05	0.05
Fertilization date	30th April 2011 (120 day-of-year)	18th May 2011 (138 day-of-year)
Fertilization rate (%)	76.3±9.5	68.4±11.9
Incubation period (hours)	137.00	137.00
Hatching date	6th May 2011 (126 day-of-year)	24th May 2011 (144 day-of-year)
Cumulative degree days of incubation (CDD)	91.30	80.50
Temperature at hatch (°C)	13.7±0.5	11.4±0.7
Hatching rate (%)	79.6±9.1	67.2±16.9
Larval mortality after 7 days of hatching (%)	10.5±6.8	18.4±15.5
Standard length at 0 dph (mm)	3.57±0.03	3.58±0.02
Myotome height at 0 dph (mm)	0.22±0.01	0.21±0.01

Ambient temperatures fluctuated but, overall, batch II was exposed to slightly higher Cumulative Degree Days (CDD) than spawning batch I (Figure 4.6). Average temperatures started out at 12.85±0.27ºC (CDD 93.60) and 11.99±0.45ºC (CDD 80.44) respectively for spawning batch I and spawning batch II during the period leading up to 7 days post hatch (dph). Temperatures increased gradually to reverse this situation between batches by the end of feeding trials (112 dph), with average temperatures of 14.72±0.65ºC (CDD 1579.73) and 13.20±0.30ºC (CDD 1615.41) respectively in batch I and II.

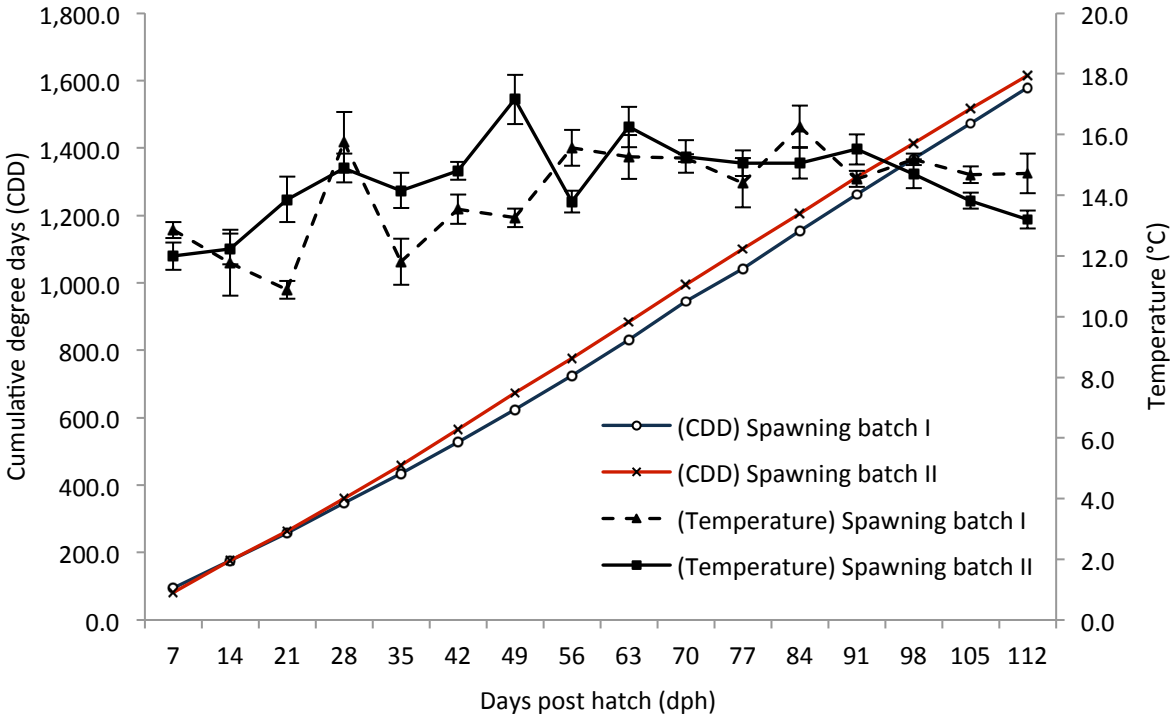


Figure 4.6: Sea water temperature and Cumulative Degree Days (CDD) profile in the feeding trials for spawning batch I and II *L. bergylta* larvae at days post-hatch (dph).

The growth of *L. bergylta* larvae was studied from hatching to 112 dph. A $\log_{10}$ dry weight versus $\log_{10}$ standard length showed a shift in the weight-length relationship corresponding to the onset of metamorphosis in both spawning batches; this is termed a 'break-point' or inflection point. Larval length-weight relationships were determined from the 'break-point' (Figure 4.7). In spawning batch I, this occurred above a log standard length of 1.079 mm, (corresponding to 11.994 mm standard length) with the linear relationship post-break point being described by y = 0.0368x - 0.0371; F = 288.99; R^2=0.7391; P<0.01. Below this value, the relationship was y = 0.0028x - 0.0021; F = 374.87; R^2=0.693; P<0.01. Spawning batch II showed almost the same pattern although the ratio was generally higher in magnitude and more sharply increasing. At the break-point which occurred at a log standard length of 1.199 mm (15.812 standard length), the larval length-weight relationship increased steeply in spawning batch II; described by the equation y = 0.0557x - 0.0603; F = 430.90; R^2=0.785; P<0.01. This compared to a relationship before the inflection point described by y = 0.0073x - 0.0053; F = 760.77; R^2=0.759; P<0.01.

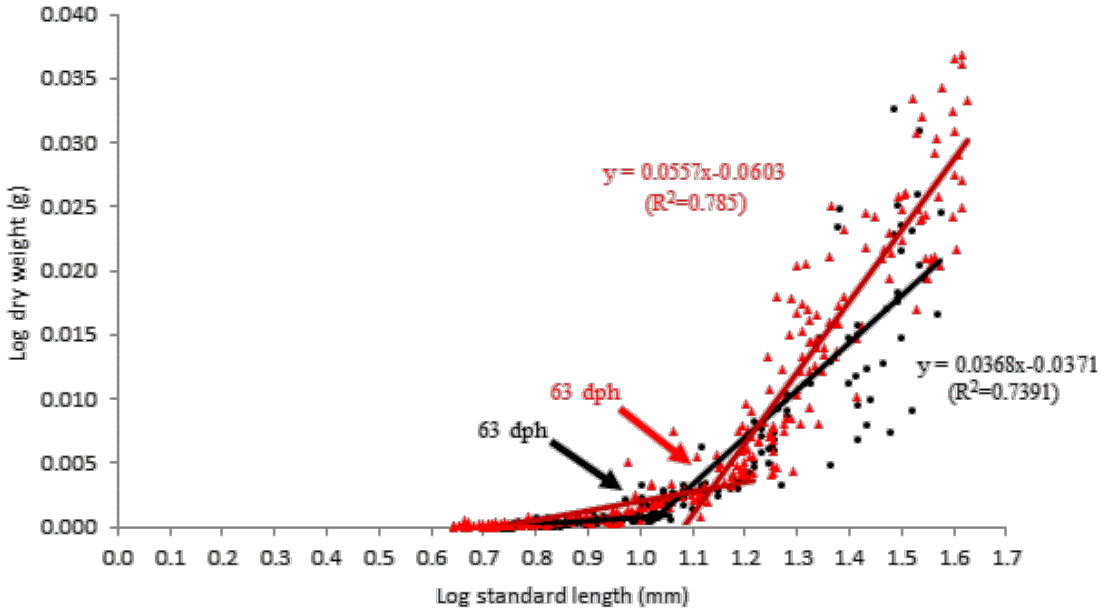


Figure 4.7: Log_{10} (standard length versus dry weight) relationships for replicate tanks together in spawning batch I (-) and II (-) *L. bergylta* larvae under co-feeding trials from 0-112 dph. Arrows with the respective colours indicate the break-point inflexions in growth rates at ~63 dph.

The change in appearance of *L. bergylta* larvae through weekly sampling from both spawning batches are presented in Figure 4.8 (0 to 49 dph) and Figure 4.9 (56 to 112 dph). Larvae development could be divided into four stages based on gross external morphology: (1) Yolk sac larva (0-6 dph); (2) Pre-flexion larva, (7-14 dph); (3) Flexion larva (21-28 dph); (4) Post-flexion larva (35-49 dph) (Figure 4.8). At hatching, the mouth and anus were closed and eyes were not pigmented. The first feed intake was observed at 7 dph, two days before the appearance of an inflated swim-bladder. Swim bladder inflation was seen between 9 to 14 dph. Larvae were fed in mixed diet (co-feeding: 49 to 84 dph) during pre-metamorphosis (1–63 dph) and post-metamorphosis (63–112 dph). Larval growth reflected the high dietary in co-feeding composition which reports the evidence with a positive larval sensitivity to mixed diet during pre-metamorphosis than post-metamorphosis. Transformation to juveniles started between 56 and 70 dph, according to fin development and increment in body depth (Figure 4.9).

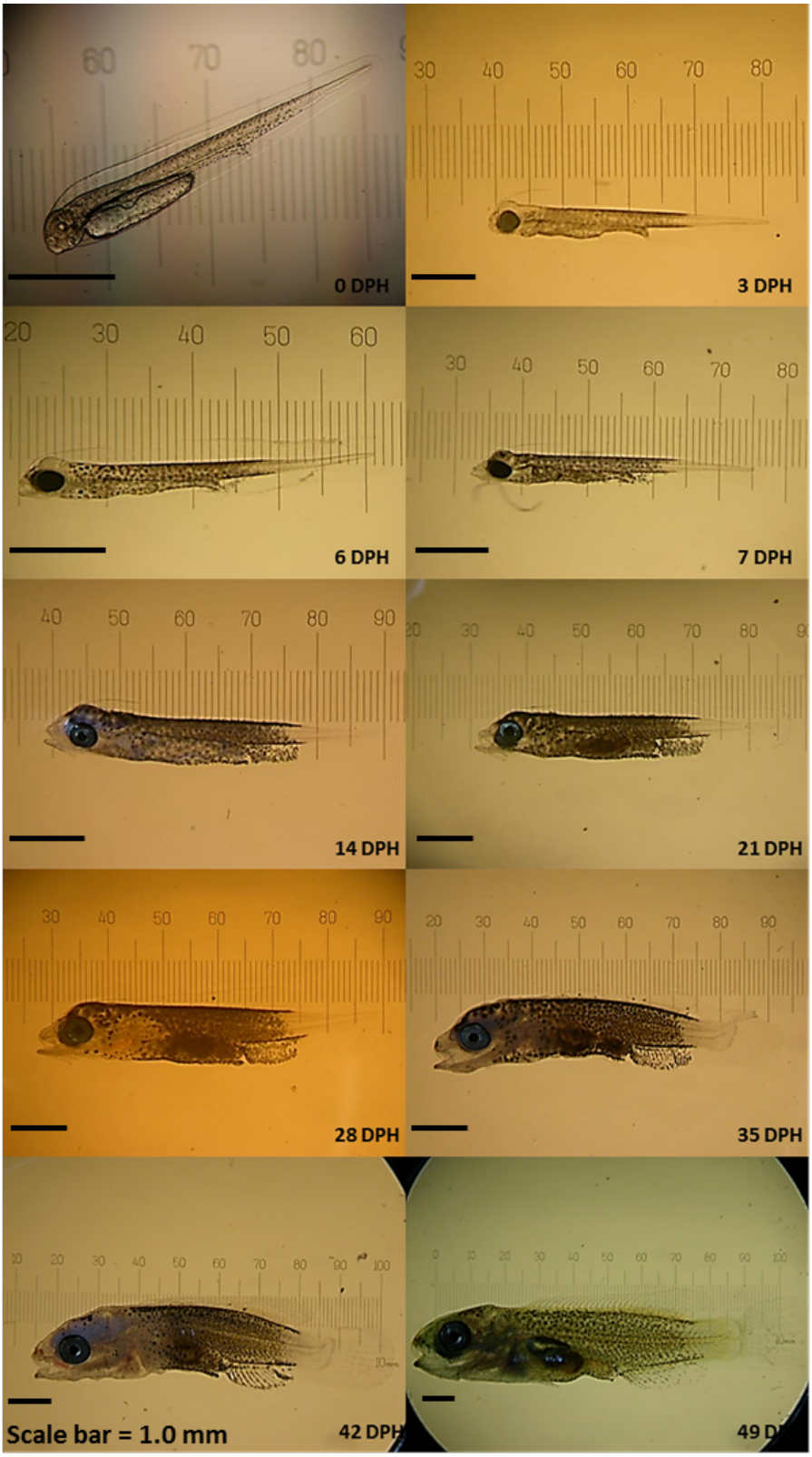


Figure 4.8: Lateral view of *L. bergylta* larvae from 0 dph to 49 dph seen under the compound microscope. The scale bars in the photo represent 1.0 mm.

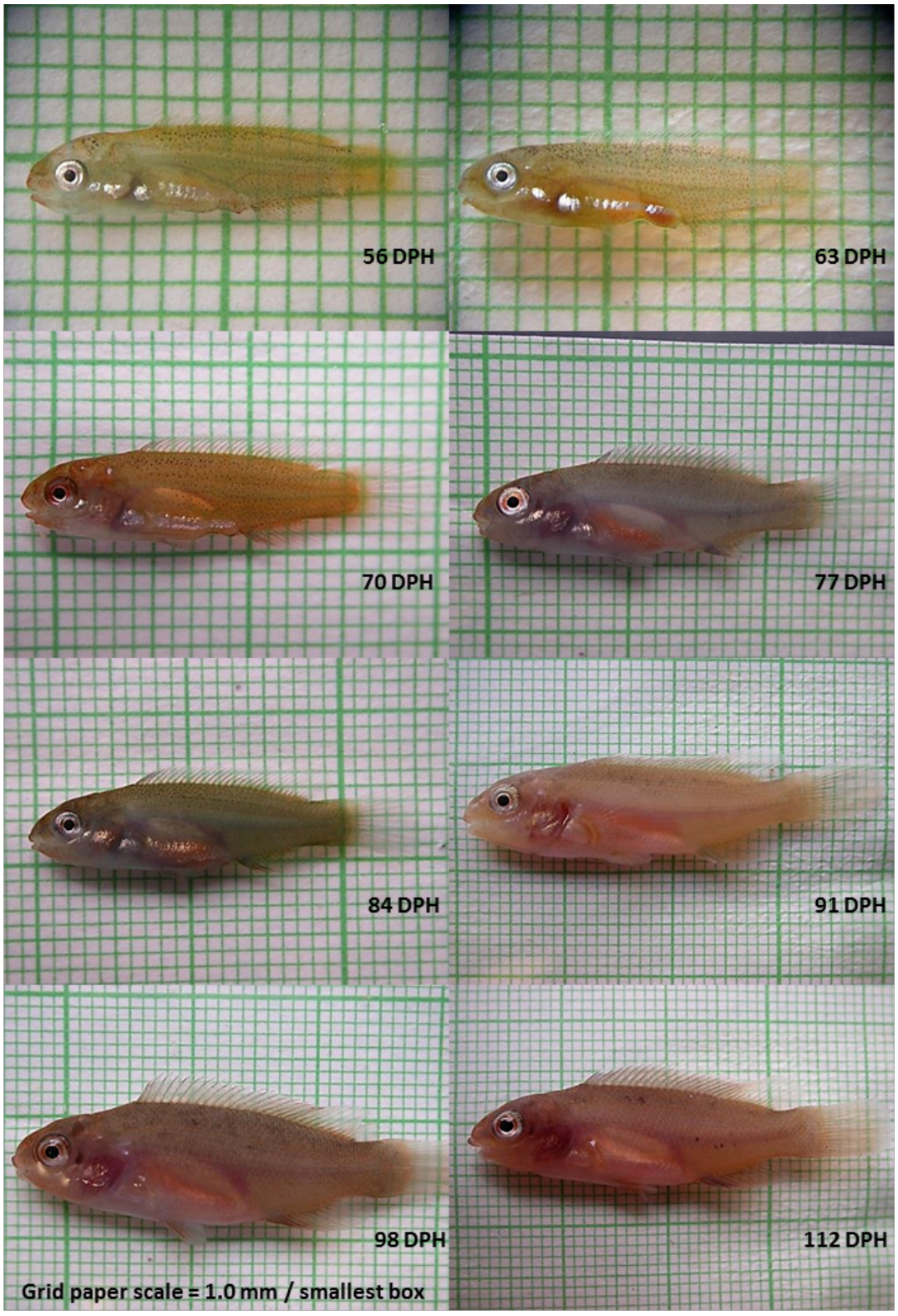


Figure 4.9: Lateral view of *L. bergylta* larvae from 56 dph to 112 dph seen under the dissecting microscope. The smallest box in a grid scale in the photos represent 1.0 mm.

4.3.2 Effect of spawning batches and diets on growth parameters

Spawning batch I and II larvae for mean standard length, myotome height, dry weight and wet weight between 7 and 112 dph on Diet A and B are shown in Table 4.3. The mean larval standard length did not vary significantly across spawning batches and Diets A or B (Table 4.3), although larvae did best on this measure in spawning batch II Diet B at mean ± s.e. of 12.84±0.78mm. Myotome height also showed no significant difference between spawning batches and Diet A or B (Table 4.3). However, there were some significant differences in dry weight, specifically between spawning batch I, Diet A (0.0070±0.0010g) and both spawning batch II Diet A (0.0125±0.0013g) and B (0.0159±0.0018g), with the latter two showing higher growth. Other differences in dry weight were for the same Diet (B) in spawning batches I and II. Mean growth for the same diet in this case was 0.0095±0.0015g in batch I and significantly higher than this at 0.0159±0.0018g in batch II. Wet weight differences were only significant between spawning batch II Diet B and the rest, with the former growing more at mean ± s.e. of 0.49±0.05g. Descriptive statistics of all growth parameters based on means and specific growth rates (SGRs) for Diet A and B in both spawning batches are displayed in Appendix 5 for the entire feeding period.

Table 4.3: Overall means and their standard errors for standard length (mm), myotome height (mm), dry weight (g) and wet weight (g) in spawning batch I and II larvae of *Labrus bergylta* on Diet A and Diet B during feeding trial. Significant differences are denoted by letters "a" to "c".

Groups	Standard length	Myotome height	Dry weight	Wet weight
Spawning batch I; Diet A	10.52±0.61[a] (n=136)	2.09±0.18[a] (n=136)	0.0070±0.0010[a] (n=136)	0.24±0.04[a] (n=33)
Spawning batch I; Diet B	11.13±0.70[a] (n=136)	2.38±0.23[a] (n=136)	0.0095±0.0015[ab] (n=136)	0.33±0.05[a] (n=33)
Spawning batch II; Diet A	11.85±0.70[a] (n=182)	2.52±0.21[a] (n=182)	0.0125±0.0013[bc] (n=182)	0.33±0.03[a] (n=70)
Spawning batch II; Diet B	12.84±0.78[a] (n=182)	2.88±0.24[a] (n=182)	0.0159±0.0018[c] (n=182)	0.49±0.05[b] (n=70)

[a, b, c] Means within a column within a group with different superscripts are significantly different at $p<0.05$.

Diet-related differences in spawning batch I and spawning batch II larvae can only be compared between day 49 to 112 dph as this was when differences in diet (Diet A versus B) were introduced. Mean standard length of larvae for this period differed significantly according to spawning batch but not according to diet: spawning batch II on Diet A (21.58±0.99mm) and B (23.86±1.04mm) were significantly higher than batch I for either diet (Table 4.4). Myotome height showed the same result of spawning batch II being significantly higher than batch I irrespective of diet. Dry weight also showed a significant difference between spawning batches with better growth in batch II than batch I; but in addition, there was a diet-related difference in the best-growing batch II. In this case, Diet B (0.0394±0.0029g) was significantly higher in dry weight than Diet A (0.0306±0.0022g). Wet weight was not different apart from spawning batch II Diet B which grew better than the rest at mean ± s.e. of 0.49±0.05g (Table 4.3 and 4.4).

Table 4.4: Means and their standard errors for standard length (mm), myotome height (mm), dry weight (g) and wet weight (g) in spawning batch I and II larvae of *Labrus bergylta* during 49 to 112 dph on receiving Diet A and Diet B in feeding trial. Significant differences are denoted by letters "a" to "c".

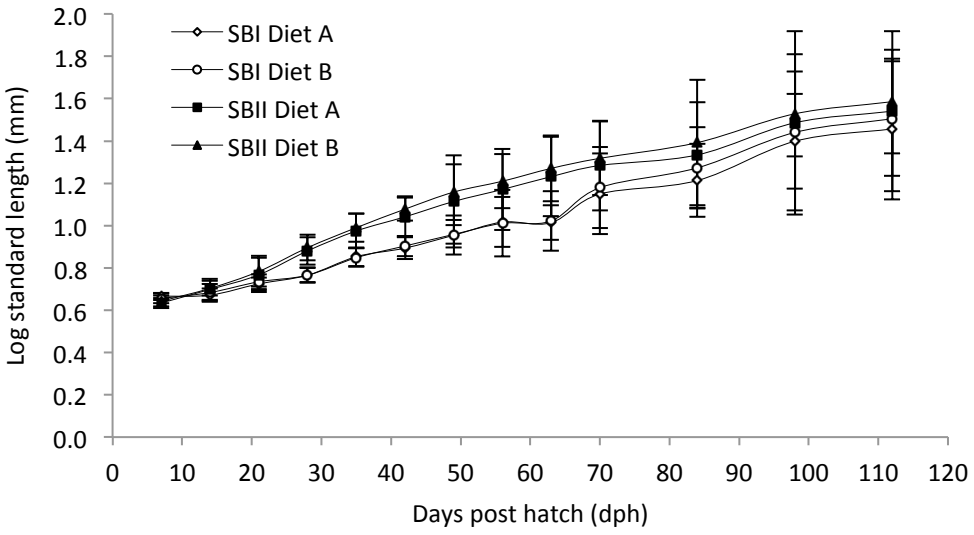
Groups	Standard length	Myotome height	Dry weight	Wet weight
Spawning batch I; Diet A	15.72±0.93[a] (n=64)	3.69±0.27[a] (n=64)	0.0139±0.0018[a] (n=64)	0.24±0.04[a] (n=33)
Spawning batch I; Diet B	16.96±1.09[a] (n=64)	4.27±0.35[a] (n=64)	0.0193±0.0026[a] (n=64)	0.33±0.05[a] (n=33)
Spawning batch II; Diet A	21.58±0.99[b] (n=70)	5.38±0.31[b] (n=70)	0.0306±0.0022[b] (n=70)	0.33±0.03[a] (n=70)
Spawning batch II; Diet B	23.86±1.04[b] (n=70)	6.19±0.33[b] (n=70)	0.0394±0.0029[c] (n=70)	0.49±0.05[b] (n=70)

[a, b, c] Means within a column within a group with different superscripts are significantly different at $p<0.05$.

4.3.3 Growth - Standard length and myotome height

During the first 112 dph, the larval growth in standard length and myotome height followed an exponential curve (Figure 4.10a and 4.11). As we saw in Table 4.4, mean standard lengths or myotome heights of larvae for the period when diets actually differed (49-112 dph), showed no effect of diet but significant effects according to spawning batch; with II growing more than I. The larvae in batch II also received higher temperatures than batch I. If we standardise this, we can observe that even at a given CDD, spawning batch II outperformed batch I throughout the experiment, having the larger mean standard length of the two batches, irrespective of diet (Figure 4.10b). Notwithstanding this fact, by 100 dph or approximately 1200 Cumulative Degree Days (CDD), spawning batch I caught-up somewhat with batch II (Figures 4.10a and 4.11).

(a)



(b)

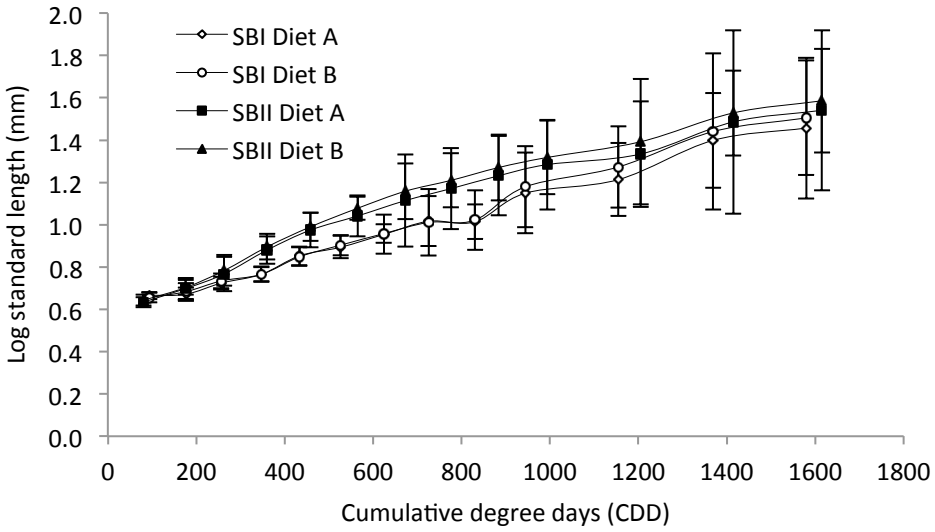


Figure 4.10: Means ± s.e. of log standard length (mm) of spawning batch I and II *L. bergylta* larvae with live feed schedule and co-feeding of Diet A (micropellet) and Diet B (micropellet and polychaete meal). (a) 7 to 112 days post hatch (dph) and (b) 0 to 1615.4°C Cumulative Degree Days (CDD).

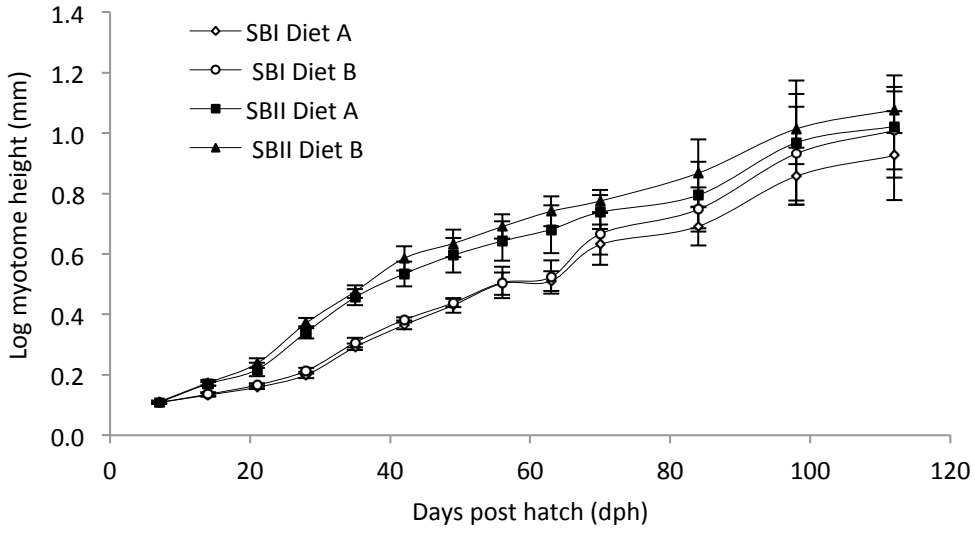


Figure 4.11: Means ± s.e. of log myotome height (mm) of spawning batch I and II *L. bergylta* larvae with live feed schedule and co-feeding of Diet A (micropellet) and Diet B (micropellet and polychaete meal) from 7 to 112 days post hatch (dph).

Specific growth rates of larvae were expressed as a percentage of standard length and myotome height. These gave different patterns from the results seen above. For example in spawning batch I, mean specific growth rate based on standard length (SGRSL) was 1.575±0.636% for Diet A and 1.666±0.469% for Diet B, while mean specific growth rate based on myotome height (SGRMH) was 1.818±0.625% for Diet A and 2.062±0.633% for Diet B. In spawning batch II, mean SGRSL was 1.473±0.240% for Diet A and 1.451±0.175% for Diet B, while mean SGRMH was 1.713±0.321% for Diet A and 1.721±0.224% for Diet B. On these measures, Diet B mostly showed better specific growth rates than Diet A (as before), however, Diet A was occasionally higher than Diet B, e.g. (spawning batch II for SGRSL). In addition, we can observe that variability due to spawning batches on specific growth measures was the opposite of previously, with spawning batch I being generally higher than II.

Weekly profiles of specific growth rates based on standard length (SGRSL) and myotome height (SGRMH) revealed a very variable pattern for both measures. Figure 4.12 and 4.13 show bar charts of weekly growth rates from 7 to 112 dph. Spawning batch I recorded the highest SGRSL on both diets at 70 dph and the lowest at 63 dph, which was similar to the SGRMH measure. Both measures for spawning batch I were also high at 35 dph. Meanwhile, spawning batch II was quite different, as this time the highest SGRSL and SGRMH was recorded on both diets at 28 dph and SGR tended to worsen after this point. Overall, spawning batch II recorded generally higher and more stable growth measures in younger larvae (~0-63 dph) relative to spawning batch I and gave better performance in total (Figure 4.12 and 4.13). Regarding diets, there was no trend in SGR between Diets A and B on a weekly basis between 49-112 dph (Figure 4.12 and 4.13).

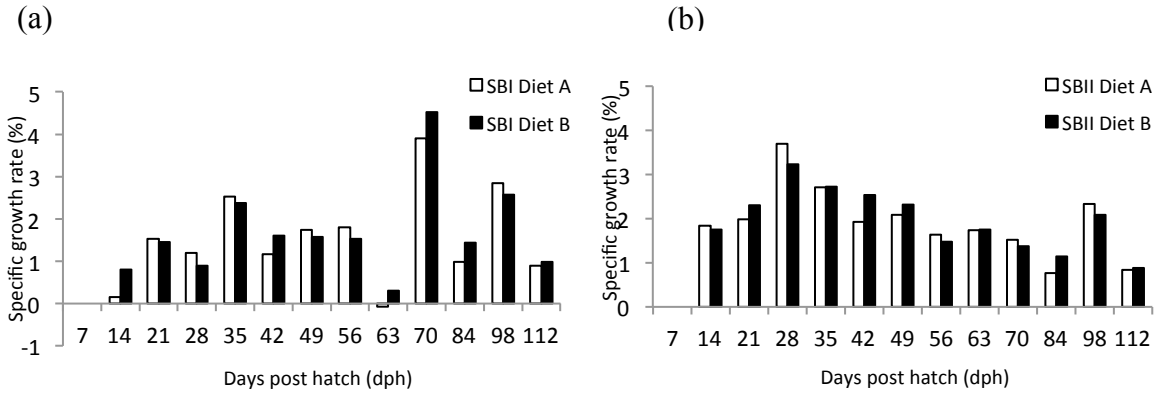


Figure 4.12: Specific growth rate expressed as a % of body mass increment for standard length (SGRSL) in (a) spawning batch I (SBI) and (b) spawning batch II (SBII) *L. bergylta* larvae on Diet A and Diet B from 7 to 112 days post hatch. Please note that Diets A and B were given at 49-112 dph.

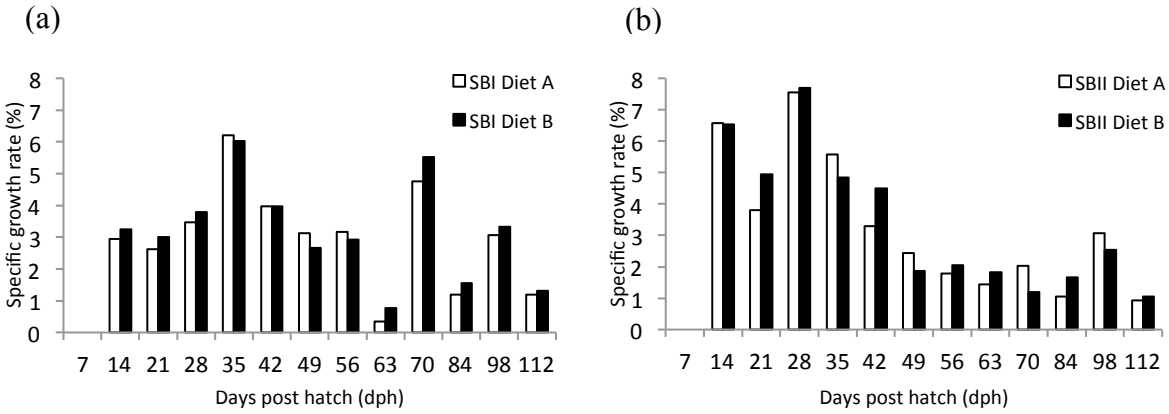


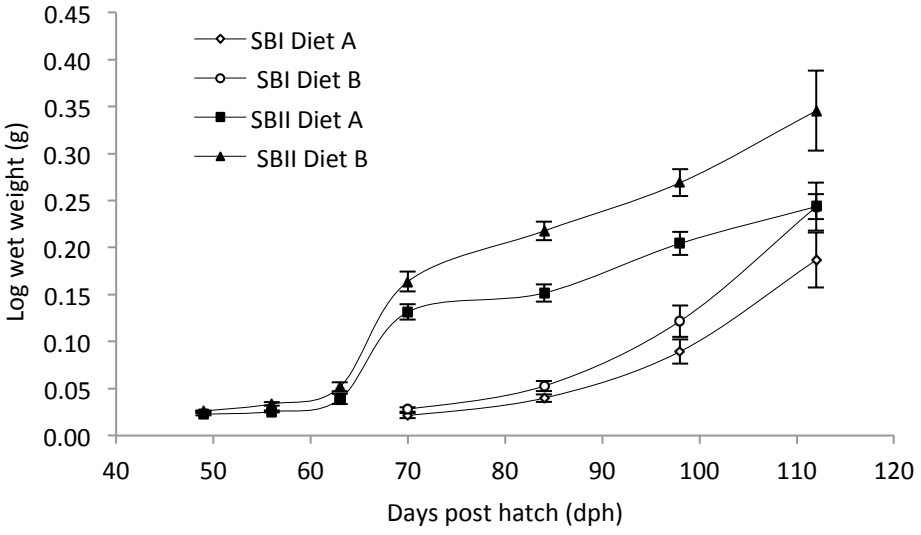
Figure 4.13: Specific growth rate expressed as a % of body mass increment for myotome height (SGRMH) in (a) spawning batch I (SBI) and (b) spawning batch II (SBII) *L. bergylta* larvae on Diet A and Diet B from 7 to 112 days post hatch. Please note that Diets A and B were given at 49-112 dph.

4.3.4 Growth - Dry and wet weight

As with length measures, larval growth measured in dry and wet weight also followed an exponential curve. Based on weight measures it was more strongly apparent that spawning batch II larvae significantly outperformed spawning batch I from approximately 28 dph onwards, although convergence between the batches was evident at 112 dph (Figures 4.14 and 4.15). Diet-specific differences were also more pronounced in the weight measure compared to length / myotome height, with dry/wet weight of Diet B larvae generally much higher than Diet A within a spawning batch.

As specified before, spawning batches were affected by temperature and cumulative degree days (CDD) difference, such that batch II received higher temperatures. Nevertheless, at a comparable CDD, batch II greatly outperformed batch I in terms of wet weight (Figure 4.14b). By ~1580 CDD, the batches had converged somewhat. At a given CDD, larvae fed Diet B had larger dry and wet weights than those fed with Diet A, particularly in spawning batch II larvae. There was a sharp rise in wet and dry weight between 63 to 70 dph, particularly SBII. The wet weight in spawning batch II larvae rose from 0.093±0.013g to 0.354±0.019g on Diet A and from 0.126±0.011g to 0.458±0.025g on Diet B in this period (Figure 4.14a). An equivalent – increase was present though less pronounced in dry weight of spawning batch II larvae (Figure 4.15).

(a)



(b)

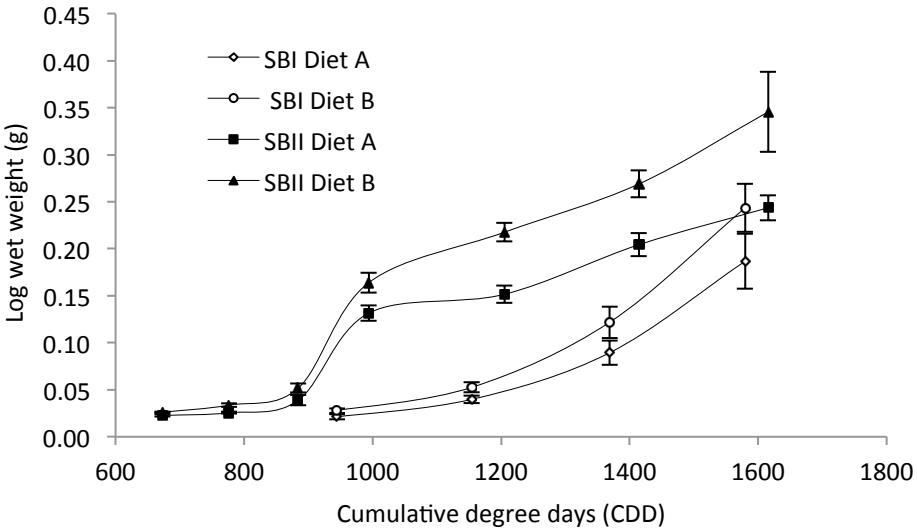


Figure 4.14: Means ± s.e. of log wet weight (g) of spawning batch I and II *L. bergylta* larvae with live feed schedule and co-feeding of Diet A (micropellet) and Diet B (micropellet and polychaete meal) from (a) 7 to 112 days post hatch (dph) and from (b) 0 to 1615.4°C cumulative degree days (CDD).

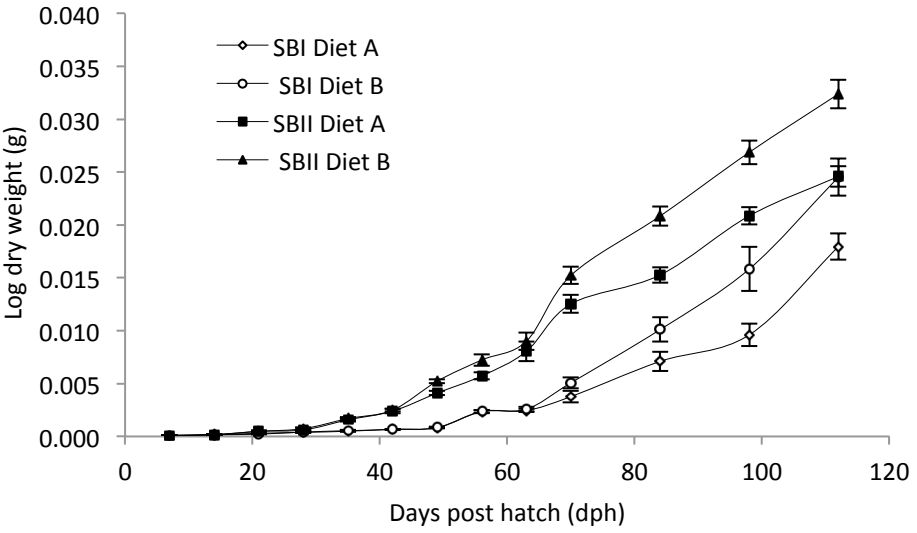


Figure 4.15: Means ± s.e. of log dry weight (g) of spawning batch I and II *L. bergylta* larvae with live feed schedule and co-feeding of Diet A (micropellet) and Diet B (micropellet and polychaete meal) from 7 to 112 days post hatch (dph).

Mean specific growth rates of larvae were expressed as a percentage of increment of dry weight (SGRDW). These SGRs were much lower than the specific growth rates measured on wet weight (SGRWW –not shown). In spawning batch I, mean SGRDW was 3.507±0.736% for Diet A and 3.554±0.542% for Diet B, meanwhile SGRWW recorded 5.267±0.472% for Diet A and 5.404±0.497% for Diet B. In spawning batch II, SGRDW recorded 3.111±0.750% for Diet A and 3.113±0.814% for Diet B, meanwhile SGRWW recorded 4.769±2.463% for Diet A and 5.256±2.239% for Diet B. As before, spawning batch I was actually higher on these measures than batch II. In addition, Diet B gave higher growth than Diet A ($p=0.010$) in SGRWW for spawning batch II, but this was not the case for spawning batch I or for dry weight.

Figure 4.16 showed weekly measures on specific growth rates for dry weight revealed a mixed pattern. Spawning batch I recorded the highest SGRDW on both diets at 56dph and the lowest at 63dph. Meanwhile, spawning batch II fluctuated a lot, but was generally higher in younger larvae before gradually declining over time. There was no particular trend in weekly specific growth rates on Diets A and B (Figure 4.16).

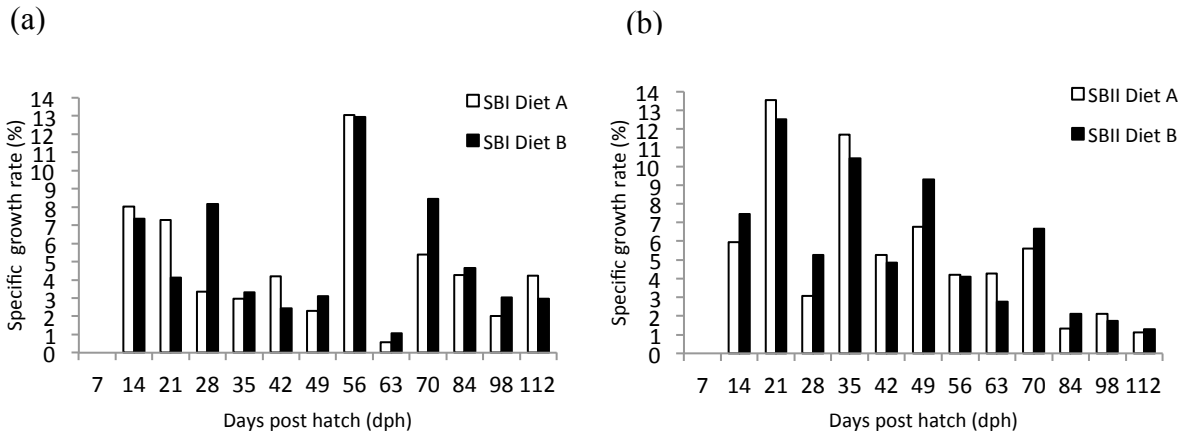


Figure 4.16: Specific growth rate expressed as a percentage of increment in dry weight (SGRDW) in (a) spawning batch I (SBI) and (b) spawning batch II (SBII) *L. bergylta* larvae on Diet A and Diet B from 7 to 112 days post hatch. Please note that Diets A and B were given at 49-112 dph.

4.3.5 Growth relationships

Growth trajectories were plotted using log transformed individual data points for various measures and applying best fit. The weight-length relationships of *L. bergylta* larvae indicated remarkably similar fit for each spawning batch and diet, so this approach did not tease-out the differences as well as previous analyses. Spawning batch II Diet B showed the strongest relationship between log dry weight and log standard length ($y = 0.032x^2 – 0.057x + 0.326$; $F = 1619$; $R^2 = 0.948$, $P<0.01$) (Figure 4.17) and log dry weight versus log myotome height ($y = 0.035x^2 – 0.029x + 0.307$; $F = 1431$; $R^2 = 0.941$, $P<0.01$) (Figure 4.18). Spawning batch I Diet A had the weakest growth relationship across both measures which described by $y = 0.021x^2 – 0.036x + 0.317$; $F = 454.60$; $R^2 = 0.87$, $P<0.01$ and $y = 0.025x^2 – 0.021x + 0.305$; $F = 446.04$; $R^2 = 0.87$, $P<0.01$, respectively. All treatment groups had $R^2 > 0.87$ in the weight-length growth relationships. The shape of the growth curve showed that larvae had proportionally larger dry weight values above log standard lengths or log myotome heights of 1.0 (10.0 mm) and 0.6 (4.0 mm), respectively. Wet weight versus length relationships were slightly weaker in terms of best fit, but these still showed that spawning batch II Diet B gave the best growth (figures not shown for brevity). The relationship between wet weight and standard length / myotome heights had a stepped shape, which was particularly pronounced in spawning batch II where values were extremely variable for mid–sized larvae and increased rapidly above 1.3 mm log standard length (19.95 mm) and 0.75 mm log myotome height (5.62 mm).

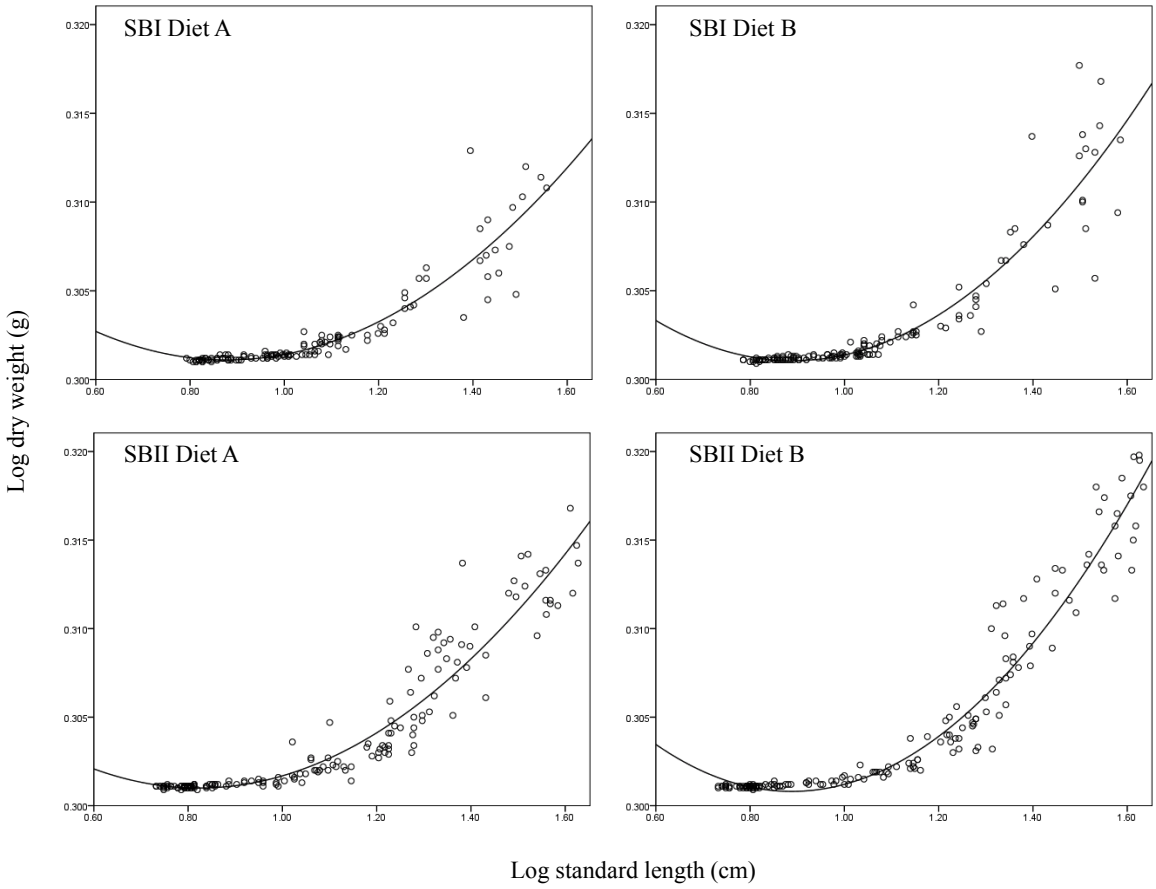


Figure 4.17: Relationship between log dry weight and log standard length of spawning batch I (SBI) and spawning batch II (SBII) *L. bergylta* larvae on Diet A and Diet B; data fitted with quadratic equation.

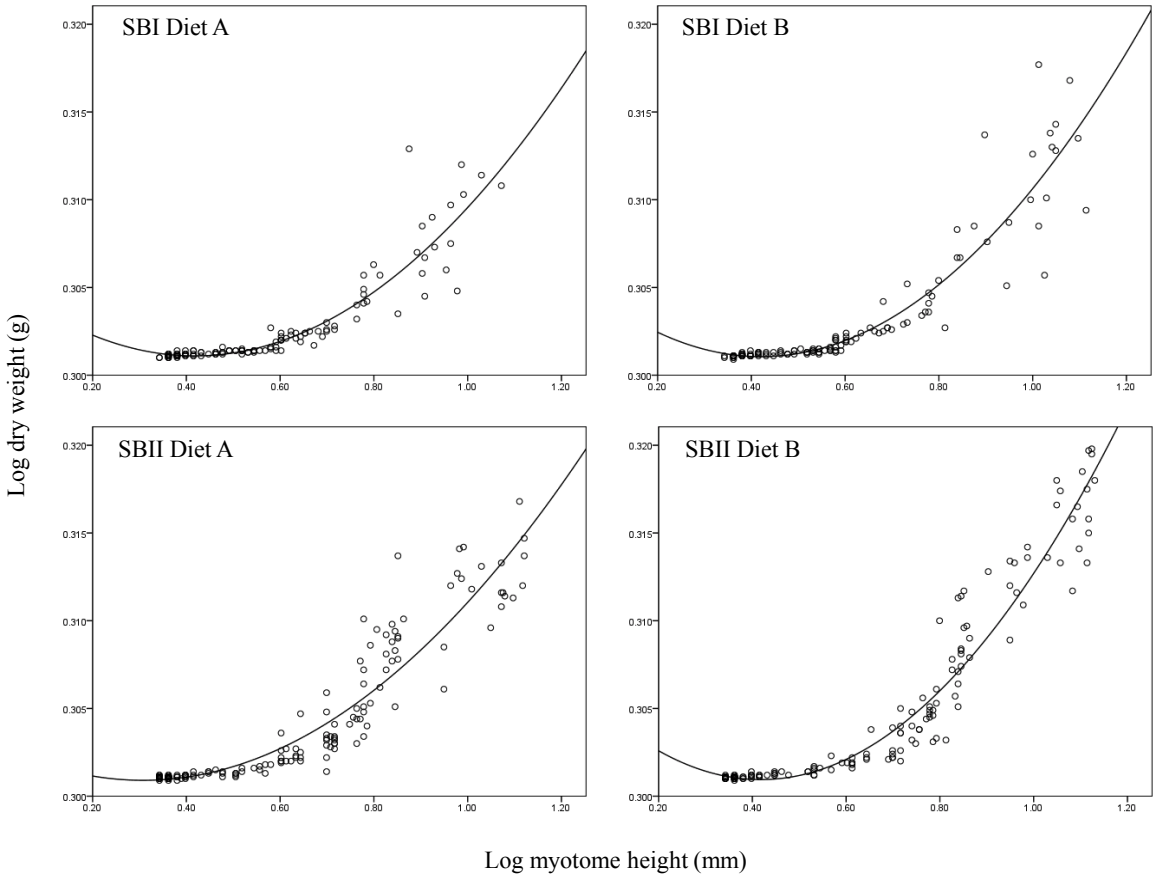


Figure 4.18: Relationship between log dry weight and log myotome height of spawning batch I (SBI) and spawning batch II (SBII) *L. bergylta* larvae on Diet A and Diet B; data fitted with quadratic equation.

4.4 Discussion

This is the first report on growth of larvae from hormonally induced *Labrus bergylta* broodstocks over 112 days post hatch and it adds information to existing studies over much shorter time periods. Some general features of larval growth were demonstrated in *L. bergylta*; in addition, specific issues relating to optimisation of larval growth and weaning were addressed.

In general, we observed that overall hatching for spawning batch I and II occurred approximately 6 days post fertilization. The first feed intake was observed at 7 dph, after the yolk sac had been absorbed, and inflation of the swim bladder occurred at 9-14 dph. Four larval development stages in relation to flexion could be observed within the period 7-49 dph, after which point we could observe the 'juveniles' at around in between day 56 and 70, in which transformation to juveniles started at 63 dph according to increment in body mass and fin development after receiving mixed diet (co-feeding: 49 to 84 dph).

Generally, early growth for both spawning batches was extremely slow, however a break-point occurred in the length-weight trajectory post-metamorphosis and after this breakpoint, the trajectory of the length-weight relationship increased exponentially. The break point occurred at a size that corresponded to 11.994 or 15.812 mm standard length in spawning batch I and II, respectively. These break-points approximately corresponded to ~63 dph in both spawning batches of these temperatures. In terms of dry weight, the break-point was 0.0058g in spawning batch I and 0.0198 g in spawning batch II. A particularly strong growth surge was observed from 63 to 70 dph, measured according to wet and dry weight; in this period, the wet weight in spawning batch II larvae rose from 0.093±0.013g to 0.354±0.019g on Diet A and from 0.126±0.011g to 0.458±0.025g on Diet B. Co-feeding of *Artemia* with dry feed (Diet A and Diet B) was given to larvae between 49 dph and 84 dph. This co-feeding period coincided with the growth surge; however, in general it was quite difficult to separate the effects of spawning batch and diet.

Dealing first with batch-specific differences, we can observe that the overall means were no different for spawning batches on several parameters (standard length, myotome length), but partial batch-specific differences were seen in dry and wet

weights. However, these data covered the entire 112 day period of larval life, not only the days between 49-112 dph when Diets A and B were actually given. Once the analysis is restricted to the period when diet actually differed, spawning batch had a very clear effect on growth on the standard length and myotome measures. Spawning batch II demonstrated significantly higher growth than batch I, to the extent that the mean SLs respectively were 15.72±0.93mm in the slower of both batch I groups and 23.86±1.04mm in the faster of both batch II groups. Only when weight measures were evaluated between 49-112 dph were we able to also detect an effect of diet. Dry weight was significantly higher in Diet B (micropellet + polychaetes) than Diet A (micropellet alone) in spawning batch II at 0.0306±0.0022g and 0.0394±0.0029g respectively. Interestingly, both diets in this batch were still superior for growth than either diet in spawning batch I. This underlines the fact that spawning batch had a greater effect than diet and that growth patterns could be broadly generalised as follows: Spawning batch II Diet B > Spawning batch II Diet A > Spawning batch I Diet B > Spawning batch I Diet A.

Batch-specific patterns were seen when plotting growth curves over time and this gave particularly strong divergence on weight rather than length measures. However, of interest was the fact that the growth patterns which were distinct for part of the growth trajectory, came somewhat towards convergence again by 112 dph. Growth convergence was particularly clear for the better-growing batch I (Diet B) and slower-growing batch II (Diet A). There is plenty evidence for the contrary view, which is that fish larvae do not make up for a slow start, for example, early diet has been found to have an effect on long term growth in Atlantic cod (Imsland *et al*., 2006; Koedijk *et al*., 2010), where after 17 months fish in the groups first fed on zooplankton were 12 to 14 % larger than those first fed on rotifers (Imsland *et al*., 2006). Imsland *et al*. proposed that this could be due to differences in the digestive tract caused by early diet affecting the feeding capacity of the larvae.

As mentioned above, when evaluated on weight measures, larvae grew best on Diet B: a diet of micropellet (O.range larval diet) with polychaete meal, which resulted in significantly higher wet weight at the end of the experimental period compared to micropellet without polychaete meal. Wet weight values for larvae receiving micropellet

+ polychaete meal were 0.493±0.052g relative to values of 0.333±0.032g for micropellet without polychaete in spawning batch II. Thus, it is hypothesised that polychaete meal was better in nutritional value and / or more attractive to younger fish larvae due to its texture and taste. The effect of diet on myotome height and standard length did not differ, however the effect of diet was marked in other ways: fish larvae fed on polychaete + micropellet appeared bigger than the fish fed on micropellet alone for both spawning batches. In addition, they appeared quicker and more reactive, being difficult to catch during sampling.

The micropellet used in the present study, was similar in composition of protein (55.6%) and lipid (16.3%) content to that used for early rearing of other marine fish species including sea bass (Cerdà *et al*., 1994; Navas *et al*., 1997; Asturiano *et al*., 2001) and sea bream (Fernández-Palacios *et al*., 1995, 1997; Zohar *et al*., 1995; Kissil *et al*., 2001). Both diets used in the present study were of equal size which excludes this variable as a factor the rate of feeding and hence growth (van der Meeren and Moksness, 2003; Busch *et al*., 2011). This leaves variation in the dry feeds' nutritional composition and difference in a feed's ability to stimulate a feeding response, based on taste and texture preferences, as the likely explanations for the observed differences in growth. In terms of nutritional composition, the quantitative amounts of lipids and proteins were similar for the polychaete meal and micropellet; however the fatty acid content varied with the polychaete meal having a greater amount of essential fatty acids (EFAs) and a higher ratio of docosahexaenoic acid (22:6*n-3*; DHA): eicosapentaenoic acid (20:5*n-3*; EPA).

EFAs are located in the polychaete's neutral lipid fraction. This has been attributed to a higher fraction of EFAs incorporated in the polar lipid fraction, and a greater amount of proteins and free amino acids (FAAs) (Evjemo and Olsen, 1997; Bell *et al*., 2003; Evjemo *et al*., 2003; Tocher *et al*., 2008; van der Meeren *et al*., 2008). The superiority of dietary phospholipids (PL) in larval fish is suggested to be associated with more efficient capacity to utilize PL than triacylglycerides (TAG) (Cahu *et al*., 2009). Their presence is also found to enhance digestion of other lipids and to aid nutrient transport from the enterocytes because they are a key component of lipoproteins

(Coutteau *et al*., 1997; Tocher *et al*., 2008). Furthermore, EFAs appear to be utilized better when provided through the polar lipid fraction.

Few studies are available on *L. bergylta* larvae for comparison with the present study. Dunaevskaya *et al*. (2012) showed growth was similar up to 21 dph for *L. bergylta* larvae fed unenriched and enriched rotifers. However, the growth during the present study was higher than in Dunaevskaya *et al*. (2012), regardless of feeding treatment. It is notable that standard length and myotome height increased slowly until approximately end of the post-flexion stage in both studies. This is probably related to the fact that most energy and building molecules obtained from early exogenous and endogenous feeding are used to construct tissues, organs and systems (Dunaevskaya *et al*., 2012). It is recognized that this period, where several essential organs are being developed, is critical in larviculture of many marine fish (Blaxter, 1988; Santamaría *et al*., 2004; Hachero-Cruzado *et al*., 2009). An exponential growth pattern based on standard length or myotome height in *L. bergylta*, agrees with growth rate patterns shown for larvae of Senegalese sole *Solea senegalensis* (Ribeiro *et al*., 1999), common dentex, *Dentex dentex* (Santamaria *et al*., 2004), common pandora, *Pagellus erythrinus* (Micale *et al*., 2006), brill, *S. rhombus* (Hachero-Cruzado *et al*., 2009).

The effects of commercial diets on larval performance in *L. bergylta* have not been studied extensively and this was the first time that the combination of an experimental freeze-dried polychaete meal and commercial larval fish feed micropellet were examined for early feeding. Overall, this study demonstrated that dried polychaete meal is good choice for weaning *L. bergylta* larvae and juveniles. This is because Diets A and B were given as co-feeding with live feed (*Artemia*) up to 84 dph, but, after this point, successful weaning was achieved onto an exclusively dry diet. An easy transition from live to dry feeds was observed, but a longer time may be needed to adapt when changing from a natural diet source. Previous studies on Atlantic cod have shown a short period of dietary change (22 to 36 dph) affected larval growth positively if changed from enriched rotifers to natural zooplankton, and negatively if the prey type change was the other way around (Koedijk *et al*., 2010). Dutton (1992) observed that prior experience to a prey type improved feeding success when the same prey was subsequently encountered, and a prolonging of the weaning period has previously given

positive results on the growth of seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) (Rosenlund *et al*., 1997). In the present study, increased duration of co-feeding with micropellet plus *Artemia* may have given the *L. bergylta* larvae a longer period to adapt to the switching dietary regimen and thus reduced the negative growth effect of this process.

The importance of environmental conditions has been suggested in several previous studies (Bergenius *et al*., 2005; Semmens and Swearer, 2011), as has nutritional quality of the diet (Wimberger, 1993; Yamasaki *et al*., 2007). While temperature is known to affect larval growth (Meekan *et al*., 2003; Green and Fisher, 2004), it is almost certain that the small difference in degree days between the larval tanks had no impact during the present experiment. Figures 4.10b and 4.14b demonstrate that even standardising for cumulative degree day (CDD) differences, we can still see spawning batch-specific differences in larval standard length, myotome heights and dry weights. From the time of hatching, spawning batch II individuals increased in size more than spawning batch I individuals and displayed a later stage of development at the same dph. This resulted in a relatively lower slope in the temperature-size relationship by the time of first-feeding (equal stage of development) in spawning batch I.

Finally, mean specific growth rates (SGRs) declined over time with growth slowing down as larvae got older on all growth measures. The shape of the decline was more pronounced in spawning batch II, presumably as these larvae were generally growing and developing faster. As with other parameters (above), spawning batch II performed better than spawning batch I larvae, which could have been for a number of reasons, including parentage, or the spawning induction procedure. The results shown here emphasise the importance of spawning batch and hence, improvements are needed in the larval culturing techniques of *L. bergylta* to increase efficiency and give more predictable growth before this can be applied in industrial contexts. In particular, further research needs to be done to understand batch variability.

References

Abdel-Warith, A. A., Russell, P. M., & Davies, S. J. (2001). Inclusion of a commercial poultry by-product meal as a protein replacement of fish meal in practical diets for African catfish *Clarias gariepinus* (Burchell 1822). *Aquaculture Research, 32*, 296-305.

Abiayad, A., & Kestemont, P. (1994). Comparison of the Nutritional-Status of Goldfish (*Carassius auratus*) Larvae Fed with Live, Mixed or Dry Diet. *Aquaculture, 128*(1-2), 163-176.

Applebaum, S. L., & Ronnestad, I. (2004). Absorption, assimilation and catabolism of individual free amino acids by larval Atlantic halibut (*Hippoglossus hippoglossus*). *Aquaculture, 230*(1-4), 313-322.

Asturiano, J. F., Sorbera, L. A., Carrillo, M., Zanuy, S., Ramos, J., Navarro, J. C., & Bromage, N. (2001). Reproductive performance in male European sea bass (*Dicentrarchus labrax*, L.) fed two PUFA-enriched experimental diets: a comparison with males fed a wet diet. *Aquaculture*, 194(1-2), 173-190.

Balon, E.K. (1979). The juvenilization process in phylogeny and the altricial to precocial forms in the ontogeny of fishes. *Environmental Biology of Fishes*, 4, 193-198.

Bell, J. G., McEvoy, L. A., Estevez, A., Shields, R. J., & Sargent, J. R. (2003). Optimising lipid nutrition in first-feeding flatfish larvae. *Aquaculture, 227*(1-4), 211-220.

Beneliahu, M. N., & Golani, D. (1990). Polychaetes (Annelida) in the Gut Contents of Goatfishes (Mullidae), with New Polychaete Records for the Mediterranean Coast of Israel and the Gulf of Elat (Red-Sea). *Marine Ecology-Pubblicazioni Della Stazione Zoologica Di Napoli I*, 11(3), 193-205.

Bergenius, M. A. J., McCormick, M. I., Meekan, M. G., & Robertson, D. R. (2005). Environmental influences on larval duration, growth and magnitude of settlement of a coral reef fish. *Marine Biology*, 147(2), 291-300.

Blaxter, J.H.S. (1988). Pattern and variety in development. In: Hoar,W.S., & Randall, D.L., (editors). Fish physiology. New York, Academic Press.

Boglino, A., Darias, M. J., Ortiz-Delgado, J. B., Ozcan, F., Estevez, A., Andree, K. B., Hontoria, F., Sarasquete, C., & Gisbert, E. (2012). Commercial products for Artemia enrichment affect growth performance, digestive system maturation, ossification and incidence of skeletal deformities in Senegalese sole (*Solea senegalensis*) larvae. *Aquaculture, 324*, 290-302.

Brock, D., Robinette, H. R., & Heinen, J. M. (1992). Culture System for Evaluating Live and Formulated Diets for Larval Fish. *Progressive Fish-Culturist, 54*(4), 270-273.

Busch, K. E. T., Peruzzi, S., Tonning, F., & Falk-Petersen, I. B. (2011). Effect of prey type and size on the growth, survival and pigmentation of cod (*Gadus morhua*, L.) larvae. *Aquaculture Nutrition, 17*(2), 595-603.

Cahu, C. L., Gisbert, E., Villeneuve, L. A. N., Morais, S., Hamza, N., Wold, P. A., & Infante, J. L. Z. (2009). Influence of dietary phospholipids on early ontogenesis of fish. *Aquaculture Research, 40*(9), 989-999.

Cahu, C., & Infante, J. Z. (2001). Substitution of live food by formulated diets in marine fish larvae. *Aquaculture*, 200(1-2), 161-180.

Cardinaletti, G., Mosconi, G., Salvatori, R., Lanari, D., Tomassoni, D., Carnevali, O., & Polzonetti-Magni, A. M. (2009). Effect of dietary supplements of mussel and polychaetes on spawning performance of captive sole, *Solea solea* (Linnaeus, 1758). *Anim Reprod Sci*, 113(1-4), 167-176.

Cavalin, F. G., & Weirich, C. R. (2009). Larval performance of aquacultured Florida pompano (Trachinotus carolinus) fed rotifers (Brachionus plicatilis) enriched with selected commercial diets. *Aquaculture, 292*(1-2), 67-73. doi: DOI 10.1016/j.aquaculture.2009.03.042

Cerda, J., Carrillo, M., Zanuy, S., Ramos, J., & Delahiguera, M. (1994). Influence of Nutritional Composition of Diet on Sea Bass, *Dicentrarchus labrax* L, Reproductive-Performance and Egg and Larval Quality. *Aquaculture*, 128(3-4), 345-361.

Charlon, N., & Bergot, P. (1984). Rearing System for Feeding Fish Larvae on Dry Diets - Trial with Carp (*Cyprinus carpio* L) Larvae. *Aquaculture, 41*(1), 1-9.

Conceicao, L. E. C., Yufera, M., Makridis, P., Morais, S., & Dinis, M. T. (2010). Live feeds for early stages of fish rearing. *Aquaculture Research, 41*(5), 613-640.

Costa, P. F. E., Narciso, L., & da Fonseca, L. C. (2000). Growth, survival and fatty acid profile of *Nereis diversicolor* (O. F. Muller, 1776) fed on six different diets. *Bulletin of Marine Science*, 67(1), 337-343.

Coutteau, P., Geurden, I., Camara, M. R., Bergot, P., & Sorgeloos, P. (1997). Review on the dietary effects of phospholipids in fish and crustacean larviculture. *Aquaculture, 155*(1-4), 149-164.

D'Arcy, J., Dunaevskaya, E., Treasurer, J. W., Ottesen, O., Maguire, J., Zhuravleva, N., Karlsen, A., Rebours, C., & FitzGerald, R. D. (2012). Embryonic development in ballan wrasse *Labrus bergylta*. *J Fish Biol, 81*(3), 1101-1110.

Drazen, J. C., Phleger, C. F., Guest, M. A., & Nichols, P. D. (2008). Lipid, sterols and fatty acids of abyssal polychaetes, crustaceans, and a cnidarian from the northeast Pacific Ocean: food web implications. *Marine Ecology Progress Series*, 372, 157-167.

Dunaevskaya, E., Amin, A. B., Ottesen, O. H. (2012). Organogenesis of Ballan Wrasse *Labrus bergylta* (Ascanius 1767) Larvae. *J Aquacult Res Dev*, 3, 142.

Dutton, P. (1992). Effects of Experience on Feeding Success by Larval White Sea-Bass, *Atractoscion nobilis*. *J Fish Biol*, 41(5), 765-773.

Evjemo, J. O., & Olsen, Y. (1997). Lipid and fatty acid content in cultivated live feed organisms compared to marine copepods. *Hydrobiologia, 358*, 159-162.

Evjemo, J. O., Reitan, K. I., & Olsen, Y. (2003). Copepods as live food organisms in the larval rearing of halibut larvae (*Hippoglossus hippoglossus* L.) with special emphasis on the nutritional value. *Aquaculture, 227*(1-4), 191-210.

Falk-Petersen, I. B. (2005). Comparative organ differentiation during early life stages of marine fish. *Fish Shellfish Immunol, 19*(5), 397-412.

Fernández-Díaz C, E Pascual & M Yúfera. (1994). Feeding behavior and prey size selection of gilthead seabream, *Sparus aurata* L., larvae fed on inert and live food. *Marine Biology* 118: 323-328.

Fernandez-Palacios, H., Izquierdo, M., Robaina, L., Valencia, A., Salhi, M., Montero, D. (1997). The effect of dietary protein and lipid from squid and fish meals on egg quality of broodstock for Gilthead seabream *Sparus aurata*. *Aquaculture* 148, 233–246.

Fernandez-Palacios, H., Izquierdo, M.S., Robaina, L., Valencia, A., Salhi, M., Vergara, J. (1995). Effect of ny3 HUFA level in broodstock diets on egg quality of gilthead seabream (*Sparus aurata*). *Aquaculture* 132, 325–337.

Finn, R. N., Fyhn, H. J., & Evjen, M. S. (1995). Physiological energetics of developing embryos and yolk-sac larvae of Atlantic cod (*Gadus morhua*). Respiration and nitrogen metabolism. *Marine Biology, 124*(3), 355-369.

Fuiman, L. A. (1983). Growth Gradients in Fish Larvae. *J Fish Biol, 23*(1), 117-123.

Furuita, H., Takeuchi, T., & Uematsu, K. (1998). Effects of eicosapentaenoic and docosahexaenoic acids on growth, survival and brain development of larval Japanese flounder (*Paralichthys olivaceus*). *Aquaculture, 161*(1-4), 269-279.

Fyhn, H. J. (1989). 1st Feeding of Marine Fish Larvae - Are Free Amino-Acids the Source of Energy. *Aquaculture, 80*(1-2), 111-120.

Galloway, T. F., Kjorsvik, E., & Kryvi, H. (1999). Muscle growth and development in Atlantic cod larvae (*Gadus morhua* L.), related to different somatic growth rates. *J Exp Biol, 202*(Pt 15), 2111-2120.

Geurden, I., Radunzneto, J., & Bergot, P. (1995). Essentiality of Dietary Phospholipids for Carp (*Cyprinus-Carpio* L.) Larvae. *Aquaculture, 131*(3-4), 303-314.

Giraldo, C., Mayzaud, P., Tavernier, E., Irisson, J. O., Penot, F., Becciu, J., Chartier, A., Boutoute, M., & Koubbi, P. (2013). Lipid components as a measure of nutritional condition in fish larvae (*Pleuragramma antarcticum*) in East Antarctica. *Marine Biology, 160*(4), 877-887.

Gisbert, E., Villeneuve, L., Zambonino-Infante, J. L., Quazuguel, P., & Cahu, C. L. (2005). Dietary phospholipids are more efficient than neutral lipids for long-chain polyunsaturated fatty acid supply in European sea bass *Dicentrarchus labrax* larval development. *Lipids, 40*(6), 609-618.

Green, B. S., & Fisher, R. (2004). Temperature influences swimming speed, growth and larval duration in coral reef fish larvae. *Journal of Experimental Marine Biology and Ecology, 299*(1), 115-132.

Grøntvedt, R.N. (2010). Oppdrett av berggylt - muligheter og utfordringer. In: Norsk Fiskeoppdrett. Norsk Fiskeoppdrett A/S, Bergen, pp. 56-57.

Grotmol, S., Kryvi, H., & Totland, G. K. (2005). Deformation of the notochord by pressure from the swim bladder may cause malformation of the vertebral column in cultured Atlantic cod *Gadus morhua* larvae: a case study. *Diseases of Aquatic Organisms, 65*(2), 121-128.

Hachero-Cruzado, I., Ortiz-Delgado, J. B., Borrega, B., Herrera, M., Navas, J. I., & Sarasquete, C. (2009). Larval organogenesis of flatfish brill *Scophthalmus rhombus* L: Histological and histochemical aspects. *Aquaculture*, 286(1-2), 138-149.

Hamre K, Sæle Ø. 2011. Oppdrett av leppefisk til lakselusbekjempelse: Hva står på menyen. Norsk Fiskeoppdrett 9/2011:70-72

Helland, S., Bæverfjord, G., & Lein, I. (2012). Produksjon av berggylt (Ballan wrasse) - Statusrapport fra Sunndalsøra. *In*: Leppeprod Dialogmøte. Nofima, Stord.

Helland, S., Terjesen, B. F., & Berg, L. (2003). Free amino acid and protein content in the planktonic copepod *Temora longicornis* compared to *Artemia franciscana*. *Aquaculture, 215*(1-4), 213-228.

Imsland, A. K., Foss, A., Koedijk, R., Folkvord, A., Stefansson, S. O., & Jonassen, T. M. (2006). Short- and long-term differences in growth, feed conversion efficiency and deformities in juvenile Atlantic cod (*Gadus morhua*) startfed on rotifers or zooplankton. *Aquaculture Research, 37*(10), 1015-1027.

Izquierdo, M. S., Socorro, J., Arantzamendi, L., & Hernandez-Cruz, C. M. (2000). Recent advances in lipid nutrition in fish larvae. *Fish Physiology and Biochemistry, 22*(2), 97-107.

Jaworski, A., & Kamler, E. (2002). Development of a bioenergetics model for fish embryos and larvae during the yolk feeding period. *J Fish Biol, 60*(4), 785-809.

Johnston, I. A., & Hall, T. E. (2004). Mechanisms of muscle development and responses to temperature change in fish larvae. *Development of Form and Function in Fishes and the Question of Larval Adaptation, 40*, 85-116.

Kestemont, P. (1995). Influence of feed supply, temperature and body size on the growth of goldfish *Carassius auratus* larvae. *Aquaculture, 136*(3-4), 341-349.

Kissil, G. W., Lupatsch, I., Elizur, A., & Zohar, Y. (2001). Long photoperiod delayed spawning and increased somatic growth in gilthead seabream (*Sparus aurata*). *Aquaculture*, 200(3-4), 363-379.

Kjorsvik, E., Olsen, C., Wold, P. A., Hoehne-Reitan, K., Cahu, C. L., Rainuzzo, J., Olsen, A. I., Oie, G., & Olsen, Y. (2009). Comparison of dietary phospholipids and neutral lipids on skeletal development and fatty acid composition in Atlantic cod (*Gadus morhua*). *Aquaculture, 294*(3-4), 246-255.

Kjørsvik, E., Pittman, K., and Pavlov, D. (2004). From fertilisation to the end of metamorphosis. Functional development. *In*: Culture of Cold-water Marine Fish, pp. 204-278. Ed. by E. Moksness, E. Kjørsvik, and Y. Olsen. Blackwell Publishing Ltd, Oxford, UK. 528 pp.

Kjorsvik, E., Vandermeeren, T., Kryvi, H., Arnfinnson, J., & Kvenseth, P. G. (1991). Early Development of the Digestive-Tract of Cod Larvae, *Gadus morhua* L, during Start-Feeding and Starvation. *J Fish Biol, 38*(1), 1-15.

Koedijk, R. M., Folkvord, A., Foss, A., Pittman, K., Stefansson, S. O., Handeland, S., & Imsland, A. K. (2010). The influence of first-feeding diet on the Atlantic cod *Gadus morhua* phenotype: survival, development and long-term consequences for growth. *J Fish Biol, 77*(1), 1-19.

Lubzens, E. (1987). Raising Rotifers for Use in Aquaculture. *Hydrobiologia, 147*, 245-255.

Luizi, F. S., Gara, B., Shields, R. J., & Bromage, N. R. (1999). Further description of the development of the digestive organs in Atlantic halibut (*Hippoglossus hippoglossus*) larvae, with notes on differential absorption of copepod and Artemia prey. *Aquaculture, 176*(1-2), 101-116.

Meekan, M. G., Carleton, J. H., McKinnon, A. D., Flynn, K., & Furnas, M. (2003). What determines the growth of tropical reef fish larvae in the plankton: Food or temperature? *Marine Ecology Progress Series, 256*, 193-204.

Micale, V., Garaffo, M., Genovese, L., Spedicato, M. T., & Muglia, U. (2006). The ontogeny of the alimentary tract during larval development in common pandora *Pagellus erythrinus* L. *Aquaculture*, 251(2-4), 354-365.

Murzina, S. A., Falk-Petersen, S., & Nemova, N. N. (2008). Lipids in the Arctic fish, *Leptoclinus maculatus* larvae. *Chemistry and Physics of Lipids, 154*, S26-S26.

Nakayama, S., & Fuiman, L. A. (2010). Body size and vigilance mediate asymmetric interference competition for food in fish larvae. *Behavioral Ecology, 21*(4), 708-713.

Navarro, J. C., Batty, R. S., Bell, M. V., & Sargent, J. R. (1993). Effects of 2 Artemia Diets with Different Contents of Polyunsaturated Fatty-Acids on the Lipid-Composition of Larvae of Atlantic Herring (*Clupea harengus*). *J Fish Biol, 43*(4), 503-515.

Navas, J. M., Bruce, M., Thrush, M., Farndale, B. M., Bromage, N., Zanuy, S., Carrillo, R., Bell, J. G., & Ramos, J. (1997). The impact of seasonal alteration in the lipid composition of broodstock diets on egg quality in the European sea bass. *J Fish Biol*, 51(4), 760-773.

Oie, G., & Olsen, Y. (1997). Protein and lipid content of the rotifer *Brachionus plicatilis* during variable growth and feeding condition. *Hydrobiologia, 358*, 251-258.

Olsen, R. E., Henderson, R. J., & Ringo, E. (1991). Lipids of Arctic Charr, *Salvelinus alpinus* (L). Dietary Induced Changes in Lipid Class and Fatty-Acid Composition. *Fish Physiology and Biochemistry, 9*(2), 151-164.

Opuszynski, K., & Shireman, J. V. (1993). Food-Habits, Feeding-Behavior and Impact of Triploid Bighead Carp, *Hypophthalmichthys nobilis*, in Experimental Ponds. *J Fish Biol, 42*(4), 517-530.

Osse, J. W. M., & Van den Boogaart, J. G. M. (2004). Allometric growth in fish larvae: Timing and function. *Development of Form and Function in Fishes and the Question of Larval Adaptation, 40*, 167-194.

Osse, J. W. M., vandenBoogaart, J. G. M., vanSnik, G. M. J., & vanderSluys, L. (1997). Priorities during early growth of fish larvae. *Aquaculture, 155*(1-4), 249-258.

Otterlei, E., Nyhammer, G., Folkvord, A., & Stefansson, S. O. (1999). Temperature- and size-dependent growth of larval and early juvenile Atlantic cod (*Gadus morhua*): a comparative study of Norwegian coastal cod and northeast Arctic cod. *Canadian Journal of Fisheries and Aquatic Sciences, 56*(11), 2099-2111.

Pedersen, G. M., Gildberg, A., & Olsen, R. L. (2004). Effects of including cationic proteins from cod milt in the feed to Atlantic cod (*Gadus morhua*) fry during a challenge trial with *Fibrio anguillarum*. *Aquaculture, 233*(1-4), 31-43.

Pena, R., & Dumas, S. (2009). Development and allometrie growth patterns during early larval stages of the spotted sand bass *Paralabrax maculatofasciatus* (Percoidei: Serranidae). *Scientia Marina, 73*, 183-189.

Person-Le Ruyet, J., Alexandre, J.C., Thébaud, L., Mugnier, C., 1993. Marine fish larvae feeding: formulated diets or live prey? *Journal of the World Aquaculture Society* 24 (2), 211–224.

Rainuzzo, J. R., Reitan, K. I., Jorgensen, L., & Olsen, Y. (1994). Lipid-Composition in Turbot Larvae Fed Live Feed Cultured by Emulsions of Different Lipid Classes. *Comparative Biochemistry and Physiology a-Physiology, 107*(4), 699-710.

Rainuzzo, J. R., Reitan, K. I., & Olsen, Y. (1997). The significance of lipids at early stages of marine fish: a review. *Aquaculture, 155*(1-4), 103-115.

Ribeiro, L., Sarasquete, C., & Dinis, M. T. (1999). Histological and histochemical development of the digestive system of *Solea senegalensis* (Kaup, 1858) larvae. *Aquaculture, 171*(3-4), 293-308.

Ronnestad, I., Conceicao, L. E. C., Aragao, C., & Dinis, M. T. (2000a). Free amino acids are absorbed faster and assimilated more efficiently than protein in postlarval Senegal sole (*Solea senegalensis*). *Journal of Nutrition, 130*(11), 2809-2812.

Ronnestad, I., Dominguez, R. P., & Tanaka, M. (2000b). Ontogeny of digestive tract functionality in Japanese flounder, *Paralichthys olivaceus* studied by in vivo microinjection: pH and assimilation of free amino acids. *Fish Physiology and Biochemistry, 22*(3), 225-235.

Ronnestad, I., Thorsen, A., & Finn, R. N. (1999). Fish larval nutrition: a review of recent advances in the roles of amino acids. *Aquaculture, 177*(1-4), 201-216.

Ronnestad, I., Tonheim, S. K., Fyhn, H. J., Rojas-Garcia, C. R., Kamisaka, Y., Koven, W., Finn, R. N., Terjesen, B. F., Barr, Y., & Conceicao, L. E. C. (2003). The supply of amino acids during early feeding stages of marine fish larvae: a review of recent findings. *Aquaculture, 227*(1-4), 147-164.

Rosenlund, G., Stoss, J., & Talbot, C. (1997). Co-feeding marine fish larvae with inert and live diets. *Aquaculture, 155*(1-4), 183-191.

Rottmann, R. W., Shireman, J. V., & Lincoln, E. P. (1991). Comparison of 3 Live Foods and 2 Dry Diets for Intensive Culture of Grass Carp and Bighead Carp Larvae. *Aquaculture, 96*(3-4), 269-280.

Sala, R., Santamaria, C. A., & Crespo, S. (2005). Growth of organ systems of *Dentex dentex* (L) and *Psetta maxima* (L) during larval development. *J Fish Biol, 66*(2), 315-326.

Sales, J. (2011). First feeding of freshwater fish larvae with live feed versus compound diets: a meta-analysis. *Aquaculture International, 19*(6), 1217-1228.

Santamaria, C. A., de Mateo, M. M., Traveset, R., Sala, R., Grau, A., Pastor, E., Sarasquete, C., & Crespo, S. (2004). Larval organogenesis in common dentex *Dentex dentex* L. (Sparidae): histological and histochemical aspects. *Aquaculture, 237*(1-4), 207-228.

Sargent, J., McEvoy, L., Estevez, A., Bell, G., Bell, M., Henderson, J., & Tocher, D. (1999). Lipid nutrition of marine fish during early development: current status and future directions. *Aquaculture, 179*(1-4), 217-229.

Segner, H., Storch, V., Reinecke, M., Kloas, W., & Hanke, W. (1994). The Development of Functional Digestive and Metabolic Organs in Turbot, *Scophthalmus maximus*. *Marine Biology, 119*(3), 471-486.

Semmens, D., & Swearer, S. E. (2011). Extended incubation affects larval morphology, hatching success and starvation resistance in a terrestrially spawning fish, *Galaxias maculatus* (Jenyns 1842). *J Fish Biol*, 79(4), 980-990.

Sharma, J. G., & Chakrabarti, R. (1999). Larval rearing of common carp *Cyprinus carpio*: A comparision between natural and artificial diets under three stocking densities. *Journal of the World Aquaculture Society, 30*(4), 490-495.

Skiftesvik, A.B., Opstad, I., Kvenseth, P.G., & Bjelland, R.M. (2011). Oppdrett av berggylte. *In*: Norsk Fiskeoppdrett. Norsk Fiskeoppdrett A/S, Bergen, pp. 42-43.

Sorgeloos, P., Dhert, P., & Candreva, P. (2001). Use of the brine shrimp, *Artemia spp*., in marine fish larviculture. *Aquaculture, 200*(1-2), 147-159.

Tocher, D. R., Bendiksen, E. A., Campbell, P. J., & Bell, J. G. (2008). The role of phospholipids in nutrition and metabolism of teleost fish. *Aquaculture, 280*(1-4), 21-34.

Treasurer, J. W. (1994). The Distribution, Age and Growth of Wrasse (Labridae) in Inshore Waters of West Scotland. J Fish Biol, 44(5), 905-918.

Trotter, A. J., Pankhurst, P. M., & Battaglene, S. C. (2005). A finite interval of initial swimbladder inflation in *Latris lineata* revealed by sequential removal of water-surface films. *J Fish Biol, 67*(3), 730-741.

Turingan R. G., Beck J. L., Krebs J. M., Licamele J. D., 2005 Development of feeding mechanics in marine fish larvae and the swimming behavior of zooplankton prey: implications for rearing marine fishes. *In*: Copepods in Aquaculture. Lee C. S., O'Bryen P. J., Marcus N. H. (eds), Blackwell Publishing, Ames, Iowa, USA, pp 234-248.

Tytler, P., & Blaxter, J. H. S. (1988). Drinking in Yolk-Sac Stage Larvae of the Halibut, *Hippoglossus hippoglossus* (L). *J Fish Biol, 32*(3), 493-494.

van der Meeren, T., & Moksness, E. (2003). Growth and mortality patterns evaluated from otolith microstructure in Atlantic cod *Gadus morhua* larvae reared on different feeding regimes in mesocosms. *Marine Ecology Progress Series*, 264, 95-107.

van der Meeren, T., Olsen, R. E., Hamre, K., & Fyhn, H. J. (2008). Biochemical composition of copepods for evaluation of feed quality in production of juvenile marine fish. *Aquaculture*, 274(2-4), 375-397.

vanSnik, G. M. J., vandenBoogaart, J. G. M., & Osse, J. W. M. (1997). Larval growth patterns in *Cyprinus carpio* and *Clarias gariepinus* with attention to the finfold. *J Fish Biol, 50*(6), 1339-1352.

Watanabe, T., & Kiron, V. (1994). Prospects in Larval Fish Dietetics. *Aquaculture, 124*(1-4), 223-251.

Whitfield, F. B., Drew, M., Helidoniotis, F., & Svoronos, D. (1999). Distribution of bromophenols in species of marine polychaetes and bryozoans from eastern Australia and the role of such animals in the flavor of edible ocean fish and prawns (shrimp). *Journal of Agricultural and Food Chemistry*, 47(11), 4756-4762.

Wiff, R., & Roa-Ureta, R. (2008). Predicting the slope of the allometric scaling of consumption rates in fish using the physiology of growth. *Marine and Freshwater Research, 59*(10), 912-921.

Wimberger, P. H. (1993). Effects of Vitamin-C-Deficiency on Body Shape and Skull Osteology in *Geophagus brasiliensis* - Implications for Interpretations of Morphological Plasticity. *Copeia*(2), 343-351.

Wold, P. A., Hoehne-Reitan, K., Cahu, C. L., Infante, J. Z., Rainuzzo, J., & Kjorsvik, E. (2009). Comparison of dietary phospholipids and neutral lipids: effects on gut, liver and pancreas histology in Atlantic cod (*Gadus morha* L.) larvae. *Aquaculture Nutrition, 15*(1), 73-84.

Yamasaki, T., Aki, T., Mori, Y., Yamamoto, T., Shinozaki, M., Kawamoto, S., & Ono, K. (2007). Nutritional enrichment of larval fish feed with thraustochytrid producing polyunsaturated fatty acids and xanthophylls. *Journal of Bioscience and Bioengineering*, 104(3), 200-206.

Yufera, M., & Darias, M. J. (2007). The onset of exogenous feeding in marine fish larvae. *Aquaculture, 268*(1-4), 53-63.

Zohar, Y., Harel, M., Hassin, S., & Tandler, A. (1995). Gilthead seabream. *In*: Bromage, N.R., Roberts, R.J. (Eds.), Broodstock Management and Egg and Larval Quality. Cambridge Univ. Press, Cambridge, 424 pp.

Chapter five

Age and Growth Patterns of *Labrus bergylta*

5.1 Introduction

The ability to determine the age structure of a population is important in managed fisheries (Boehlert, 1985) to understand the population dynamics (Radtke and Shafer, 1992) or construct age-structured population models for sustainable exploitation (Fossum *et al*., 2000). Hence, deriving the age of individual fish within populations during stock assessment exercises remains an integral component of fisheries ecology and management (Sideleva and Zubina, 1990; Cailliet *et al*., 2001; Hernandez-Miranda *et al*., 2009; Beardsley and Britton, 2012), as this forms the basis for calculations of growth rate, mortality rate and productivity (Campana, 2001; Campana and Thorrold, 2001).

Age interpretation is, however, a difficult and subjective discipline and combining this with body size to interpret growth patterns can be complex (Campana, 2001). This is a result of both the stochasticity of environmental factors that can influence fish growth (Wetherington *et al*., 1989; Maceina, 1992; Coggins and Pine, 2010; Morrongiello *et al*., 2014) and the accuracy of age estimates, which are partly a function of the skill and experience of the person making the estimate, and partly reliant on the ability to carry out proper validation of methods (Maceina *et al*., 2007; Bostanci, 2008; Francis *et al*., 2010).

In temperate regions, fish ageing methods may be based on incremental changes on hard parts of the body, particularly scales and otoliths, which frequently reveals the presence of ‘rings’ that are correlated with seasonal changes in growth (Jones, 1992, Jones, 2000; Britton *et al*., 2004). The ageing of fish based on bony structures assumes that the examination of growth increments in the structures will result in the correct determination of age (Britton *et al*., 2004). Consequently, the usefulness of the derived data is dependent on validating the periodicity of structure deposition (usually annually) (Beamish and McFarlane, 1983; Campana, 2001). Age validation ensures that the banding patterns are properly interpreted, either by using animals of known age, or an independent set of observations, or some other technique (Campana, 2001). This is rarely carried out, as known-age information for adults involves holding animals in captivity over long periods –in the knowledge that

captivity itself will affect growth (and possibly deposition) rates. Alternatively, validation requires onerous mark-recapture exercises or regular sampling of fish over a relatively extended period of time (Ewing *et al*., 2007; Britton *et al*., 2010).

Regarding larval growth, the discovery of daily increment deposition in the otoliths of freshwater (Morioka and Matsumoto, 2007) and cold-water fish larvae (Dulcic, 1995; Mendiola and Alvarez, 2008) has provided a reliable method for estimating larval age over short larval timespans. Although daily micro-increment deposition has been shown for many species of young fish under controlled growth conditions, some studies have failed to validate daily micro-increment deposition at low growth rates (Geffen, 1982; Rice *et al*., 1985; Campana *et al*., 1987). To accurately age fish larvae, periodicity of micro-increment deposition must first be determined. The date of first increment deposition must also be determined. Both objectives are best achieved by rearing known-age larvae in the laboratory (Jones, 1992), albeit the feeding regimen in the laboratory may differ from the wild. A review of larval age validation studies cites that more than 90% use laboratory-reared larvae (Campana, 2001). This approach has also been adopted in the present study, using otoliths from larvae of known age (from Chapter four) to determine when the first increments are laid down in larval *L. bergylta*.

It is more difficult to validate growth in adults as we are generally not dealing with known–age adults. As such, validation of adult growth can be examined using two methods: marginal increments and edge analysis of time series such as monthly captures of individuals; and also by mark-recapture. Marginal increment and edge analysis is actually similar to mark-recapture because the recent growth at the otolith margin is used to validate mark periodicity with respect to time elapsed in the wild (e.g. monthly samples) or the time between mark and recapture (including physical marking of the otolith with fluorescent markers such as oxytetracycline). In addition, back-calculation is used to test the validity of aging e.g., relationships describing fish lengths at various otolith sizes can be compared against the length at age data.

Once validation is complete, the most accurate and certainly the most laborious method to evaluate adult growth is to count the number of increments denoting ‘age’ in a large sample of otoliths (Jones, 1992) and relate this to body length. Otoliths must generally be polished before the increments become visible (Secor and Dean, 1992) but once validation is complete and counting is complete, these data can be incorporated into predictive models (Katsanevakis and Maravelias,

2008; Bar and Radde, 2009). For example, it is possible to utilise a significant relationship between fish age according to banding patterns and otolith weight or length to derive growth models (Pawson, 1990; Fletcher, 1991; Worthington *et al*., 1995; Bermejo, 2007). Thresholds in the growth trend can arise because otolith weight increases throughout life, and bands are laid down even in older fish, whereas body length may reach a threshold (Fowler and Doherty, 1992; Bermejo, 2007). The age in years can be plotted versus body length to derive length-at-age and growth function co-efficients. Rather than depending on annular band determination and validation, it has been postulated that otolith weight and length may be valid alternatives for the determination of growth patterns as these may eliminate subjectivity and inaccuracies associated with the traditional annuli interpretation (Bermejo, 2007). Combining the latter approach with otolith banding is also possible.

Several mathematical models have been found to be suitable for describing growth in the early life of fishes (Kaufmann, 1981). In principle, calculation of growth rate is based upon the growth trajectories or equations derived from fitted growth model curves (Campana and Jones, 1992). Non-linear models such as exponential models have mainly been used to describe the variation in fish size (generally length) with age (Campana and Jones, 1992; Chih, 2009); but though growth begins exponentially, a slowing down of growth is generally observed once a threshold length has been reached. The level of this threshold (L_{max} or L_{∞}) as well as the rate at which the threshold is reached (k) are key parameters in modelling adult growth e.g. in the von Bertalanffy growth function (De Graaf and Prein, 2005; He *et al*., 2005; He and Bence, 2007). Meanwhile for larval growth, the intrinsic growth-pattern in the early stages of many fish species is often exponential (Bartsch, 2002). This pattern has been successfully described by the Gompertz, Laird-Gompertz, logistic and von Bertalanffy mathematical functions (Simard *et al*., 1992; Jones, 2002; van der Meeren and Moksness, 2003).

Age and growth has been determined in *L. bergylta* larvae by analysing daily growth increments in otoliths (D'Arcy, 2013). Meanwhile, growth in adult fish has been examined in the Atlantic waters of NW Spain; also using various otolith measures including deposition patterns, length and weight of otoliths (Villegas-Rios *et al*., 2013). There is still a lack of information on *L. bergylta* growth throughout its range, hence the main purpose of this study was to determine the age and growth

patterns of larvae, juvenile and adult phases of *L. bergylta* on the west coast of Ireland. The growth rate of *L. bergylta* is of particular interest because aquaculture production of this potential cleaner fish species depends on this information and selective breeding for faster growth is potentially possible.

In the present study, hatchery-reared known-age larvae were used to describe early growth. Since growth increments in the larval otoliths of *L. bergylta* are suggested to form on a daily basis (D'Arcy, 2013), counts of daily rings and diameters of the sagittal otoliths from larvae were used to provide accurate estimates of otolith diameter at age (dph), as well as describing features in embryonic otoliths, such as hatching marks . For wild *L. bergylta* adults, otoliths were sampled and the margin examined over successive months to validate deposition periodicity before describing age-at-length. Various otolith size and fish size parameters were explored before finally generating von Bertalanffy growth function co-efficients for west of Ireland wild *L. bergylta* adults. The latter provides the means of comparison across growth studies in various parts of the range as information to be used in potential cleaner fish evaluations.

5.2 Materials and Methods

5.2.1 Fish sampling and otolith extraction for juveniles and adults

Labrus bergylta were sampled according to methods described during previous experiments (Chapters two and four) for the purposes of growth analysis. Monthly samples of adults and juveniles (i.e. age four years old and under; Muus and Nielsen, 1999) were captured in trammel nets close to Carna, in the west of Galway Bay over 17 months between 18 April 2010 and 12 August 2011. Individual body weight, total length and sampling date were recorded at capture.

Juveniles (n=19) and adults (n=234) of *L. bergylta* were euthanized prior to otolith removal using an overdose of MS-222. *L. bergylta* juvenile and adult otoliths are very small (Figure 5.1N) and their extraction is not as straight-forward as other species. For juveniles and adults, both sagittal otoliths were removed from an area between the base of the skull and the first vertebrae, adjacent to right and left medulla oblongata. The otoliths were removed by means of a capitation of cervical bone in order to separate the head from the body (Figure 5.1H). Both gills were completely removed together with the gill arches using a cutter. Sagittal incision was made by a knife from the base of the cranium and was split into two parts. A vertical incision of the brain was made before removing the left and right otoliths. At this point, the otoliths were easy to locate beside the inner right and left medulla oblongata (Figure 5.1K). Each otolith was protected by a membranous layer of sac and was removed by fine forceps and dissection needles to gently tease otoliths out of their holding vesicles.

Otoliths were cleaned immediately after being removed of adhering tissues and sterilized with 70% ethanol. They were then rinsed and labelled according to the tagged number of sampled fish. All cleaned otoliths were air dried before being measured. Otolith weight (±0.001g) and length (±0.01cm) (Figure 5.3) was measured using a microscale (Mason Technology, Metler Toledo [AB204-S]) and vernier callipers, respectively. They were stored dry in a 1.5 ml tube for later examination.

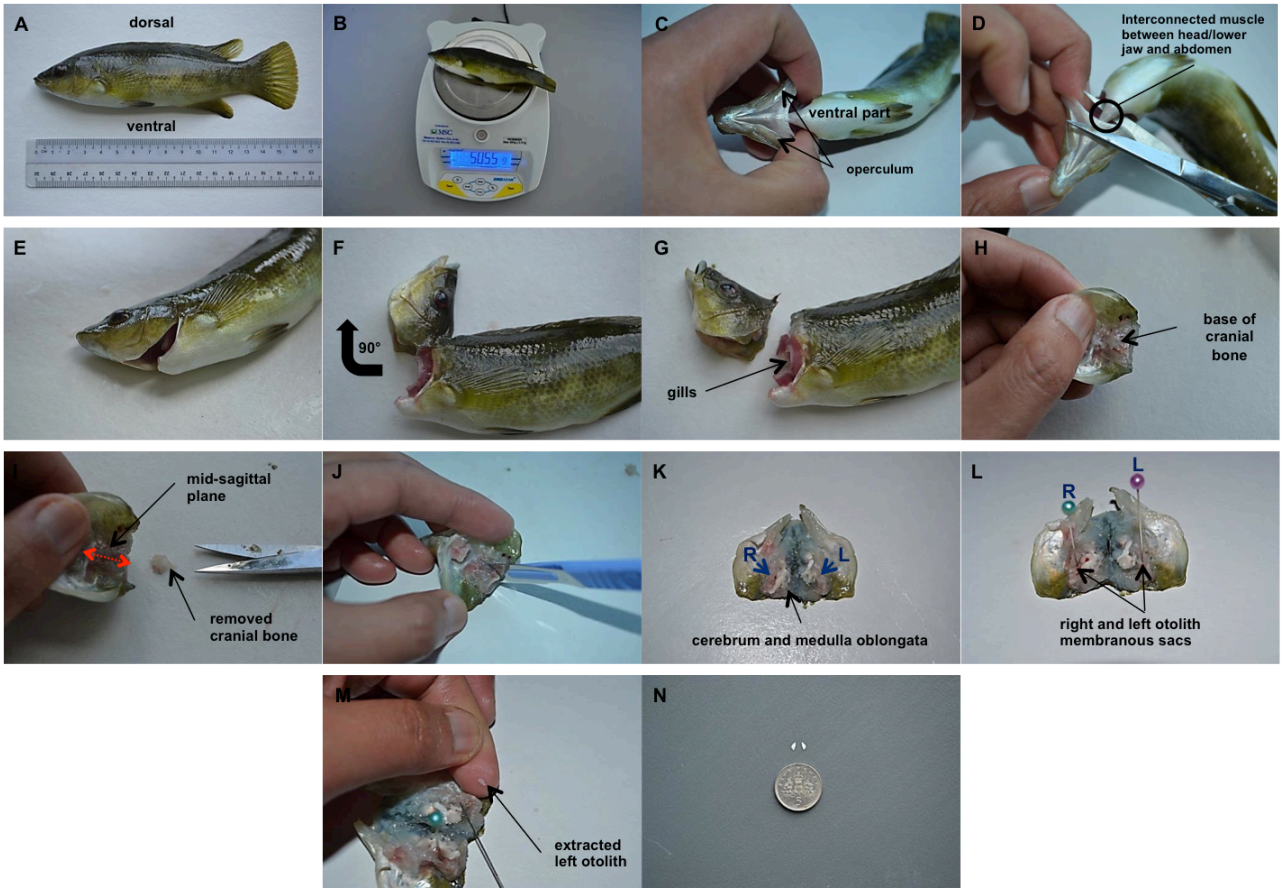


Figure 5.1: A quick (less than a minute) and simple procedure for right and left otolith extraction of juvenile and adult *L. bergylta*; (A) measurement of fish total length; (B) measurement of fish weight; (C, D and E) opening of both operculums for cutting down the interconnected muscle between head and ventral abdomen; (F and G) bending of the head and decapitation; (H and I) removing the base of cranial bone; (J, K and L) splitting the cranium at the mid-sagittal plane in order to observe the right and left otoliths within membranous sacs adjacent to medulla oblongata; (M and N) otolith extraction.

5.2.2 Embedding and polishing otoliths

To embed adult sagittal otoliths, 0.1 g solid tripod wax was placed at the centre of a glass slide and heated on a hotplate (Stuart Scientific) for 15 seconds with the heating intensity adjusted to number 6 (120ºC) until the tripod wax turned semi-solid. A light touch showed when the tripod wax became a gummy liquid. Melting point was controlled by reducing the heating adjustment to number 2 (40ºC) to prevent the wax from forming bubbles. Fine forceps were used to place an otolith on top of the melted tripod wax, with the sulcus side facing up. Only one otolith was mounted per slide to ensure that polishing could be optimised (Figure 5.2). The orientation of the otolith was adjusted while the wax was still soft to ensure that the plane of the growth increments was parallel to the microscope slide. A gentle push was applied downwards on the otolith to ensure that the otolith was sufficiently embedded to withstand polishing. The tripod wax set quickly after removal from the hotplate. When it was necessary to remove the otolith, or adjust its position or orientation for any reason, the slide was simply reheated until the tripod wax had liquefied and adjustments were made.

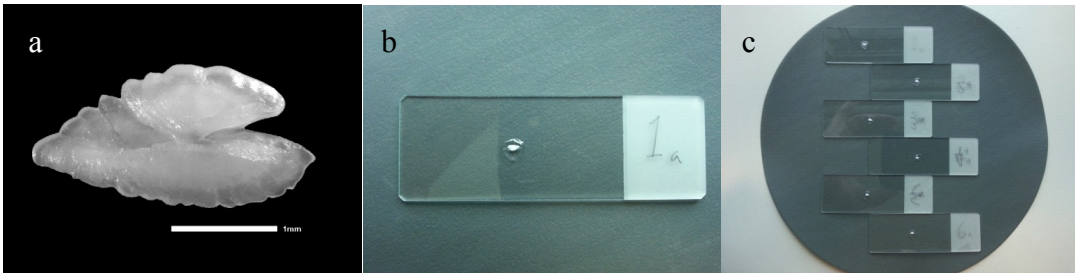


Figure 5.2: a) 'Cuniform' sagittal otoliths of *Labrus bergylta*; b) fixed into tripod wax on labelled glass slides; c) ready fixed otoliths on a sand paper (scale bar = 1 mm).

Otoliths from adult *L. bergylta* were polished to make the growth bands more easily visible. Three different grades of sand paper, coarse (P#1200), medium (P#2000) and fine (P#4000), were used in sequence for polishing; beginning with coarse sand paper and progressing with medium and fine grades. To polish, the mounted otolith was centred between the index and middle fingers and the least possible pressure was applied. The slide was moved in a rapid circular motion on wet sand paper. The otolith was frequently checked (every few seconds) under low magnification (10x) during polishing until the polishing plane was slightly above the plane of the growth

increments. At this point, the incremental growth rings of the juvenile and adult otoliths were ready for counting.

5.2.3 Incremental growth and age validation

A stereomicroscope (Olympus, BX51) with Image Pro Plus Analyser software version 6.3 was used to capture and store the images. During seasonal growth, each 'annulus' is constituted by one opaque and one translucent zone. The incremental growth terminology was based on that recommended by Secor *et al*. (1995), whereby the term 'opaque zone' refers to the area that appeared milky or dark under transmitted light in unstained samples; this alternates with a 'translucent' zone within a given growth cycle. Age was determined by counting the number of opaque bands from the core to the edge (Oxenford and Hunte, 1983). Counts began at the first clearly defined opaque zone that encircled the primordium, defined by the outer edge of the nucleus and representative of the first Winter growth check.

Validation of annual counts was carried out by analysing marginal increments over successive months of wild captured *L. bergylta* adults. Difficulties in observing incremental rings at the margins of older (therefore slower growing) individuals meant that validation of marginal analysis was easier in individuals aged (putatively) 10 years old or less (n=29). Therefore validation took place in the 10 years old group, as follows:- marginal increments were measured along the anterior apex of the sagittal otolith, beginning at the outer edge of the most recent opaque band (i.e. the band before the margin) and stretching to the otolith margin (Appendix 7 and 8). For individuals sampled over consecutive months, these measurements (in micrometers) corresponded to the average monthly otolith deposition at the margin over a period of 17 months.

The number of annuli, weight and length from left and right otolith pairs was noted in a subsample of 50 random individual fish to establish whether there was any left/right bias. All other otoliths were read by two independent readers and an agreed age was reached based on the number of annuli in the right sagittal otolith for each individual fish. More specifically, for all readable (right-sided) otoliths, four separate incremental counts were undertaken by both readers along three axes (-also see axes counts, below). All counts were performed blind, for example, the tagged otolith sample

and age of fish was not revealed to either reader. Age was determined only when the same result was achieved or their difference was less than 5% of the estimated age. Larger bands could be observed under 4x magnification while the finest bands required 40x and 60x magnifications, especially the bands that were laid down between the primordium and the first observable band; similarly, higher magnifications were required to observe the distance between the last of the opaque bands and the edge of the otolith.

Figure 5.3 illustrates how the formation of rings was stretched towards three dominant axes/apexes (oa (anterior apex), ob and oc (posterior apices)). Annuli are more distinctly spaced and easier to count along these apices. Therefore the number of annuli was counted along each of the three axes from the primordium with the expectation that the number of annuli should be the same for all axes. Thus oa=ob=oc in terms of the number of annuli but oa≠ob≠oc in terms of distance from the nucleus (Jones, 1992).

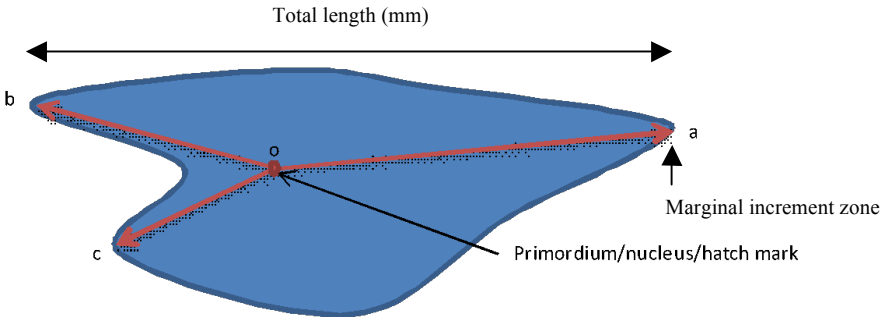


Figure 5.3: Axis for total length of an otolith and the three axes from the nucleus (o), oa, ob and oc for counting the number of annual rings.

In unusual circumstances, one of the paired (left or right) otoliths had structural defects; for example, one of the otoliths occasionally displayed altered axes of growth (Figure 5.4). Very occasionally, one of three types of otolith variations in shape and size were found; i.e. abnormal shape (b - c) and abnormal size or dwarf (d). As this would affect otolith weight and length measurements and might result in a change in the relationship between otolith size and fish size, otoliths (b-d) were omitted from the study and only the 'normal' ones (a) from a pair was analysed further.

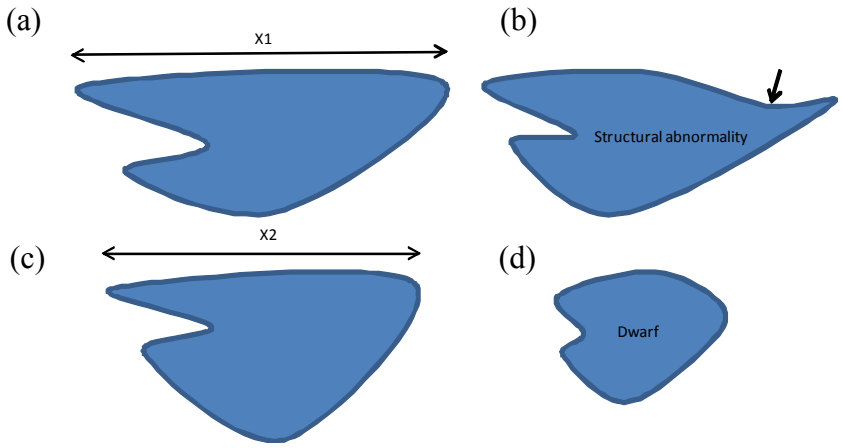


Figure 5.4: Occasional variation of shape and size between right and left otolith pair from same adult *Labrus bergylta*; (a) normal shape and size, (b - c) abnormal shape and different size and (d) abnormal size/dwarf. X1 and X2 indicate difference in total length even though the weights were the same.

5.2.4 Larval otolith preparation and diameter measurements

L. bergylta larvae were reared in the laboratory from fertilization to 49 days post hatch (dph). These larvae were fed live feed, initially once a day (at 1dph), increasing to three times daily by 9-49 dph, with the live feed items changing according to larval development (–see Chapter four for more details). To establish when the first band was deposited and describe early otolith growth, larvae were sampled on a total of nine sampling days (1, 3, 7, 14, 21, 29, 35, 42 and 49 dph). Four larvae were sampled on each sampling day (total n=36). These individuals of known age were sacrificed to enable examination of growth patterns and to establish whether these were daily. Larvae were anaesthetised using Tricaine methanesulfonate (MS 222) and preserved in buffered ethanol (80%) to ensure no damage to the larvae prior to otolith extraction. In the larva's head, the sagittae are located posterior to both eyes within the otolith membranous sacs in which are medial and ventral to the lapilli (Figure 5.5; Secor *et al*., 1992). Sagittal otoliths from left and right sides were removed using a fine dissecting needle and dissecting microscope with cross-polarized light.

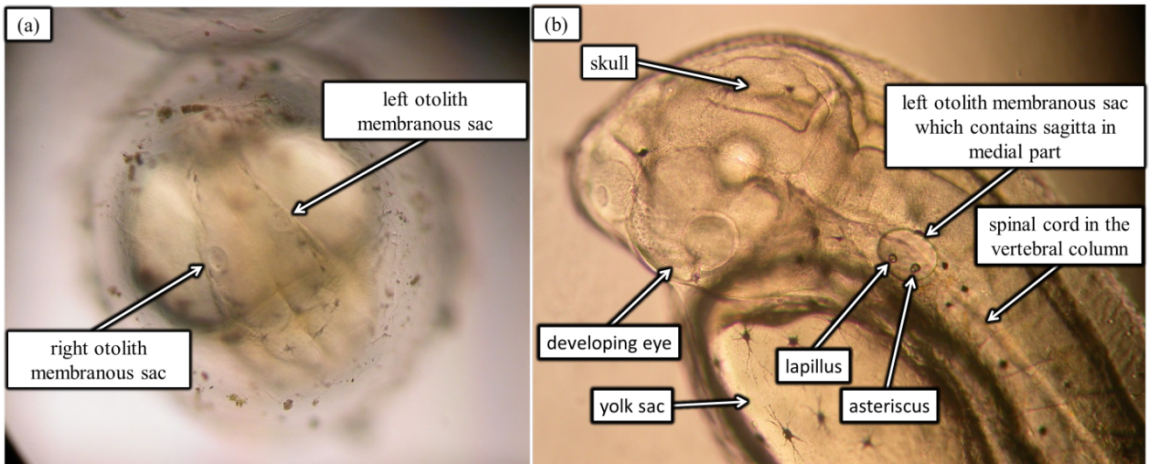


Figure 5.5: (a) Superior view of *L. bergylta* embryo in a developing egg with prominent right and left otolith membranous sacs. Pairs of lapilli and asterisci otoliths can be seen; (b) Lateral left view of 1 dph *L. bergylta* larvae with prominent left asteriscus and lapillus. Sagitta cannot be seen as this is located at the medial part of the membranous sac that contains the otoliths.

The larval sagittal otoliths were transferred to clean slides by picking them up on the end of a fine dissecting needle that had been pre-wetted with immersion oil. The otoliths were examined in immersion oil (56822-50ml; Sigma–Aldrich) after having been permanently mounted under a cover slip in a quick drying, neutral Acrytol mounting medium (3801700-20ml; Leica Biosystems). Each otolith was imaged and stored at magnification 1000x for daily ring counting using an Olympus BX51 microscope and Image Pro Plus Analyzer software version 6.3. The average diameters of each sagittal otolith were measured through the core of the otolith at the longest, shortest and intermediary axis. Measurements were made to the nearest micrometer using either image analysis of stored images, or (initially) using an ocular graticule on a compound microscope (60x) (Olympus BX51).

The fact that bands were deposited at a daily rate was established by counting the number of rings in known-age larvae (n=36). The number of opaque rings (right-sided sagittal otoliths only) were read along the anterior apex from the hatch-check to the otolith edge. To ensure reading accuracy, two independent readers conducted a 'blind-count' for all the readable right-sided sagittal otoliths, as before. Four separate increment counts were taken by both readers, as before.

5.2.5 Data analyses and growth calculation

For larvae, daily band deposition was validated using increment counts from larvae whose age post-hatch was known. The relationship between otolith diameter and larval age was described by plotting individual otolith diameters against the fish age, expressed in number of days post-hatch (dph) and carrying out regression analysis in SPSS version 21 (2011). For adult age validation, mean monthly marginal increments were examined over 17 consecutive months in ~10 year-old individuals (n=29) to validate the edge-increments of the catch-year.

Various otolith and fish size data were taken for adult (n=234; 150 fish sampled in 2010 and 84 sampled in 2011) and juvenile (n=19) *L. bergylta*. Hence, the total number of sampled fish was 253. All statistical analyses were carried out in SPSS version 21 (2011). A t-test was carried out to determine if there were differences between left and right otolith pairs; thereafter, right-sided sagittal otolith variables were analysed. Normality of model residuals was assessed using the Kolmogorov-Smirnov goodness-of-fit test (Feltz, 2002) after which the number of annuli (age) was $\log_{10}$-transformed ($\log_{10}$ (x)). The assumption of homogeneity of variance of responses was assessed using Levene's test. Regression analysis was used to explore relationships between fish weight (g), fish length (cm), otolith weight (g), otolith length (mm) and number of annual rings (age) of the juveniles and adults *L. bergylta*. Linear, quadratic, polynomial, power and cubic expressions fits were explored. The model was fitted to length-at-age of both otolith and fish data from juveniles to 30 years old adults. Residual plots were examined following regression analyses. The Statistical Package for the Social Sciences (SPSS) was used to obtain the estimates for the growth functions.

The von Bertalanffy growth function (VBGF) introduced by von Bertalanffy in 1938 predicts the length of a fish as a function of its age. The VBGF is given in several forms, including an expression of the age-length relationship as follows:

$[L_t = L\infty\ (1 - e^{-k(t - t_0)})]$

L_t = length at time t

$L\infty$ = maximum theoretical length (mathematical asymptote of the curve or the asymptotic length at which growth is zero)

k = growth constant (growth rate at which the growth curve approaches $L\infty$)

t_0 = theoretical age at zero length

L_∞ or $L_{infinity\ or}$ L_{max} is the mean maximum length that individuals may reach given unlimited growing time (t = infinity), k is a rate constant (how fast L_∞ is reached), e is the constant 2.718, t = age of fish and finally, t_0 = the age at which fish have theoretical length of 0 cm. The latter is included to adjust the equation for the initial size of the organism and is defined as age at which the organisms would have had zero size.

To fit the VBGF to *L. bergylta* data, three parameters (L_∞, k and t_0) were fitted by nonlinear regression. VBGF parameters were calculated for *L. bergylta* based on analysis of length-at-age data, with age (in years) determined by otolith annuli and fish length given in cm. Estimated VBGF parameters can be sensitive to the range of ages and sizes used and no fish of age 1 were collected, so the intercept was constrained to 0 cm. VBGF parameters were calculated using the 'length-at-age' procedure in FISAT II software (version 1.2.2, FAO, Rome, Italy). All fish (male and female) were analysed together before analysing females separately.

5.3 Results

5.3.1 Validation of daily growth rings in larval otoliths

36 viable larval otoliths, each representing a wrasse individual were examined for various parameters. The otoliths were first observed inside developing larvae three days after fertilisation and were round in basic appearance. Larvae hatched ~6 days post-fertilisation (chapter four) and the hatch mark, or 'hatch-check', was clearly apparent in all sagittae examined. The embryonic micro-increments, which were laid down prior to hatch, were difficult to measure due to the necessity of changing the focal point to observe pre- and post-hatch zones. Since they would have been dubious, these measurements were not made. All counts of larval rings began after the hatch-check. The number of rings counted after the hatch-check aligned with known age up to 49 dph, confirming that one growth increment was formed per day, at least through to 49 dph. This result is illustrated in Figure 5.6. Additional microscope images of developing larval otoliths from hatch to 42 dph are shown in Figure 5.7.

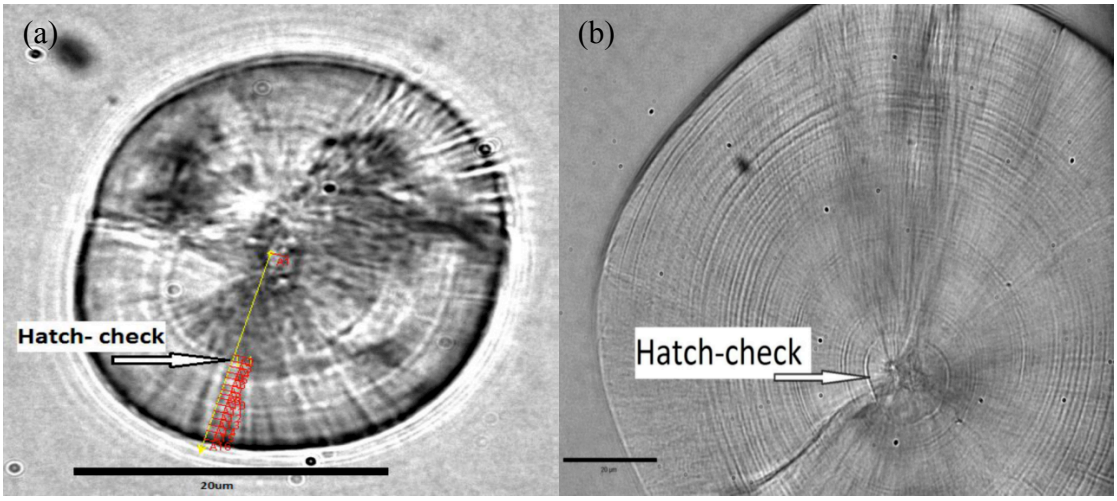


Figure 5.6: Micro-incremental counts and measurements began at the hatch-check. (a) Hatch-check with 14 daily rings on a sagitta from *L. bergylta.* The larva was of known age (14 dph), so this image illustrates the fact that there was daily ring formation in *L. bergylta* larvae; (b) hatch-check and daily growth rings on a *L. bergylta* larva of 49 dph. Scale bar = 20 µm.

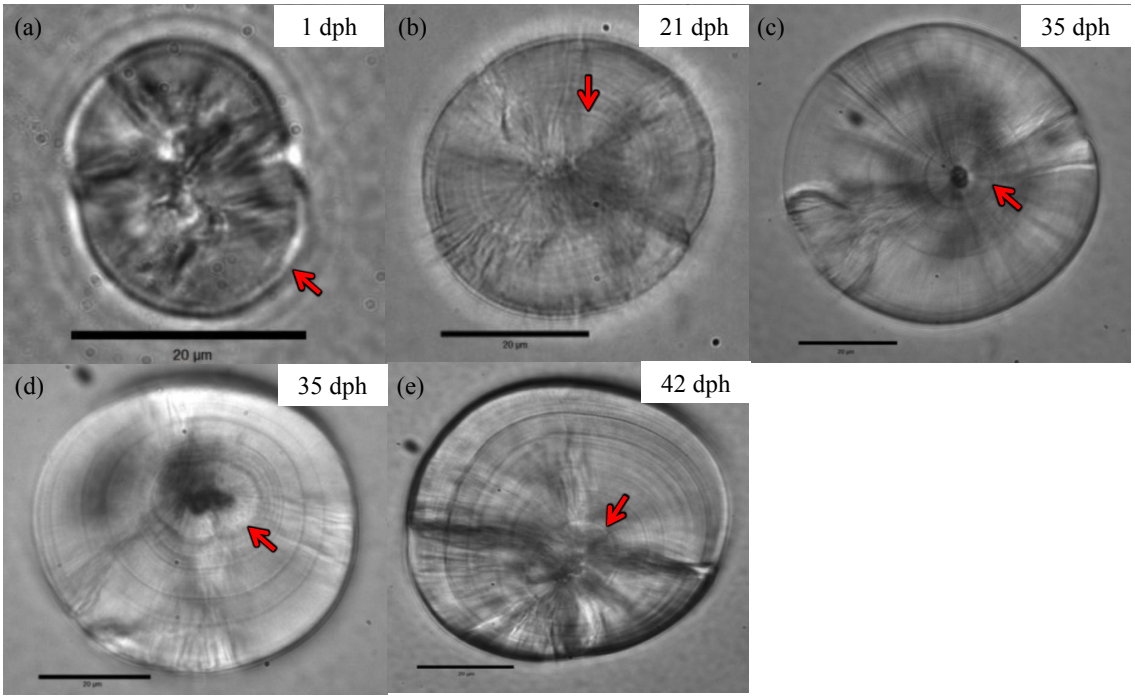


Figure 5.7: Sagittal otolith illustrating larval growth over time. The hatch-check is shown by a red arrow in each case (a) 1 dph; (b) 21 dph; (c) and (d) 35 dph; (e) 42 dph. Scale bar = 20 µm.

The mean diameter of all replicates of 1 dph larval otoliths was 19.5±1.3µm (n=4). Individual larval otolith diameters (n=36) were plotted against the respective age from 0 to 49 dph (Figure 5.8). The otoliths displayed an exponential growth pattern described by a 3rd order polynomial growth curve (R^2=98.3%, $p<0.01$). The otolith growth pattern was negligible for the first 21 days but a minor increase in growth occurred between days 21 - 30 and, thereafter, a steady increase in growth was noted until the final sampling at 49 dph. An analysis of mean otolith diameters between paired specimens (left and right otolith) via paired t-test found no significant difference between left and right otoliths both weight ($t=0.57$, $p>0.05$, $df=50$) and length ($t=1.84$, $p>0.05$, $df=50$) (Table 5.1).

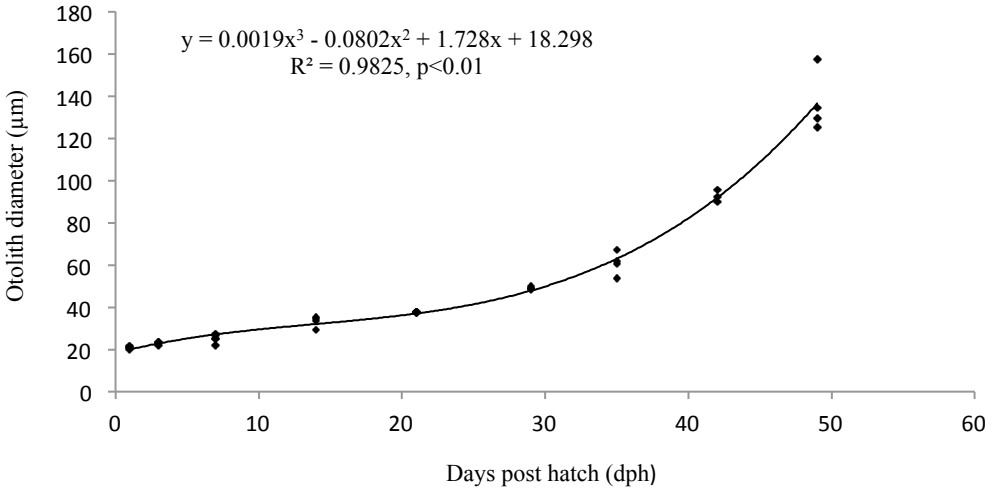


Figure 5.8: Individual diameter of larval otolith from known age larvae between 0 to 49 days post hatch (dph) in *Labrus bergylta.* Sample size was n=4 larvae at each dph.

5.3.2 Validation of annual growth rings in juvenile and adult otoliths

Preliminary examination was carried out on otoliths from *L. bergylta* of suitable sizes of >500g adults (n=30) and juveniles (n=19) were also examined by measuring the distance from the central part (nucleus) to the first, second and third observable annual rings. Figure 6.9 illustrates the central part of an adult *L. bergylta* otolith known as the nucleus (= "primordium") approximately 20µm in diameter, which is synonymous with the hatch mark. The zone between the nucleus and the first year band or 'winter check', towards the centre of the otolith showed fading of micro-increment rings. Thus, it was not possible to produce clear micro-incremental rings at the centre of the otolith for most adult and juvenile individuals, even after polishing. However, annual bands were much clearer, in particular after the first year band (Figure 5.9). A graphic to illustrate the relative size of otoliths from a one-day old larva and a three-year old juvenile is presented in Figure 5.10 and an eight-year old adult in Appendix 6.

In addition to fading of the otolith micro-increments prior to the first winter check, some further observations can be made at a macroscopic level in sagittal otoliths of juvenile and adult *L. bergylta*. The third annual band and onwards were very prominent compared to first and second bands, as structurally, the otolith is thicker at the centre. The greatest distance between the annual bands was between third and fourth

year bands, indicating a higher growth rate in the three and four-year-old individuals. In contrast, the distance tended to decrease from the 11th band onwards, indicating that growth rate slowed down after this age. Figure 5.11 shows the distance (μm) of the observable annual rings from the nucleus to the third annual ring through the axis of anterior apex (Figure 5.3) in a subsample of juvenile and adult *L. bergylta*.

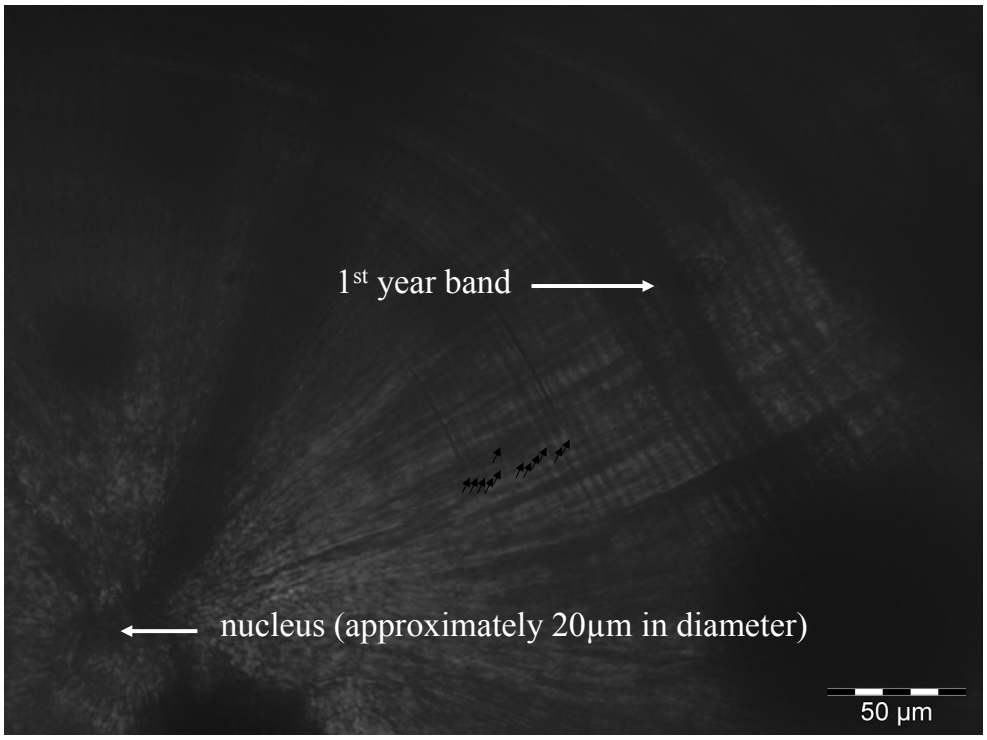


Figure 5.9: Incremental growth from central part of an adult *L. bergylta* otolith, known as the nucleus or primordium. The '1st year' band (indicated) represents the winter check in an annual growth cycle. Fading of rings (black arrows) is evident towards the otolith centre (scale bar = 50 μm).

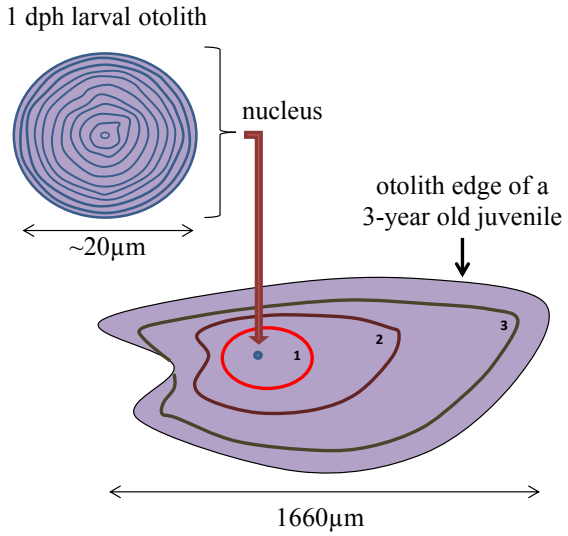


Figure 5.10: Size of one-day post-hatch (dph) larval otolith relative to three-year old juvenile otolith in *L. bergylta*. The nucleus (representing the hatch mark) is indicated in blue. Numbered coloured bands represent annular increments in a juvenile.

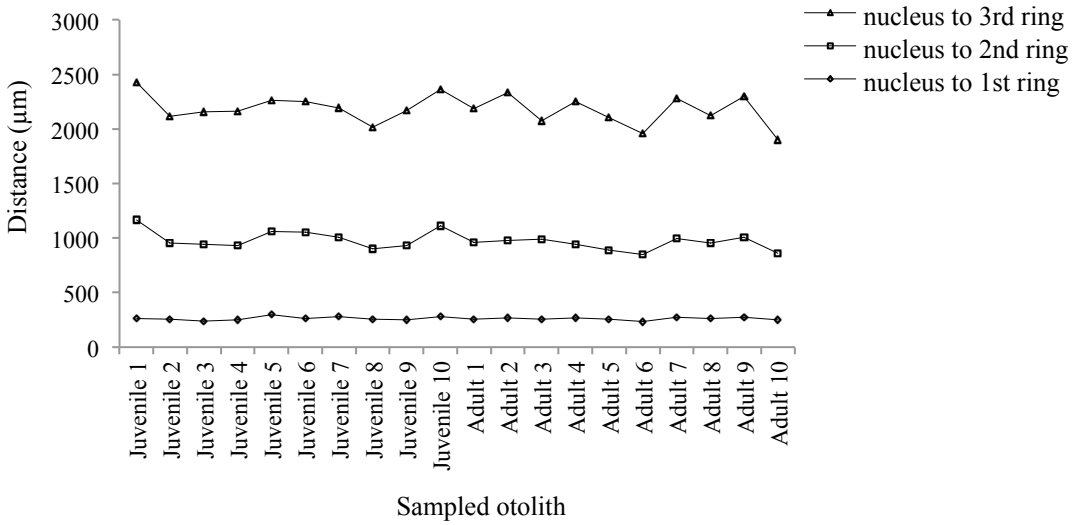


Figure 5.11: Distance from nucleus to annual rings in a subsample of juvenile and adult *L. bergylta* otoliths (n=10 included for brevity).

An important element of aging fish using otoliths is validation of the observation that growth bands represent one year. This can be done via examination of consecutive monthly samples to ensure that marginal increments that accrue over the course of 12 months correspond to a single winter (opaque) and summer (translucent) band, i.e., one annulus per growth cycle. It was difficult in the older individuals to observe monthly increments that constituted the formation of annual rings as these became narrower and closer to the margin. Thus, individuals which did not present this problem were chosen for validation. A total of 29 individuals with a medium body size (500g), i.e. individuals of approximately 10 years old, were used for validation purposes. Validation using marginal increments took place through a period of 17 months between April 2010 and August 2011. However there were gaps in monthly sampling of *L. bergylta* in the months of August, September and December in 2010 and January and June in 2011, as appropriate size classes were not available in these months.

There was a gradually increasing trend in the average marginal increment between April and October 2010, with 2011 showing a similar pattern; while mean increments decreased sharply between November and March/April (Figure 5.12). In addition, formation of translucent deposition was observed from April to October, whereas opaque deposition took place between November and March/April (Figure 5.12). It can be concluded that the formation of growth rings followed a seasonal pattern and that an annulus is formed over an approximately annual period.

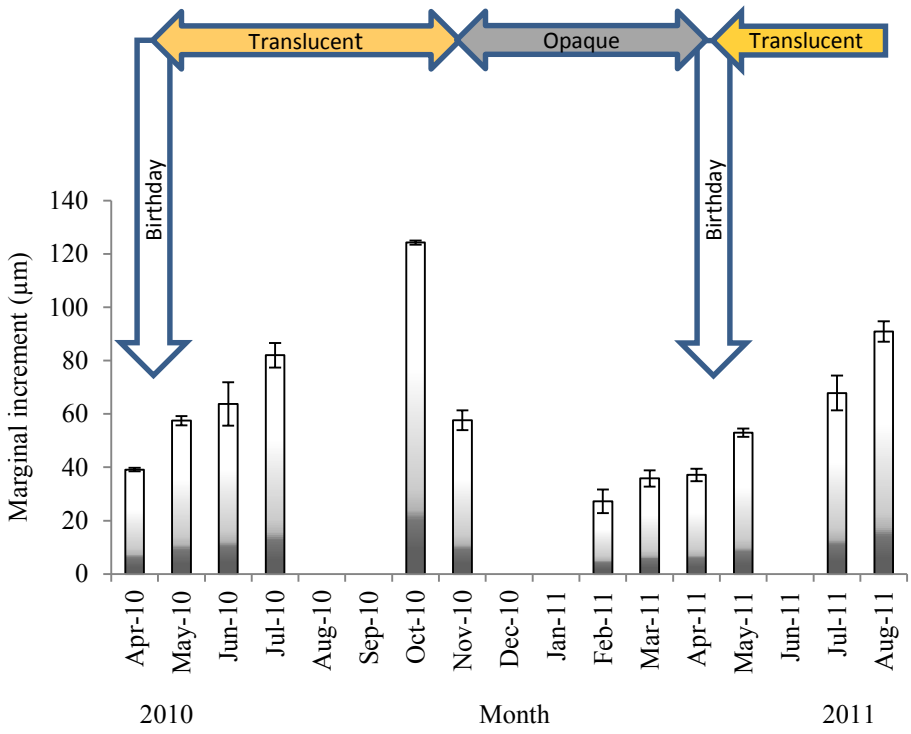


Figure 5.12: Mean monthly marginal increment in *L. bergylta* otoliths measured in micrometers (µm) and plotted with one standard error of the mean (±s.e.) for ~500g individuals (approximately 10 year-old adults), n=29. The timeline illustrating the seasonal birth dates of wrasse during April of each year is indicated. Please note that no *L. bergylta* samples of suitable size were available for some of these months.

Sagittal otoliths were easy to read with clearly identifiable annuli and percentage agreements between the readings of the independent readers were >95%. Tests for differences between left and right sagittal otoliths in $\log_{10}$ transformed weights and lengths (n=50 randomly sampled *L. bergylta* individuals) were not statistically significant (paired t-test, Table 5.1). Likewise, there were similar counts of annual rings between left and right otoliths.

The age of *L. bergylta* was determined in 253 individuals that were wild-caught between April 2010 and August 2011. Table 5.2 shows estimated age distribution of the wild caught *L. bergylta* varied from 3 to 30 years old; however the majority of individuals from the wild were 13 years old, which contributed 12.3% of the total sample number. The distribution of ages of wild *L. bergylta* is illustrated in Figure 5.13. Sagittal otoliths from a juvenile (three years old) and adult (eight years old) *L. bergylta* are shown in Figure 5.14.

Table 5.1: Paired t-test of mean size of left and right otoliths in *L. bergylta* (log_{10} transformed otolith data in each case).

	N	Right mean ± s.e	Left mean ± s.e	t
Otolith weight	50	0.00324 ± 0.00030	0.00323 ± 0.00030	$0.57^{n.s}$
Otolith length	50	0.15646 ± 0.00585	0.15476 ± 0.00575	$1.84^{n.s}$

$^{(n.s)}$ not significant

Table 5.2: Age distribution of n=253 wild *L. bergylta* sampled between April 2010 and August 2011. Shading indicates juvenile age classes (Muss and Nielsen, 1999).

age	male	female	frequency	cumulative frequency	percentage (%)	cumulative percentage (%)
3	0	15	15	15	5.9	5.9
4	0	4	4	19	1.6	7.5
6	0	3	3	22	1.2	8.7
7	0	12	12	34	4.7	13.4
8	0	23	23	57	9.1	22.5
9	0	25	25	82	9.9	32.4
10	0	29	29	111	11.5	43.9
11	2	26	28	139	11.1	55.0
12	2	19	21	160	8.3	63.3
13	1	30	31	191	12.3	75.6
14	0	23	23	214	9.1	84.7
15	1	11	12	226	4.7	89.4
16	3	6	9	235	3.6	93.0
17	0	2	2	237	0.8	93.8
18	2	6	8	245	3.2	97.0
19	0	1	1	246	0.4	97.4
20	0	3	3	249	1.2	98.6
21	1	0	1	250	0.4	99.0
23	0	1	1	251	0.4	99.4
24	0	1	1	252	0.4	99.8
30	1	0	1	253	0.4	100.2

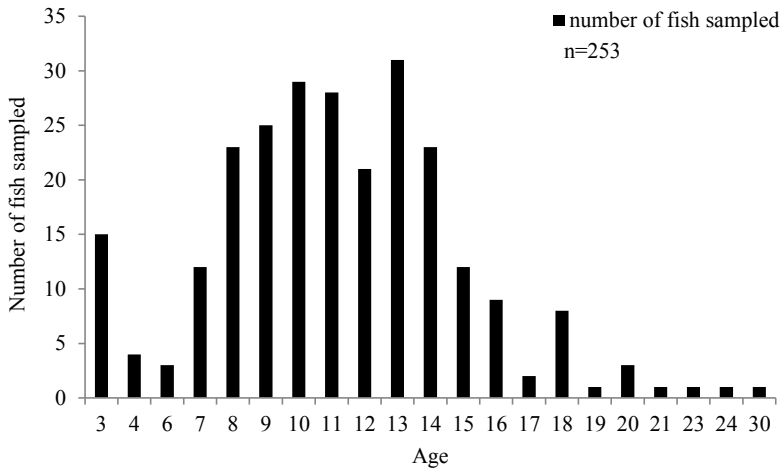


Figure 5.13: Age structure of n=253 *L. bergylta* sampled between April 2010 and August 2011.

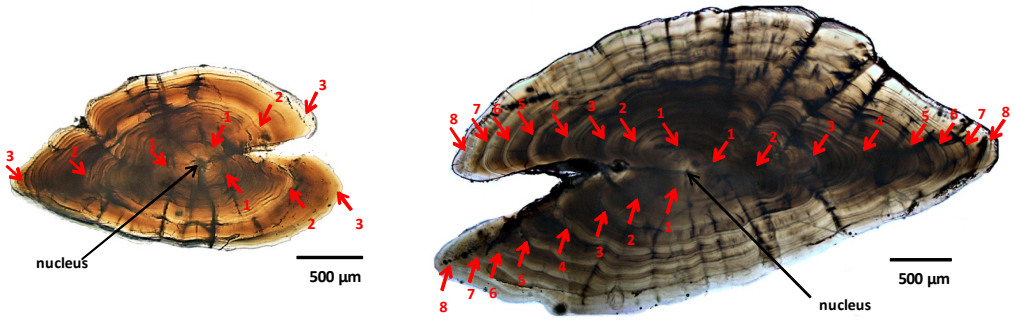


Figure 5.14: A comparison of polished sagittal otoliths in transverse section from three year old (left) and eight year old (right) *L. bergylta*. Numbered arrows show the respective complete annual rings (scale bar = 500µm).

5.3.3 Data exploration in *Labrus bergylta* growth and aging parameters

As left and right otoliths were similar in length and weight, only the right sagittal otoliths were included for data exploration. In all analyses, residuals were normally distributed. Homogeneity of variance was confirmed where the test assumption required it. The growth pattern of *L. bergylta* (both sexes), as described by the von Bertalanffy growth function (VBGF) from otoliths, gave function co-efficients of $L\infty = 42.48 \pm 0.884$ cm and $k = 0.16 \pm 0.013$ per annum in this study (all individuals, male and female). When 14 males were removed from the dataset, the VBGF model was run again, but this did not change the $L\infty/k$ parameter values.

Size relationships between somatic and otolith variables were best described by quadratic curves in all cases (Figure 5.15 - 5.18; n=253). A significant positive quadratic relationship was observed between fish length and age ($\log_{10}$ number of annuli), taking the form $y = -14.92 + 70.19x - 21.09x^2$ (Figure 5.15). The coefficient of multiple determination was 0.844; therefore, about 84.4% of the variation in the fish length was explained by its quadratic relationship with the age of the fish ($R^2 = 0.84$, $F= 678.58$, intercept = -14.92, $b_1 = 70.19$, $b_2 = -21.09$, $p<0.001$). A significant positive quadratic relationship was also obtained between otolith length and fish age, $y = -0.059 + 0.779x - 0.182x^2$ (Figure 5.16) ($R^2 = 0.80$, $F= 507.25$, intercept = -0.059, $b_1 = 0.779$, $b_2 = -0.182$, $p<0.001$).

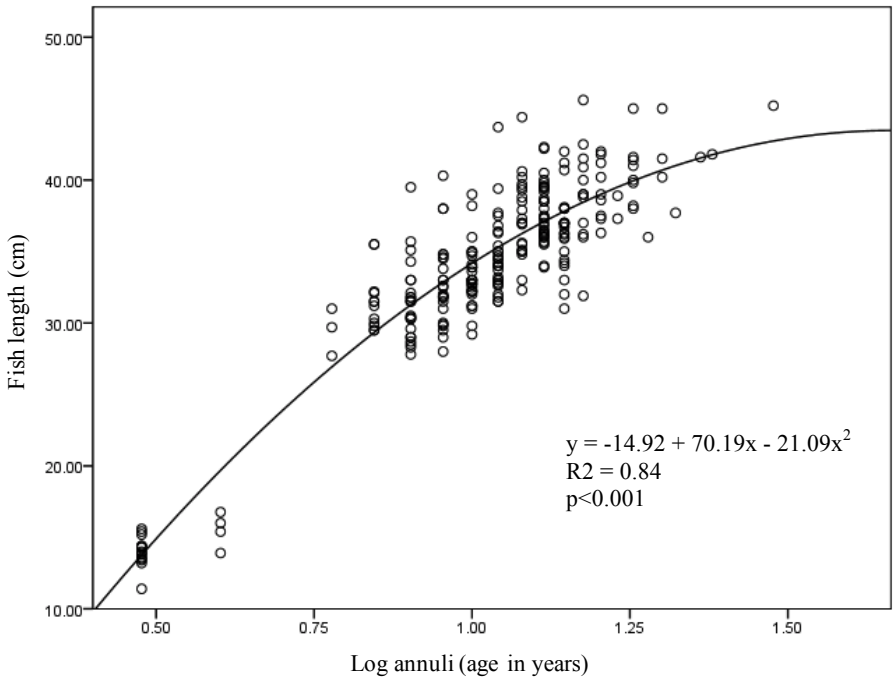


Figure 5.15: Relationship between fish length and $\log_{10}$ number of annuli (age in years) in juvenile and adult *Labrus bergylta.*

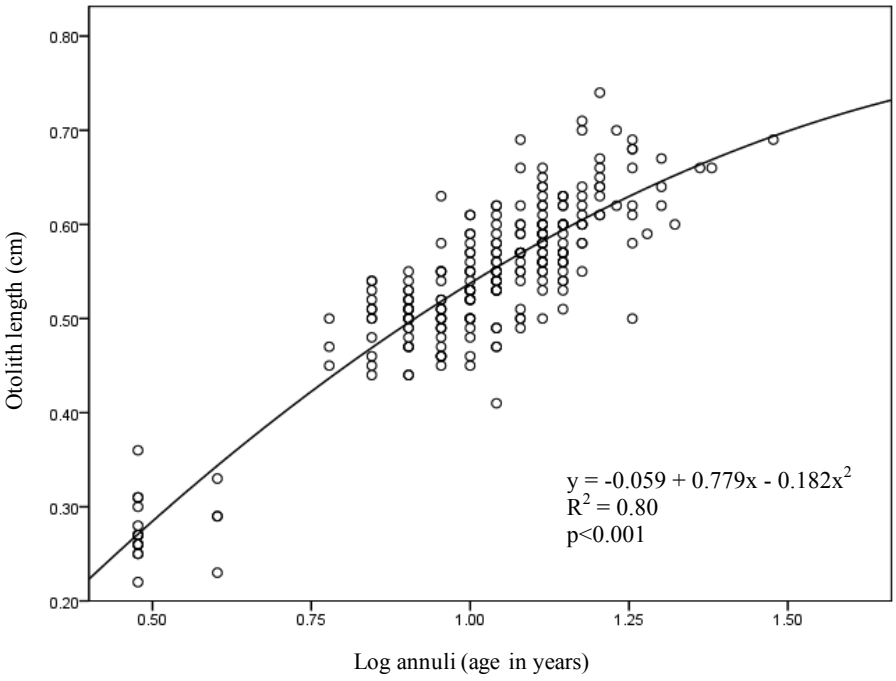


Figure 5.16: Relationship between otolith length and $\log_{10}$ number of annuli (age in years) in juvenile and adult *Labrus bergylta*.

A further significant positive quadratic relationship was obtained between fish length and otolith length ($R^2 = 0.85$, F = 695.97, intercept = -16.76, $b_1 = 136.14$, $b_2 = -75.71$, $p<0.001$), $y = -16.76 + 136.14x - 75.71x^2$ (Figure 5.17). In comparison with other parameters, otolith weight versus fish age shows a significant convex relationship ($R^2 = 0.83$, F = 592.76, intercept = 0.001, $b_1 = -0.007$, $b_2 = 0.016$, $p<0.001$), $y = 0.001 - 0.007x + 0.016x^2$ (Figure 5.18). However, otolith weight versus fish length showed slightly less fit ($R^2 = 0.78$, F = 431.04, $p<0.001$ –plot not shown) and plots of fish weight and age produced higher data scatter and are not presented here.

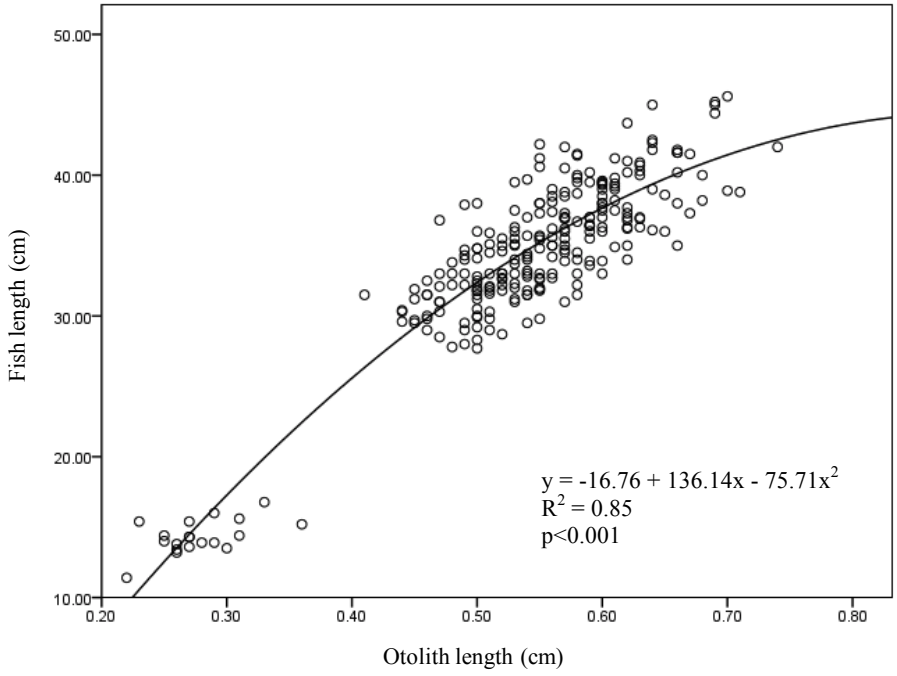


Figure 5.17: Relationship between fish length and otolith length in juvenile and adult *Labrus bergylta*.

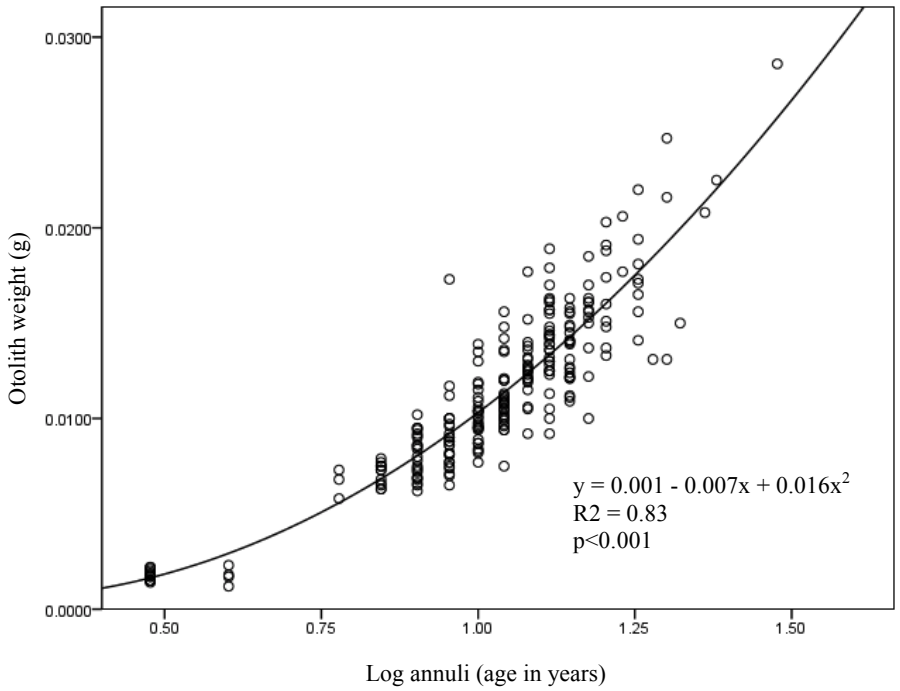


Figure 5.18: Convex relationship between otolith weight and $\log_{10}$ number of annuli (age in years) in juvenile and adult *Labrus bergylta.*

5.4 Discussion

This study presents the first description of growth parameters in *Labrus bergylta* adults at the northwest west of its range, in the west of Ireland, for comparison with recent work in the Biscay area (Galicia; Villegas-Rios *et al*., 2013). It is the first attempt at validating annular increments in *L. bergylta* sampled monthly in the west of Ireland in juveniles and adults. Some otolith data for early (<49 dph) larvae is also presented.

To estimate age from field-captured adult fish, two pieces of information must be known: (a) the age at first increment formation and (b) the accuracy of increment counts. Dealing with these in reverse order, *L. bergylta* otoliths from adults in the present study exhibited clear 'annuli'. Under reflected light, the whole otoliths showed a clear internal structure of alternating wide translucent and narrow opaque increments; as described in other labrids (Gordoa *et al*., 2000). In addition, marginal increments in adult otoliths over consecutive months validated the monthly accretion of calcium carbonate rings, but with varying degrees of opacity in different seasons. In many fish species, opaque zones representing winter growth appear as darker bands and alternate with translucent zones which correspond to the hyaline or summer growth zones (Hayashi *et al*., 1995; Newberger and Houde, 1995; Stephenson and Hall, 2003). This analysis indicated that alternating opaque/translucent zones clearly represented one year's growth with a clear seasonal trend relative to the birth-date of *L. bergylta* in Carna in late April (Chapters two and three). This is significant because it indicates that individuals can be aged by counting the annuli (an individual opaque plus translucent zone represents a year's growth). Mean sizes of consecutive monthly marginal increments indicated that accretion of marginal growth increases slowly from March and decrease again in November, reflecting *L. bergylta* growth patterns over the months. Growth estimation based on sagittal otoliths is preferred to scales and opercular bones as these structures are not reabsorbed or metabolically reworked and are not exposed to external damage (Campana and Neilson, 1985).

Turning our attention to (a); *L. bergylta* larvae were ideal candidates for otolith micro-structure analysis due to the clarity of micro-increments which could be read without the need for polishing or added preparations (this was not the case for juvenile and adult otoliths). The present study examined the initiation of micro-increment

deposition in sagittal otoliths for *L. bergylta* larvae after hatching, as well as the rate of accretion of rings in larvae of known age while these grew under laboratory culture. Embryonic rings (i.e. pre-hatching) were difficult to count and measure but these were definitely present, with either two or three embryonic rings being apparent in the larvae sampled. In a review of wrasse otolith micro-structure, Victor (1982) cited by Cowen (1991) stated that two embryonic rings are typical of labrids. Although the embryonic rings were impossible to measure in the present study, a clearly visible hatch-check was present enabled precise core diameter measurements to be made (mean ± s.e. of 19.5±1.3μm). This result indicates how much material begins to accrete in the otolith before the fish is 'born' (i.e. hatches); although the corresponding length of embryonic *L. bergylta* was not recorded.

The next analysis was of growth increment counts in larvae - this study confirmed that the first daily growth increment after the hatch-check occurs at approximately 1 dph. Thereafter, daily increments could be observed from 1- 49 dph; these were clearly apparent as rings beyond the hatch check. Thus the results confirmed the daily deposition of otolith growth increments in the first 49 days of life for *L. bergylta* larvae. Larvae of other temperate wrasse species e.g. banded parrot fish *Notolabrus fucicola* (Richardson, 1840) and the blue throated parrotfish *N. Tetricus* (Richardson, 1840), have also shown that the first micro-increments were not laid down until the beginning of exogenous growth (i.e. after hatching), producing a single daily ring (Welsford, 2003). A clearly defined hatch-check followed by daily ring production was also reported in Pacific cod, *Gadus macrocephalus* (Narimatsu, 2007), and Nile tilapia *Oreochromis niloticus* (Zhang and Runham, 1992).

Growth increment measurements over this period were made from the hatch-check in all cases, as this was the most reliable and consistent reference point. Although the numbers of larvae sacrificed to growth analysis was small, the measurements of larval micro-increments in the present study revealed that growth of *L. bergylta* larvae started slowly but rapidly increased at ~35dph. Development of *L. bergylta* larvae may be divided into four stages based on gross external morphology: (1) Yolk sac larva (0 - 6 dph); (2) Pre-flexion larva (7 - 14 dph); (3) Flexion larva (21 - 28 dph) and (4) Post - flexion larva (35 – 49 dph) (Artuz, 2005 and Chapter four). The otolith growth pattern

agrees well with the results in Chapter five which showed growth was increased (specific growth rates; SGRs) at the onset of post-flexion (35 - 49 dph), in which the dorsal, anal, caudal and pelvic fins were initially developed or separated. Post-flexion was mainly characterized by increases in the size and complexity of pre-existing organs and structures and metamorphosis was normally complete at 49 dph (Dunaevskaya *et al*., 2012). The same trend has been reported in previous studies in which increased growth began at 35 dph (D'Arcy, 2013; Dunaevskaya *et al*., 2012; Ottesen *et al*., 2012).

When the diameter of the otolith was examined in relation to standard length of larvae, a near linear ($y = 10.413x - 24.945$; $R^2 = 0.93$; shown in bold) or quadratic relationship ($y = 0.7954x^2 - 3.5694x + 25.862$; $R^2 = 0.97$) was observed (Figure 5.19). A similar relationship was also established by Aguilera *et al*., (2009). In terms of variability of early growth, the distance measured between the 'nucleus' i.e. hatch-check and the first, second and third annular ring was fairly consistent across replicate fish; however, such variations as there were in individuals became more pronounced in individuals as time went on to older years.

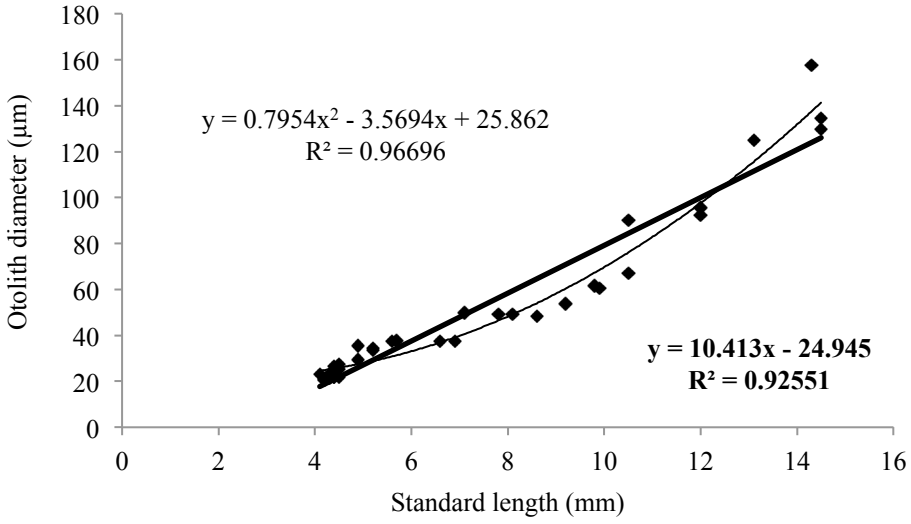


Figure 5.19: Individual diameter of larval otolith versus standard length from 0 to 49 days post hatch in *Labrus bergylta*.

Having validated the aging methods, the age of wrasse in the current study varied from 0-30 years however most individuals captured from the wild were ~13 years

old. Age 8-14 years old group contributed the majority of *L. bergylta* in the sampling area at >70% of all individuals. Juveniles (<4 years old) individuals were very rare in the present population (n=19). The reason for this is because the sampling area was away from the natural habitat of juvenile *L. bergylta* where they usually hide in shallow water among the seaweeds rather in the open sea. Another pattern was that the population sampled at Carna was dominated by females at each age group with a total of only 13 males plus 1 transitional individual seen overall. No specific age classes were associated with male *L. bergylta* at Carna as male gonads were seen in individuals of 3-8 years old, n=7/14, as well as middle-aged groups (10-18 years old, n=7/14).

Once the allometric relationship between growth variables is known, then size at prior age can be back-calculated, e.g. from the otolith size variables, and the average aging pattern can be validated with respect to average length. Therefore, the form of the relationship between the size of the otolith, fish size and age, as deduced by the number of annuli, was explored. The relationship between fish length as a predictor (independent variable) with otolith length, otolith weight and age (in years) (dependent variables) for *L. bergylta* indicated strong positive correlations (Appendix 9). Most relationships between the growth variables for *L. bergylta* were best described by regression with a quadratic fit and hence, a curvilinear relationship. Nevertheless, a quadratic relationship between otolith weight versus fish age was convex instead of concave (as seen in all other comparisons). The relationships between otolith size and fish age (otolith weight and length), showed that individuals accreted calcium carbonate in the otoliths throughout the lifetime of the fish over 30 years span, on average. However, otolith weight clearly accretes much slower than otolith length as *L. bergylta* get older, since the curves differ in shape. Skeljo *et al*., (2012) recommended the use of otolith length for ease of interpretation and the best predictor of age rather than weight.

The robust relationships between fish and otolith characteristics in *L. bergylta* permits the back calculation of fish body size at younger ages to predict individual growth or validate the ageing method (Pilling *et al*., 2002). When *L. bergylta* reaches 10 years old (log annuli = 1), the calculated fish length would be 34.18 cm based from the generated growth equations of fish length versus age ($y = -14.92 + 70.19x - 21.09x^2$) in Figure 5.15. Meanwhile, the calculated otolith length is 0.54 cm based from the

generated growth equations of otolith length versus age ($y = -0.059 + 0.779x - 0.182x^2$) in Figure 5.16. Similarly, according to the equation of fish length versus otolith length ($y = -16.76 + 136.14x - 75.71x^2$) in Figure 5.17, 34.80 cm of fish length is obtained (for an otolith size of 0.54 cm), which is approximate to the 34.18 cm value from growth equations above. Therefore, the result from these equations may predict fish size and otolith size in various ways and may validate the aging method in *L. bergylta*.

Many other studies also found that otolith length relationships were an objective, fast, repeatable and accurate method of describing fish growth (Fletcher and Blight, 1996; Pilling *et al*., 2003; Cardinale and Arrhenius, 2004; Bermejo, 2007; Sallami *et al*., 2013; Yilmaz *et al*., 2014). Deformations in otoliths affect otolith dimensions and may change the relationships between otolith and fish size. Total length of the otolith is probably more robust to deformations and a better parameter to use for allometric relationship determination on that basis. Notwithstanding its utility, the intercept in otolith length plots was not as realistic as that provided by age in no. of annuli versus fish length plots, as the x-axis intercept showed that size zero otoliths belonged to 0.87 years old fish (antilog of intercept -0.059), which is too high. –see next.

There was a very good concave quadratic fit ($R^2 = 0.84$) between *L. bergylta* age (in years measured by the no. of annuli) and body length. Overall, the results suggest that fish length was a good predictor of age. A significant, albeit weaker relationship, was observed between *L. bergylta* age and weight (not shown). Based on fish length-age equation ($y = -14.92 + 70.19x - 21.09x^2$), at age 2 ($\log_{10}$(age)), an individual could reach 4.24 cm, and could reach up to 42.76 cm in total length at 30 years of age. The observed maximum age in Galway in this study (30 year-old) is larger than previously reported in the Irish Sea (29 years old) (Dipper *et al*., 1977) and in the south of Galicia, North-West Spain (22 years old) (Villegas-Rios *et al*., 2013); yet still, this represents a modest longevity within the family Labridae (Choat *et al*., 2006; Cossington *et al*., 2010). *L. bergylta* is generally not a commercially fished species, at least in the west of Ireland, so reasons behind differences in longevity may be related to the lower water temperature in the Irish Sea. It is recognized that adult longevity increases when there is a decline in average water temperature during the individuals' development (Atkinson, 1994; Munch and Salinas, 2009). But *L. bergylta* is apparently commercially fished in Galicia, since

historical records are available from fish markets (Villegas-Rios *et al*., 2013); which may also explain a shorter lifespan in that location. Although the largest *L. bergylta* in terms of total length recorded in the present study was 45.6 cm, a historical record of 74.0 cm total length was reported at the north of the *L. bergylta* range (Muncaster *et al*., 2010).

The modelled maximum length from von Bertalanffy growth function (VBGF) in the present study gave $L_\infty = 42.48 \pm 0.884$ cm and growth rates (k) of 0.16 ± 0.013 year^{-1} for all *L. bergylta* (male plus female). Hence 42.48 cm is the approximate length where growth is predicted to cease, according to west of Ireland data. This is a slight under estimate since an individual of 45.6 cm was captured, however this threshold L_∞ value shows reasonably good agreement with the largest individuals observed in the study area. In comparison, *L. bergylta* sampled in the south of Galicia, north-west Spain showed smaller $L_\infty = 38.99$ cm and approximately double the rate $k = 0.39$ for the equivalent data (males plus females) (Villegas-Rios *et al*., 2013). In another study from the Sea of Marmara, Artuz, 1995 estimated that from scales a $L_\infty = 72.79$ cm, $k = 0.141$ and $t_0 = 1.35$ mm, but this is dubious since individuals of only 0^+ - 13 years old were sampled using scales rather than otoliths. Variation in L_∞ and k may be related to differing selective pressures between populations from sample locations, different ambient temperatures, different hard structures used for ageing (otoliths vs. opercular bones vs. scales) or sampling bias (Meekan *et al*., 2001).

Size-at-age plots reveal that *L. bergylta* invests a relatively small proportion of its life span in initial rapid somatic growth, as seen in other labrid species (Choat *et al*., 1996; Gordoa *et al*., 2000; Gust *et al*., 2002). But it is also shown that growth of *L. bergylta* is considerably slower than other fish species like cod (*Gadus morhua*), with e.g. $L_\infty = 122$ cm and $k = 0.304$ (Magnussen, 2007). Some growth comparisons between sexes in protogynous wrasses showed the existence of sex-specific differences; with males attaining larger sizes (L_∞) than females, but at a slower growth rate (k) (Choat *et al*., 1996; 2006). This was also seen in *L. bergylta* in galician waters (Villegas-Rios *et al*., 2013). By contrast, axillary wrasse, *Symphodus mediterraneus* (L.) from the eastern Adriatic Sea found more and faster growth in males ($L_\infty = 16.09$ cm, $k = 0.50$ and $t_0 = -0.83$) and females ($L_\infty = 14.11$ cm, $k = 0.33$ and $t_0 = -1.42$) (Skeljo *et al*., 2015). Sex-

specific growth differences could, in part, be explained by a growth spurt after sex change (Dipper *et al*., 1977). Or the differences in growth could be related to differences in social organization, which in turn, are related to differences in the sex change mechanisms (Walker and McCormick, 2004). For example, in a mediterranean species of wrasse and protogynous hermaphrodite, *Coris julis*, the fish that become males are already the larger individuals in their age group, which are associated with a higher growth rates relative to females ($k_{males} = 0.199$ and $k_{females} = 0.161$), and *Coris julis* also experiences a growth spurt after sex change (Linde *et al*., 2011). In *L. bergylta* from the west of Ireland, males were usually amongst the largest individuals (10/14 were >29cms in length), even though these large-ish fish were often rather young (as seen above). What this demonstrates is that size (not age) is an important element of sex change in *L. bergylta* and that growth must be very rapid in males. In Galician waters, males grew more than females, but at a lower rate ($L_{\infty males} = 40.36$ cm, $L_{\infty females} = 37.51$ cm, $k_{males} = 0.34$, $k_{females} = 0.44$; Villegas-Rios *et al*., 2013). In the present study only 13 males plus one transitioning individual were observed out of 253 individuals, so that it was not possible to generate a male growth function. The female only growth in the west of Ireland was exactly the same as 'all' individuals due to the fact that the 14 males were dwarfed in the population overwhelmingly biased towards females (n=239). Interestingly, colour morphs of *L. bergylta* (plain versus spotted) were also shown to have different growth rates (spotted grew to a larger size but at a slower rate) (Villegas-Rios *et al*., 2013). Demonstration of this intra-specific difference shows the great variability which exists in *L. bergylta* and shows that different selective pressures are very likely in this species. The findings of VBGF could also be used to evaluate the fecundity or spawning performance of *L. bergylta* according to their size and age in particular populations. In Chapter three, there might be some evidence that correlates spawners' age with spawning and fecundity as we do not know what range of age, which contributes in spawning.

Analysis of growth using annuli has the advantage over otolith size parameters of also being to supply data on metrics such as age-specific growth rates or life history trait parameters including maturity (Cardinale and Arrhenius, 2004). For example, an interesting observation in this study, the greatest distance between the annual bands was

between third and fourth year bands, indicating a higher growth rate in the three and four-year-old individuals. In contrast, the distance tended to decrease from the 11th band onwards, indicating that growth rate slowed down after this age. Otolith size data may not, therefore, produce data that may be relied on for setting fisheries management targets. For example, Pilling *et al*., 2003 showed that otolith weight-age relationships could not be used to estimate individual ages accurately, due to the level of overlap in otolith weight between age classes. Sagittal otoliths only were used in this analysis because *L. bergylta* otoliths are rather small, even in adults, thus the larger sagittal otoliths the best option. Notwithstanding this fact, otolith extraction and preparation could be carried out with moderate ease, albeit the process itself was quite time-consuming. Although it would be unusual for round fish to have asymmetrical pairs of otoliths, nonetheless, comparisons were made between left and right pairs in the present study. No significant differences were found in a random sample of 50 left/right pairs of adult sagittal otoliths in terms of i) weight ii) length and iii) number of annuli.

References

Abecasis, A. R. C., Canha, A., Reis, D., Pinho, M. R., & Gil-Pereira, J. (2009). Age and growth of the forkbeard *Phycis phycis* (Gadidae) from the Azorean archipelago, North Atlantic. *Journal of the Marine Biological Association of the United Kingdom*, 89(3), 629-633.

Aguilera, B., Catalan, I. A., Palomera, I., & Olivar, M. P. (2009). Otolith growth of European sea bass (*Dicentrarchus labrax* L.) larvae fed with constant or varying food levels. *Scientia Marina, 73*(1), 173-182.

Anderson, J. R., Morison, A. K., & Ray, D. J. (1992). Age and Growth of Murray Cod, *Maccullochella peelii* (Perciformes, Percichthyidae), in the Lower Murray-Darling Basin, Australia, from Thin-Sectioned Otoliths. *Australian Journal of Marine and Freshwater Research*, 43(5), 983-1013.

Artüz, L. (2005). Age, growth and feeding behavior of the *Labrus bergylta* Ascanius 1767 in Sea of Marmara. *Hidrobiologica*, Publications Scientifiques, 2005/2, p. 98-100.

Atkinson, D. (1994). Temperature and Organism Size - a Biological Law for Ectotherms. *Advances in Ecological Research*, Vol 25, 25, 1-58.

Bar, N. S., & Radde, N. (2009). Long-term prediction of fish growth under varying ambient temperature using a multiscale dynamic model. *Bmc Systems Biology, 3*, 107.

Bartsch, J. (2002). Modelling the temperature mediation of growth in larval fish. *Fisheries Oceanography, 11*(5), 310-314.

Beamish, R. J., & Mcfarlane, G. A. (1983). The Forgotten Requirement for Age Validation in Fisheries Biology. *Transactions of the American Fisheries Society, 112*(6), 735-743.

Beardsley, H., & Britton, J. R. (2012). Contribution of temperature and nutrient loading to growth rate variation of three cyprinid fishes in a lowland river. *Aquatic Ecology, 46*(1), 143-152.

Bermejo, S. (2007). Fish age classification based on length, weight, sex and otolith morphological features. *Fisheries Research, 84*(2), 270-274.

Boehlert, G. W. (1985). Using Objective Criteria and Multiple-Regression Models for Age-Determination in Fishes. *Fishery Bulletin, 83*(2), 103-117.

Bostanci, D. (2008). A comparison of calcified structures for aging of pikeperch (*Sander lucioperca*) in Bafra Fish Lake, Turkey. *Journal of Freshwater Ecology, 23*(3), 485-486.

Boughamou, N., Derbal, F., & Kara, M. H. (2014). Otolithometry and Scalimetry-Two Valid Methods to Describe the Growth of Peacock Wrasse, *Symphodus tinca* (Actinopterygii: Perciformes: Labridae) from Eastern Algeria. *Acta Ichthyologica Et Piscatoria*, 44(4), 285-293.

Britton, J. R., Cowx, I. G., & Peirson, G. (2004). Sources of error in the ageing of stocked cyprinids. *Fisheries Management and Ecology, 11*(6), 415-417.

Britton, J. R., Harper, D. M., & Oyugi, D. O. (2010). Is the fast growth of an equatorial *Micropterus salmoides* population explained by high water temperature? *Ecology of Freshwater Fish, 19*(2), 228-238.

Cailliet, G. M., Andrews, A. H., Burton, E. J., Watters, D. L., Kline, D. E., & Ferry-Graham, L. A. (2001). Age determination and validation studies of marine fishes: do deep-dwellers live longer? *Experimental Gerontology, 36*(4-6), 739-764.

Campana, S. E. (2001). Accuracy, precision and quality control in age determination, including a review of the use and abuse of age validation methods. *J Fish Biol, 59*(2), 197-242.

Campana, S. E., & Jones, C. M. (1992). Analysis of otolith microstructure data. *In* D. K. Stevenson and S. E. Campana (eds.), Otolith microstructure examination and analysis. p. 73-100. *Can. Spec. Publ. Fish. Aquat. Sci*. 117.

Campana, S. E., Gagne, J. A., & Munro, J. (1987). Otolith Microstructure of Larval Herring (*Clupea harengus*) - Image or Reality. *Canadian Journal of Fisheries and Aquatic Sciences, 44*(11), 1922-1929.

Campana, S. E., & Neilson, J. D. (1985). Microstructure of Fish Otoliths. Canadian Journal of *Fisheries and Aquatic Sciences,* 42(5), 1014-1032.

Campana, S. E., & Thorrold, S. R. (2001). Otoliths, increments, and elements: keys to a comprehensive understanding of fish populations? *Canadian Journal of Fisheries and Aquatic Sciences, 58*(1), 30-38.

Cardinale, M., & Arrhenius, F. (2004). Using otolith weight to estimate the age of haddock (*Melanogrammus aeglefinus*): a tree model application. *Journal of Applied Ichthyology, 20*(6), 470-475.

Chih, C. P. (2009). The Effects of Otolith Sampling Methods on the Precision of Growth Curves. *North American Journal of Fisheries Management, 29*(6), 1519-1528.

Choat, J. H., Axe, L. M., & Lou, D. C. (1996). Growth and longevity in fishes of the family Scaridae. *Marine Ecology Progress Series*, 145(1-3), 33-41.

Choat, J. H., Davies, C. R., Ackerman, J. L., & Mapstone, B. D. (2006). Age structure and growth in a large teleost, *Cheilinus undulatus*, with a review of size distribution in labrid fishes. *Marine Ecology Progress Series*, 318, 237-246.

Coggins, L. G. Jr., & Pine, W. E. III. (2010). Development of a temperaturedependent growth model for the endangered humpback chub using capture-recapture data. *The Open Fish Science Journal,* 3, 122–131.

Cossington, S., Hesp, S. A., Hall, N. G., & Potter, I. C. (2010). Growth and reproductive biology of the foxfish *Bodianus frenchii*, a very long-lived and monandric protogynous hermaphroditic labrid. *J Fish Biol*, 77(3), 600-626.

Cowen, R. K. (1991). Variation in the Planktonic Larval Duration of the Temperate Wrasse *Semicossyphus pulcher*. *Marine Ecology Progress Series, 69*(1-2), 9-15.

D'Arcy, J. Studies on the biology of the Ballan wrasse, *Labrus bergylta*: Macro- and micro-structure analysis of larval ballan wrasse otoliths, including age validation [dissertation]. Galway: National University of Ireland, Galway; 2013.

D'Arcy, J., Dunaevskaya, E., Treasurer, J. W., Ottesen, O., Maguire, J., Zhuravleva, N., Karlsen, A. Rebours, C., & FitzGerald, R. D. (2012). Embryonic development in ballan wrasse *Labrus bergylta*. *J Fish Biol, 81*(3), 1101-1110.

De Graaf, G., & Prein, M. (2005). Fitting growth with the von Bertalanffy growth function: a comparison of three approaches of multivariate analysis of fish growth in aquaculture experiments. *Aquaculture Research*, 36(1), 100-109.

Dipper, F.A., Bridges, C.R., & Menz, A. (1977). Age, growth and feeding in the ballan wrasse *Labrus bergylta* Ascanius 1767. *J Fish Biol*, 11: 105–120.

Dulcic, J. (1995). Estimation of Age and Growth of Sardine, *Sardina pilchardus* (Walbaum, 1792), Larvae by Reading Daily Otolith Increments. *Fisheries Research, 22*(3-4), 265-277.

Dunaevskaya, E., Amin, A. B., & Ottesen, O.H. (2012). Organogenesis of Ballan Wrasse *Labrus Bergylta* (Ascanius 1767) Larvae. *J Aquacult Res Dev*, 3:142.

Ewing, G. P., Lyle, J. M., Murphy, R. J., Kalish, J. M., & Ziegler, P. E. (2007). Validation of age and growth in a long-lived temperate reef fish using otolith structure, oxytetracycline and bomb radiocarbon methods. *Marine and Freshwater Research, 58*(10), 944-955.

Feltz, C. J. (2002). Customizing generalizations of the Kolmogorov-Smirnov goodness-of-fit test. *Journal of Statistical Computation and Simulation, 72*(2), 179-186.

Fletcher, W. J. (1991). A Test of the Relationship between Otolith Weight and Age for the Pilchard *Sardinops neopilchardus*. *Canadian Journal of Fisheries and Aquatic Sciences, 48*(1), 35-38.

Fletcher, W. J., & Blight, S. J. (1996). Validity of using translucent zones of otoliths to age the pilchard *Sardinops sagax neopilchardus* from Albany, western Australia. *Marine and Freshwater Research, 47*(4), 617-624.

Fossum, P., Kalish, J., & Moksness, E. (2000). Special issue: 2nd International Symposium on Fish Otolith Research & Application, Bergen, Norway, 20-25 June 1998 - Foreword. *Fisheries Research, 46*(1-3), 1-2.

Fowler, A. J., & Doherty, P. J. (1992). Validation of Annual Growth Increments in the Otoliths of 2 Species of Damselfish from the Southern Great-Barrier-Reef. *Australian Journal of Marine and Freshwater Research, 43*(5), 1057-1068.

Francis, R. I. C. C. (1990). Back-Calculation of Fish Length - a Critical-Review. *J Fish Biol, 36*(6), 883-902.

Francis, R. I. C. C., Campana, S. E., & Neil, H. L. (2010). Validation of fish ageing methods should involve bias estimation rather than hypothesis testing: a proposed approach for bomb radiocarbon validations. *Canadian Journal of Fisheries and Aquatic Sciences, 67*(9), 1398-1408.

Geffen, A. J. (1982). Otolith Ring Deposition in Relation to Growth-Rate in Herring (*Clupea harengus*) and Turbot (*Scophthalmus maximus*) Larvae. *Marine Biology, 71*(3), 317-326.

Gordoa, A., Moli, B., & Raventos, N. (2000). Growth performance of four wrasse species on the north-western Mediterranean coast. *Fisheries Research*, 45(1), 43-50.

Gunther, C. C., Temming, A., Baumann, H., Huwer, B., Mollmann, C., Clemmesen, C., & Herrmann, J. P. (2012). A novel length back-calculation approach accounting for ontogenetic changes in the fish length - otolith size relationship during the early life of sprat (*Sprattus sprattus*). *Canadian Journal of Fisheries and Aquatic Sciences, 69*(7), 1214-1229.

Gust, N., Choat, J. H., & Ackerman, J. L. (2002). Demographic plasticity in tropical reef fishes. *Marine Biology*, 140(5), 1039-1051.

Hayashi, A., Dotsu, K., & Ohta, M. (1995). Reliability of Surface Reading Methods to Count Otolith Opaque Zones on Aging in Marbled Rockfish *Sebastiscus marmoratus* Compared with the Cutaway Reading Method. *Nippon Suisan Gakkaishi*, 61(1), 1-5.

He, J. X., & Bence, J. R. (2007). Modeling annual growth variation using a hierarchical Bayesian approach and the von Bertalanffy growth function, with application to lake trout in southern Lake Huron. *Transactions of the American Fisheries Society*, 136(2), 318-330.

He, J. X., Rudstam, L. G., Forney, J. L., VanDeValk, A. J., & Stewart, D. J. (2005). Long-term patterns in growth of Oneida Lake walleye: a multivariate and stage-explicit approach for applying the von Bertalanffy growth function. *J Fish Biol*, 66(5), 1459-1470.

Hernandez-Miranda, E., Veas, R., Espinoza, C. V., Thorrold, S. R., & Ojeda, F. P. (2009). The use of otoliths and larval abundance for studying the spatial ecology of the blenny *Scartichthys viridis* (Valenciennes, 1836) in coastal central Chile. *Revista De Biologia Marina Y Oceanografia, 44*(3), 619-633.

Horn, P. L. (1996). Age and growth of red cod (*Pseudophycis bachus*) off the south-east coast of South Island, New Zealand. *New Zealand Journal of Marine and Freshwater Research*, 30(2), 151-160.

Jones, C. 1992. Development and application ofthe otolith increment technique. *Can. Spec. Publ. Fish. Aquat. Sci*. 117:1-11.

Jones, C. M. (2000). Fitting growth curves to retrospective size-at-age data. *Fisheries Research, 46*(1-3), 123-129.

Jones, M. (2002). The US Fish and Wildlife Service Fisheries Program - A historical summary. *Fisheries, 27*(2), 34-35.

Katsanevakis, S., & Maravelias, C. D. (2008). Modelling fish growth: multi-model inference as a better alternative to a priori using von Bertalanffy equation. *Fish and Fisheries, 9*(2), 178-187.

Kaufmann, K. W. (1981). Fitting and Using Growth-Curves. *Oecologia, 49*(3), 293-299.

Li, L., Hoie, H., Geffen, A. J., Heegaard, E., Skadal, J., & Folkvord, A. (2008). Back-calculation of previous fish size using individually tagged and marked Atlantic cod (*Gadus morhua*). *Canadian Journal of Fisheries and Aquatic Sciences, 65*(11), 2496-2508.

Lin, Y. J., & Tzeng, W. N. (2009). Validation of annulus in otolith and estimation of growth rate for Japanese eel *Anguilla japonica* in tropical southern Taiwan. *Environmental Biology of Fishes*, 84(1), 79-87.

Linde, M., Palmer, M., & Alos, J. (2011). Why protogynous hermaphrodite males are relatively larger than females? Testing growth hypotheses in Mediterranean rainbow wrasse *Coris julis* (Linnaeus, 1758). *Environmental Biology of Fishes*, 92(3), 337-349.

Maceina, M. J. (1992). A Simple Regression-Model to Assess Environmental-Effects on Fish Growth. *J Fish Biol, 41*(4), 557-565.

Maceina, M. J., Boxrucker, J., Buckmeier, D. L., Gangl, R. S., Lucchesi, D. O., Isermann, D. A., Jackson, J. R., & Martinez, P. J. (2007). Current status and review of freshwater fish aging procedures used by state and provisional fisheries agencies with recommendations for future directions (vol 32, pg 329, 2007). *Fisheries, 32*(10), 508-508.

Magnussen, E. (2007). Interpopulation comparison of growth patterns of 14 fish species on Faroe Bank: are all fishes on the bank fast-growing? *J Fish Biol*, 71(2), 453-475.

Manooch, C. S., & Drennon, C. L. (1987). Age and Growth of Yellowtail Snapper and Queen Triggerfish Collected from the United-States-Virgin-Islands and Puerto-Rico. *Fisheries Research, 6*(1), 53-68.

Mason, D. L., & Manooch, C. S. (1985). Age and Growth of Mutton Snapper Along the East Coast of Florida. *Fisheries Research, 3*(2), 93-104.

Meekan, M. G., Ackerman, J. L., & Wellington, G. M. (2001). Demography and age structures of coral reef damselfishes in the tropical eastern Pacific Ocean. *Marine Ecology Progress Series*, 212, 223-232.

Mendiola, D., & Alvarez, P. (2008). Validation of daily increments in the otolith microstructure of Northeast Atlantic mackerel fish larvae. *Fisheries Research, 89*(3), 300-304.

Milton, D. A., Short, S. A., Oneill, M. F., & Blaber, S. J. M. (1995). Aging of 3 Species of Tropical Snapper (Lutjanidae) from the Gulf of Carpentaria, Australia, Using Radiometry and Otolith Ring Counts. *Fishery Bulletin*, 93(1), 103-115.

Morioka, S., & Matsumoto, S. (2007). Otolith development and daily increment formation in larvae of the Kabyabya, a Malawian cyprinid, *Opsaridium tweddleorum*. *Ichthyological Research, 54*(1), 44-48.

Morrongiello, J. R., Walsh, C. T., Gray, C. A., Stocks, J. R., & Crook, D. A. (2014). Environmental change drives long-term recruitment and growth variation in an estuarine fish. *Global Change Biology, 20*(6), 1844-1860.

Mugiya, Y., & Muramatsu, J. (1982). Time-Marking Methods for Scanning Electron-Microscopy in Goldfish Otoliths. *Bulletin of the Japanese Society of Scientific Fisheries, 48*(9), 1225-1232.

Muncaster, S., Andersson, E., Kjesbu, O. S., Taranger, G. L., Skiftesvik, A. B., & Norberg, B. (2010). The reproductive cycle of female Ballan wrasse *Labrus bergylta* in high latitude, temperate waters. *J Fish Biol, 77*(3), 494-511.

Muncaster, S., Andersson, E., Skiftesvik, A. B., Opstad, I., Taranger, G. L., & Norberg, B. (2008). Seasonal reproductive cycle of Ballan wrasse (*Labrus bergylta*) in Norway. *Cybium, 32*(2), 199-199.

Muncaster, S., Norberg, B., & Andersson, E. (2013). Natural sex change in the temperate protogynous Ballan wrasse *Labrus bergylta*. *J Fish Biol, 82*(6), 1858-1870.

Munch, S. B., & Salinas, S. (2009). Latitudinal variation in lifespan within species is explained by the metabolic theory of ecology. *Proceedings of the National Academy of Sciences of the United States of America*, 106(33), 13860-13864.

Muus, B.J. & Nielsen, J. G. (1999). Sea fish. Scandinavian Fishing Year Book. Hedehusene: Denmark, 340 p.

Narimatsu, Y., Hattori, T., Ueda, Y., Matsuzaka, H., & Shiogaki, M. (2007). Somatic growth and otolith microstructure of larval and juvenile Pacific cod *Gadus macrocephalus*. *Fisheries Science, 73*(6), 1257-1264.

Newberger, T. A., & Houde, E. D. (1995). Population Biology of Bay Anchovy *Anchoa mitchilli* in the Mid-Chesapeake Bay. *Marine Ecology Progress Series*, 116(1-3), 25-37.

Nitschke, P., Burnett, J., & Kelly, B. C. (2001). Age and growth verification for cunner in Western Cape Cod Bay, Massachusetts, using tag-recapture data. *Transactions of the American Fisheries Society*, 130(6), 1150-1163.

Ottesen, O.H., Dunaevskaya, E., & D'Arcy, J. D. (2012). Development of *Labrus bergylta* (Ascanius 1767) Larvae from Hatching to Metamorphosis. *J Aquac Res Development*, 3, 127.

Oxenford, H. A., & Hunte, W. (1983). Age and Growth of Dolphin, *Coryphaena hippurus*, as Determined by Growth Rings in Otoliths. *Fishery Bulletin, 81*(4), 906-909.

Panfili, J., & Tomas, J. (2001). Validation of age estimation and back-calculation of fish length based on otolith microstructures in tilapias (Pisces, Cichlidae). *Fishery Bulletin, 99*(1), 139-150.

Pawson, M. G. (1990). Using Otolith Weight to Age Fish. *J Fish Biol, 36*(4), 521-531.

Pilling, G. M., Grandcourt, E. M., & Kirkwood, G. P. (2003). The utility of otolith weight as a predictor of age in the emperor *Lethrinus mahsena* and other tropical fish species. *Fisheries Research, 60*(2-3), 493-506.

Pilling, G. M., Kirkwood, G. P., & Walker, S. G. (2002). An improved method for estimating individual growth variability in fish, and the correlation between von Bertalanffy growth parameters. *Canadian Journal of Fisheries and Aquatic Sciences*, 59(3), 424-432.

Radtke, R. L., & Shafer, D. J. (1992). Environmental Sensitivity of Fish Otolith Microchemistry. *Australian Journal of Marine and Freshwater Research, 43*(5), 935-951.

Rice, J. A., Crowder, L. B., & Binkowski, F. P. (1985). Evaluating Otolith Analysis for Bloater *Coregonus hoyi* - Do Otoliths Ring True. *Transactions of the American Fisheries Society, 114*(4), 532-539.

Ruzicka, J. J., & Radtke, R. L. (1995). Estimating the Age of Antarctic Larval Fish from Otolith Microstructure Using Light and Electron-Microscopy. *Polar Biology, 15*(8), 587-592.

Sallami, B., Bearez, P., & Ben Salem, M. (2013). Allometric relationships between fish length and otolith length for three Anguilliformes in northern coast of Tunisia (Central Mediterranean). *Cybium, 37*(3), 159-163.

Sardenne, F., Dortel, E., Le Croizier, G., Million, J., Labonne, M., Leroy, B., Bodin, N., & Chassot, E. (2015). Determining the age of tropical tunas in the Indian Ocean from otolith microstructures. *Fisheries Research*, 163, 44-57.

Secor, D. H., & Dean, J. M. (1992). Comparison of Otolith-Based Back-Calculation Methods to Determine Individual Growth Histories of Larval Striped Bass, *Morone saxatilis*. *Canadian Journal of Fisheries and Aquatic Sciences, 49*(7), 1439-1454.

Secor, B.W., Dean, J.M., & Laban, E.H. (1992). Otolith Removal and Preparation for Microstructural Examination. *In*: Otolith Microstructure Examination and Analysis, Stevenson, D.K. and S.E. Campana (Eds.). Canadian Special Publication of Fisheries and Aquatic Sciences, Canada, pp: 19-57.

Secor, D. H., Trice, T. M., & Hornick, H. T. (1995). Validation of Otolith-Based Aging and a Comparison of Otolith and Scale-Based Aging in Mark-Recaptured Chesapeake Bay Striped Bass, *Morone saxatilis*. *Fishery Bulletin, 93*(1), 186-190.

Sideleva, V. G., & Zubina, L. V. (1990). Peculiar Structure of Otoliths in Ecologically Different Species of Baikal Sculpins (Cottoidei) .2. The Otolith Structure in Connection with the Ecology and Phylogenetic Relations of the Species. *Zoologichesky Zhurnal, 69*(10), 77-85.

Simard, P., Castonguay, M., Damours, D., & Magnan, P. (1992). Growth Comparison between Juvenile Atlantic Mackerel (*Scomber scombrus*) from the 2 Spawning Groups of the Northwest Atlantic. *Canadian Journal of Fisheries and Aquatic Sciences, 49*(11), 2242-2248.

Skeljo, F., Ferri, J., Brcic, J., Petric, M., & Jardas, I. (2012). Age, growth and utility of otolith morphometrics as a predictor of age in the wrasse *Coris julis* (Labridae) from the eastern Adriatic Sea. *Scientia Marina, 76*(3), 587-595.

Skeljo, F., Ferri, J., Brcic, J., & Vuletina, V. (2015). Age and growth of the axillary wrasse, *Symphodus mediterraneus* (L.) from the eastern Adriatic Sea. *Marine Biology Research*, 11(7), 780-784.

Stephenson, P. C., & Hall, N. G. (2003). Quantitative determination of the timing of otolith ring formation from marginal increments in four marine teleost species from northwestern Australia. *Fishery Bulletin*, 101(4), 900-909.

van der Meeren, T., & Moksness, E. (2003). Growth and mortality patterns evaluated from otolith microstructure in Atlantic cod *Gadus morhua* larvae reared on different feeding regimes in mesocosms. *Marine Ecology Progress Series, 264*, 95-107.

Victor, B. C. (1982). Daily Otolith Increments and Recruitment in 2 Coral-Reef Wrasses, *Thalassoma bifasciatum* and *Halichoeres bivittatus*. *Marine Biology, 71*(2), 203-208.

Villegas-Rios, D., Alonso-Fernandez, A., Fabeiro, M., Banon, R., & Saborido-Rey, F. (2013). Demographic variation between colour patterns in a temperate protogynous hermaphrodite, the ballan wrasse *Labrus bergylta*. *PLoS One, 8*(8), e71591.

Walker, S. P. W., & McCormick, M. I. (2004). Otolith-check formation and accelerated growth associated with sex change in an annual protogynous tropical fish. *Marine Ecology Progress Series*, 266, 201-212.

Welsford, D. C. (2003). Interpretation of otolith microstructure in the early life history stages of two temperate reef wrasses (Labridae). *Marine and Freshwater Research, 54*(1), 69-75.

Wetherington, J. D., Weeks, S. C., Kotora, K. E., & Vrijenhoek, R. C. (1989). Genotypic and Environmental Components of Variation in Growth and Reproduction of Fish Hemiclones (Poeciliopsis, Poeciliidae). *Evolution, 43*(3), 635-645.

Worthington, D. G., Doherty, P. J., & Fowler, A. J. (1995). Variation in the Relationship between Otolith Weight and Age - Implications for the Estimation of Age of 2 Tropical Damselfish (*Pomacentrus moluccensis* and *Pomacentrus wardi*). *Canadian Journal of Fisheries and Aquatic Sciences, 52*(2), 233-242.

Worthington, D. G., Fowler, A. J., & Doherty, P. J. (1995). Determining the most efficient method of age determination for estimating the age structure of a fish population. *Canadian Journal of Fisheries and Aquatic Sciences, 52*(11), 2320-2326.

Yilmaz, S., Yazicioglu, O., Saygin, S., & Polat, N. (2014). Relationships of Otolith Dimensions with Body Length of European Perch, *Perca fluviatilis* L., 1758 From Lake Ladik, Turkey. *Pakistan Journal of Zoology, 46*(5), 1231-1238.

Zhang, Z., & Runham, N. W. (1992). Otolith Microstructure Pattern in *Oreochromis niloticus* (L). *J Fish Biol, 40*(3), 325-332.

Chapter six
General Discussion and Future Research

The field of physiology and research on reproduction of *Labrus bergylta* has experienced phenomenal growth and now forms the basis for numerous studies on early life history, reproduction, growth and ageing. This thesis represents the first effort to compile and summarize many procedures and analyses associated with basic and applied research areas on the biology and life history of *L. bergylta*. The basic regulation of sex hormones and gonadal development in a reproductive cycle (Chapter two) has revealed the first insight into the status of wild populations of *L. bergylta* in Galway, west Ireland, with implications for reproductive development. In Chapter three, applied research is stressed on the hormonal manipulation of *L. bergylta* for gonad maturation and egg production in captive stocks by formation of breeding groups or territories. The establishment of spawning induction protocol and the nature of the species in terms of sex hormone levels and spawning has been described. This has implications for hatchery production protocols and fishery assessments. In fact, the result of this study is the first report on manipulated and artificial breeding of *L. bergylta* in Ireland. In Chapter four, larvae from induced spawning *L. bergylta* were used for diet modifications in co-feeding of dry diets with live prey to determine their effect on growth performance. The growth comparison on diet adaptation was successfully proven until 112 days post hatch (dph) which could be prolonged but limit on time constraints. The feeding regime could also be a module to hatchery up to early juveniles. Finally, ageing and growth validation also has been described in the analysis of larval, juveniles and adults otolith from micro to macro-structure in Chapter five. It has resulted in a confirmation of the number of daily and yearly increments which described strong relationships between fish size and age. The prediction of age has proven the rate of growth through growth trajectory of *L. bergylta* from sagittal otolith sampled. The age structure of *L. bergylta* in Carna, west Ireland, was determined using sagittal otolith. This is the first documented *L. bergylta* age structure determination in this region. The information gathered in this thesis will hopefully aid hatchery operations, as well as fishery management and aquaculture policy especially for *L. bergylta* production.

The pattern of reproductive parameters including glucose, total protein, albumin and globulin levels in a cycle of wild *L. bergylta* has been documented. In a recent study, the concentrations of the androgen testosterone were much lower than estradiol (E_2). This is probably because most of the individuals were females and this reflects the promotional effects of E_2 on ovary development. The gonadal changes were accompanied by characteristic fluctuations in plasma sex steroid concentrations, especially E_2, testosterone (T) and progesterone with increase in oocyte diameters and GSI which peaked in April 2010 and 2011. Concentrations of E_2 decreased sharply in May and June during atretic gonads when both female and male fish were in a post-spawned state (Muncaster *et al*., 2010) and remained low thereafter. This appears to be a key event in the initiation of gonad restructure together with HSI which helped in yolk protein synthesis as in other protogynous fishes. Gonadal restructure in *L. bergylta* is characterized by oocyte degeneration and ovarian apoptosis. In view of the evidence for the role of E_2 in driving sex change, however, it may be that post-spawned fish have low enough concentrations of circulating E_2 to allow for the progression of gonadal inversion at the physiological level. It is highly likely that behaviour and size are important mediators in this process. Although there was an overlap of sizes between male and female fish, the majority of female fish were smaller than males. There are, however, strong indications that both size and season contribute towards the timing of sex change in this species.

The coincidence of transitional fishes post-spawning is a common seasonal pattern in many protogynous hermaphrodites (Jones, 1980; Shapiro, 1989; Bhandari *et al*., 2003a; Bhandari *et al*., 2003b; Kokokiris *et al*., 2006). Shapiro (1989) proposed that the post-spawning sex change of socially inducible sex-changing species is primarily an artefact of environmental alterations such as increased adult mortality and juvenile recruitment associated with season. This could explain the existence of the sex-changing individuals present in June, July, August and September; these data are not published in other studies of *L. bergylta*. Sex change occurred almost exclusively at the end of the breeding season and most often in fish between 34 and 41 cm in total length (Muncaster *et al*., 2013). It is, therefore, best to conduct manipulative experiments such as hormonal induction (androgen) to induce sex change during the post-spawning period when

steroid levels are low and cease to be a confounding factor. The manipulation of sex ratios to maintain breeding groups or the administration of interventions such as aromatase inhibitors could be done in order to produce males.

A combination of LHRHa, hCG and Ovaprim® had been designed to resemble the hypothalamo-pituitary-gonad axis of reproductive steroids regulation. A successful spawning induction of *L. bergylta* had been reported for the first time in this species in both trials showing that hCG is a potent spawning inducer. It is not possible to accurately quantify the baseline steroid levels as this process has intervened stress during handling. Captivity in tanks irrespective of either wild or captive fish would be a stressor as well. Cortisol remained higher before the injection until the second injection of spawning trial. Basal plasma cortisol levels were high in wild fish when compared to the captive fish. However, cortisol response to post spawning was relatively low (3.7 fold decrease). The positive spawning results from the first and second trials of the wild fish on hormonal induction were very similar, suggesting that hormonal manipulation of hCG was the potent spawning inducer and had no adverse effect on egg viability. Unfortunately, the captive fish in the hormonal induction trial in 2011 were not responsive. It is recommend that further monitoring of the broodstock nutrition and rearing condition will give a positive impact on fish health and spawning. The artificial fertilisation (stripping) of *L. bergylta* was conducted using eggs and milt from adults. The progress of the eggs was monitored with a brief practical description of embryonic development from fertilisation to hatch. Chapter four provide a valuable assessment of *L. bergylta* larval growth.

It is well known that different diets may result in highly different somatic growth rates between fish larvae of the same genetic origin (Claramunt and Wahl, 2000). Using *Artemia sp.* as early live feed for the *L. bergylta* larvae resulted in increased larval growth and survival while in certain extend co-feeding with micropellet (Diet A) and dried polychaeta (Diet B) were supplied through the diet as the *Artemia sp.* did not have sufficient dietary quality necessary to obtain optimal growth up to 112 dph. There were no negative growth of both Spawning batch I (SBI) and Spawning batch II (SBII) larvae observed after the dietary switches from *Artemia sp.* to polychaeta and micropellet, strongly suggesting that *L. bergylta* larvae have no problems accepting a different dried

feed, after being introduced to either polychaeta or micropellet. The larvae from different co-feeding treatments had no body deformities of newly-hatched fish larvae in Atlantic cod (*Gadus morhua*) such as axial (lordosis or kyphosis) or lateral (scoliosis) curvature of the vertebrae in the abdominal or caudal region (Kjorsvik *et al*., 2009). Darcy *et al*. (2012) stated that severe malformations of *L. bergylta* larvae tended to increase with increasing temperature and 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 were observed.

Overall, the results of this study show that co-feeding with dried polychaeta at the beginning of 49 days after hatching clearly improved the growth and quality of *L. bergylta* larvae in terms of dry weight (DW) and wet weight (WW) at the end of the experimental period when compared to feeding with micropellet. However, there were no significant differences in standard length (SL) and myotome height (MH). The increased growth of larvae from the polychaeta treatment is likely due to spawning batch rather than diet. The effect of spawning batch on larvae of *L. bergylta* may be different under various temperatures. SBII had significantly better growth compared to SBI in all growth parameters on both Diet A and Diet B. This was observed when dried polychaeta were supplied only for a short period of time (49 to 112 dph) at the end of exogenous feeding, and when fed exclusively with the formulated feeds (91 to 112 dph). SL and MH increased in growth rate post 91 dph (exclusively polychaetas and micropellet). Specific growth rates (SGRs) of WW showed significantly different growth between Diet A and B in SBII larvae. Higher SGRs were observed when dried polychaeta were used as larval feed while micropellet was the least suitable first feed affecting the larval growth. Further studies are needed to clarify the combined effect of temperature in order to determine the optimal temperature range for embryo and larval development of *L. bergylta*. It can be concluded that polychaeta has nutritional composition more suited as early mixed feed for the rearing of *L. bergylta* larvae resulting in increased larval somatic growth compared to micropellet. This was expected because the texture and taste of polychaeta make them natural prey organisms for marine fish.

There is a chance by prolonging the co-feeding periods, gradually increasing the fraction of the new dried feed organism. In future studies, regimes with longer periods of polychaeta feeding should be investigated to determine how far into the larval development, polychaeta would be the superior dried feed organism. However, considering the nutritional benefits of dried polychaeta, the future experiments should test diets solely consisting of polychaeta; and how the size of polychaeta can be adjusted to larval size to find a way to reduce the negative effect on growth observed when switching from dried polychaeta to another bigger size dried feed. Dried polychaeta were found to have the following advantages over live *Artemia sp.* and mixed dry micropellets as the polychaeta last longer by floating in seawater and require less time to fall to the bottom. At present, the availability of dried polychaeta is not adequate to supply the amount that would be needed for a large scale intensive cultivation of *L. bergylta*. There is need for a large scale production of intensively-cultured polychaeta, where the harvested worms can be stored and shipped off to commercial buyers. The farmer could then process the needed amount in a similar manner that is common for the production of dried feed today. However, improvements in polychaeta culturing techniques must be made more efficient before this can be commercially viable. Preferably, freeze-dried polychaeta could be easily ordered and quickly shipped to the farmers as with *Artemia sp.* cysts today. The farmers can then store the larvae and feed them when the required size is achieved. The production need not be any more time consuming complicated than that of *Artemia sp.* or micropellets but will give larvae of a higher growth quality. The result of this study can be useful in improving the production of this species through incubation and larval culture.

A practical guide for ageing *L. bergylta* from Carna, west Ireland is to provide current otolith sampling, processing and validating methodologies and techniques which generally describe specifically for *L. bergylta*. This manual should serve as a valuable training tool for new laboratory personnel and used as a guide for ageing *L. bergylta*. I believe that this manual will facilitate the adoption of these techniques and standards for this and other similar species beyond the Ireland region. This study produced the first estimates of the age-based growth pattern parameters among larvae, juveniles and adults using whole sagittal otoliths with the polishing method and revealed striking growth

characteristics of the species. This is one of the most rapid and widespread methods for fish age determination (Campana and Thorrold, 2001). The whole otoliths under reflected light showed a clear internal structure of alternating wide translucent and narrow opaque increments. The result was confirmed by the presence of a single yearly and marginal increment analysis. The analysis confirmed that *L. bergylta* produced 1 annulus per year on the otoliths. The maximum percentage of fish with opaque margins found in winter (slow growth period) corresponds to the coldest water temperatures in the study area. The observed maximum age in Carna in this study is 30 years, more or less similar than previously reported (29 years) and represents a modest longevity within the family Labridae (Villegas-Rios *et al*., 2013). Variations to this could be related to different hard structures used for ageing (otoliths vs. scales vs. opercular bones) or sampling bias.

This ageing methodology first began with a validation of the number of micro-increments that were deposited daily. This technique, which was first described by Pannella (1971 and 1974), has become a keystone feature of larval ecology because it allows the estimation of larval age in days due to the daily formation of micro-increments in otoliths. The reason for validating this daily occurrence is because of instances of non-daily micro-incremental deposition and variability in the timing of initial ring deposition some species. Thus, micro-incremental counts of the sagittal otoliths from laboratory-reared larvae were undertaken as a means of validating the number of rings deposited daily. All counts began from the "hatch-check" which was clearly visible in most sagittae used in the study. The findings indicate that a single ring was deposited daily. The average core diameter (diameter of hatch-check) was 19.5±1.3µm. The availability of known-age larvae presented an opportunity to improve age estimates of wild-caught larvae.

Simultaneous to the age validation, the relationship between the micro and macrostructure of the larval otoliths was examined with regard to the larval, juvenile and adult weight, length and age. These descriptors, in conjunction with the validated daily and yearly increment deposition, provided an insight into the profile and nature of *L. bergylta* otoliths and form the basis for Chapter five of this thesis. It is hoped that this information will be useful to future fisheries studies (Campana and Thorrold, 2001;

Long and Stewart, 2010; Ramsat *et al*., 2011). The relationship between otolith weight and fish age and between otolith length and fish length showed that individuals accreted calcium carbonate in the otoliths throughout their lifetime. The relationship between otolith (sagittal) diameters or lengths and the standard length and age of *L. bergylta* was examined. Best subset regression analysis suggests that otolith length, by itself, was the best predictor of age. The use of the Statistical Analysis System (SAS) and the von Bertalanffy growth function (VBGF) using the FAO-ICLARM Stock Assessment Tool (FISAT) package has enabled the growth rates of *L. bergylta* to be described using the robust relationship between fish length and otolith length. This has enabled the back calculation of fish body size at younger ages in individual growth studies. The estimated ultimate length and growth rates for *L. bergylta* sampled using this program was $L\infty$ = 42.48±0.884 cm and k = 0.16±0.013 per annum in this study, relatively low for k values and high for $L\infty$ values, typical of long-lived tropical fishes.

Conclusions, recommendations and future perspectives

Although the broad-scale approach taken in this study focused on different aspects of the life history of *L. bergylta*, links have been made between the subject areas, using well-established laboratory techniques. It has provided a deeper insight into the life history of *L. bergylta*. The strength of the recent study is on the hatcheries to improve production to meet growing demand. The inclusion of the co-feeding trials and the larval growth studies in Chapter four should make a valuable contribution to future hatchery protocol development and optimization. The addition of the data on otolith macro- and microstructure from larvae, juveniles and adults, including the age validation verification and growth model in Chapter five, as derived from the availability of known-age larvae, should prove very useful to fisheries biologists when the decision is made to undertake a more detailed study of wild *L. bergylta*. The outcomes of this study have highlighted a number of points that should be considered in future studies. For example, Chapters two, three and four outlined the basic reproductive framework of *L. bergylta* production. Efforts should to be continued to establish and identify critical aspects of captive spawning, egg incubation and larval development systems, in order to develop optimal hatchery techniques. Fisheries managers should

consider a more detailed examination of the reproductive status in relation to the spawning success of either wild or captive broodstocks in order to achieve synchronization and completion of final maturation via hormonal induction like hCG.

In future research, several knowledge gaps need to be filled to improve the culture and development of *L. bergylta*. Four important research areas have been identified: (i) broodstock nutrition and rearing conditions, (ii) spawning and egg development, (iii) larval nutrition, growth and rearing conditions and (iv) use of *L. bergylta* in salmon cages. Broodstock nutrition was not examined here but it should cover the effects of easily digestible protein sources on feed intake in relation to growth and intestinal health. Further studies should look into the requirements of arachidonic acid (ARA), docosahexaenoic acid (DHA), iodine, vitamin A, D and K, zinc, bone minerals and 2-aminoethanesulfonic acid (taurine). High protein intake is a very important component for the development and maturing eggs and spawning. In addition, rearing conditions have to be designed in order to provide optimal shelters as which closely imitate the natural habitats. In addition, a better understanding of the sex change process and how to control it in captive populations will probably be valuable to the growing aquaculture industry. Further development of anti-fouling materials as part of the rearing facility should be included. Practical rearing system improvements in tank design and water treatment with regard to *L. bergylta* health, growth and survival need to be studied and developed.

Spawning and egg developments are critical areas in reproduction which need technical improvements. Even though eggs incubated in a recent study did not have any contamination and hatching problem, further development of procedures for removal of the gelatinous layer on the egg surface and egg disinfection methodology may be beneficial to ease the handling and incubation of the eggs. In preservation of the gametes, further development of procedures for short and long term storage of sperm can be realized in order to secure availability of good quality sperm especially when there is a limited sample of male *L. bergylta*. In hormonal induction study of *L. bergylta*, further development of procedures for synchronization and completion of final maturation need to be investigated to pin-point the perfect timing of spawning with different dosage of hCG or Ovaprim®.

Larval nutrition, growth and rearing conditions are known to be one of the core subjects in *L. bergylta* production. Further development of culture and enrichment protocols for rotifers and feeding strategies for larvae to supply all nutrients at adequate levels is needed. In the recent study, co-feeding with polychaeta is able to initiate growth better than the commercial micropellet. Overall, there is potential for increasing growth rates through improved diets and rearing techniques, particularly during the larval stage. Thus, test of cultivated polychaeta and formulated as start feeding in commercial hatcheries would give better growth and survival. In addition, further improvement of water quality during start feeding need to be done in order to avoid any water-borne infection within the rearing tanks. Finally, the reared and graded *L. bergylta* need to be tested in salmon cages compared with wild *L. bergylta* as cleaner fish. In the rearing facility, the use of different hides or territories has to be designed and in different types and densities. Observations have to be made via video monitoring of behavior in order to look on delousing efficiency of *L. bergylta* in cages with large salmon. Nevertheless, the optimal size relationship between *L. bergylta* and salmon for efficient delousing has to be investigated. The impact of temperature (high, low and temperature changes) on delousing efficiency of *L. bergylta* should be investigated as to provide the optimum environment for sea lice control and not to forget the cost benefits of delousing methods has to be ruled out for better practice in terms of economical wise. Finally, integrating this knowledge may help to improve the *L. bergylta* culture and also to provide further information on the species which may be useful for developing new management strategies which are continually challenged by over exploitation for sea lice control in salmon cages.

References

Bhandari, R. K., Higa, M., Komuro, H., Nakamura, S., & Nakamura, M. (2003). Treatment with an aromatase inhibitor induces complete sex change in the protogynous honeycomb grouper (*Epinephelus merra*). *Fish Physiology and Biochemistry, 28*(1-4), 141-142.

Bhandari, R. K., Komuro, H., Nakamura, S., Higa, M., & Nakamura, M. (2003). Gonadal restructuring and correlative steroid hormone profiles during natural sex change in protogynous honeycomb grouper (*Epinephelus merra*). *Zoological Science, 20*(11), 1399-1404.

Campana, S. E., & Thorrold, S. R. (2001). Otoliths, increments, and elements: keys to a comprehensive understanding of fish populations? *Canadian Journal of Fisheries and Aquatic Sciences, 58*(1), 30-38.

Claramunt, R. M., & Wahl, D. H. (2000). The effects of abiotic and biotic factors in determining larval fish growth rates: A comparison across species and reservoirs. *Transactions of the American Fisheries Society, 129*(3), 835-851.

D'Arcy, J., Dunaevskaya, E., Treasurer, J. W., Ottesen, O., Maguire, J., Zhuravleva, N., Karlsen, A.,Rebours, C., & FitzGerald, R. D. (2012). Embryonic development in ballan wrasse *Labrus bergylta*. *J Fish Biol, 81*(3), 1101-1110.

Jones, G. P. (1980). Growth and Reproduction in the Protogynous Hermaphrodite *Pseudolabrus celidotus* (Pisces, Labridae) in New-Zealand. *Copeia*(4), 660-675.

Kjorsvik, E., Olsen, C., Wold, P. A., Hoehne-Reitan, K., Cahu, C. L., Rainuzzo, J., Olsen, A. I., Oie, G., &Olsen, Y. (2009). Comparison of dietary phospholipids and neutral lipids on skeletal development and fatty acid composition in Atlantic cod (*Gadus morhua*). Aquaculture, 294(3-4), 246-255.

Kokokiris, L., Fostier, A., Athanassopoulou, F., Petridis, D., & Kentouri, M. (2006). Gonadal changes and blood sex steroids levels during natural sex inversion in the protogynous Mediterranean red porgy, *Pagrus pagrus* (Teleostei : Sparidae). *Gen Comp Endocrinol, 149*(1), 42-48.

Long, J. M., & Stewart, D. R. (2010). Verification of Otolith Identity Used by Fisheries Scientists for Aging Channel Catfish. *Transactions of the American Fisheries Society, 139*(6), 1775-1779.

Muncaster, S., Andersson, E., Kjesbu, O. S., Taranger, G. L., Skiftesvik, A. B., & Norberg, B. (2010). The reproductive cycle of female Ballan wrasse *Labrus bergylta* in high latitude, temperate waters. *J Fish Biol, 77*(3), 494-511.

Muncaster, S., Norberg, B., & Andersson, E. (2013). Natural sex change in the temperate protogynous Ballan wrasse *Labrus bergylta*. *J Fish Biol, 82*(6), 1858-1870.

Pannella, G. (1971). Fish otoliths: daily growth layers and periodical patterns. *Science* (Wash., D.C.) 173:1124-1127.

Pannella, G . (1974). Otolith growth patterns: An aid in age determination in temperate and tropical fishes. *In*: T. B. Bagenal (editor). The ageing of fish, p. 28-39. Unwin Brothers, La., Surrey.

Ramsay, A. L., Milner, N. J., Hughes, R. N., & McCarthy, I. D. (2011). Comparison of the performance of scale and otolith microchemistry as fisheries research tools in a small upland catchment. *Canadian Journal of Fisheries and Aquatic Sciences, 68*(5), 823-833.

Shapiro, D. Y. (1989). Inapplicability of the Size-Advantage Model to Coral-Reef Fishes. *Trends in Ecology & Evolution, 4*(9), 272-272.

Villegas-Rios, D., Alonso-Fernandez, A., Fabeiro, M., Banon, R., & Saborido-Rey, F. (2013). Demographic variation between colour patterns in a temperate protogynous hermaphrodite, the ballan wrasse *Labrus bergylta*. *PLoS One, 8*(8), e71591.

Appendices

Appendix 1 (Chapter two)

Heidenhain's AZAN

Materials for general staining of cells:

Acidified water		**Aniline - alcohol**	
Glacial acetic acid	5ml	Ethanol, 70%	400ml
Water	995ml	Aniline	4ml
Acetic – alcohol		**Phosphotungstic acid, 5%**	
95% alcohol	200ml	Phosphotungstic acid	10g
Glacial acetic acid	2ml	Water	200ml
Azocarmine solution		**Heidenhain's blue – orange (stock)**	
Azocarmine G (C.I. 50085)	2g	Aniline blue (C.I. 42755)	1g
Water	200ml	Orange G (C.I. 16230)	4g
Glacial acetic acid	2ml	Water	200ml
		Glacial acetic acid	16ml

Heidenhain's blue – orange	
Stock	10ml
Water	20ml

Protocol:		Time
1.	Deparaffinise and bring to water	
2.	Azocarmine G – 60oC	20 minutes
3.	Aniline – alcohol	3 seconds
4.	Acid – alcohol	30 seconds
5.	Phosphotungstic acid	30 minutes
6.	Phosphotungstic acid	30 minutes
7.	Phosphotungstic acid	30 minutes
8.	Acidified water	3 minutes
9.	Aniline blue – Orange G working solution	20 minutes
10.	3x 96% alcohol	3 minutes
11.	2x 100% alcohol	3 minutes
12.	Clear and mount in DPX	

Results:
Nuclei and erythrocytes bright red, cytoplasm orange-red. Collagen, reticular fibres, basement membrane blue.

Reference: Kiernan (2008)

Appendix 2 (Chapter three)

Standard calculation of hormonal dilution for LHRHa, hCG and injection for Ovaprim®

a) LHRHa (1mg [1,000µg])

Recommended dosage: 60µg/kg

Desired volume of injection: 0.1ml (0.1cc)

Fish weight: 500g

Hormone concentration given to 500g body weight fish:

60µg/kg x 0.5kg / 0.1ml = 300µg/ml

Required volume of liquid for dilution:

1,000µg / 300µg/ml = 3.333ml

b) hCG (10,000IU)

Recommended dosage: 500IU/kg

Desired volume of injection: 0.2ml (0.2cc)

Fish weight: 1000g

Hormone concentration given to 1000g body weight fish:

500IU/kg/1kg/0.2ml = 2,500IU/ml

Required volume of liquid for dilution:

10,000IU / 2,500IU/ml = 4ml

c) Ovaprim® (0.5ml/kg)

Fish weight: 600g

Required volume of injection: 0.5ml/kg x 0.6kg = 0.3ml

Appendix 3 (Chapter three)

Summary of hormonal induction:

Material
LHRHa, hCG, Ovaprim®, saline (0.9%), ms222, 1.0ml syringes (hormone), 5.0ml syringes (blood), glass plate with grid layout (egg counter), 100ml glass beaker, weighing scale, camera, dissecting microscope with camera on top and incubation facilities.

Method
[fish must be anaesthetized first before sampling bloods and stripping eggs by using ms222]

Day 1
15.00 to 19.00 : 1st blood pre-treatment (1ml each) and
1st Treatment (LHRHa/hCG/Ovaprim®/placebo)
Day 2
15.00 to 19.00 : next 24h for 2nd treatment @ stripping @ bloods

Day 3
15.00 to 19.00: next 24h for 3rd treatment @ stripping @ bloods

Or

Method

A) Treatment Tanks : Tank 1 (8 fish) and Tank 2 (8 fish)

- First line : LHRHa
  Date : Monday @ 16/5/2011

- Second line : hCG
  Date : Tuesday @ 17/5/2011

- Third line : Ovaprim®
  Date : Wednesday @ 18/5/2011

B) Control Tanks : Tank 3 (8 fish) and Tank 4 (8 fish)

- Saline : Monday @ 16/5/2011
  : Tuesday @ 17/5/2011
  : Wednesday @ 18/5/2011

Appendix 4 (Chapter three)

List of spawnings Trial 1

Day	Egg volume (ml)	Incubator tag no.	Temperature (°C)
Saturday	25	1	13.0
Saturday	1	*2* (control)	12.9
Saturday	20	3	12.9
Saturday	20	4	13.1
Sunday	15	5	13.1
Saturday	15	6	13.2
Saturday	10	7	12.8
Saturday	25	8	12.8
Sunday	8	9	13.0
Saturday	8	10	12.9
Saturday	12	11	12.9
Saturday	8	12	13.0
Saturday	5	13	13.0
Saturday	8	14	12.9

1st egg batch record: Fertilized on 30th April 2011 and hatched on 6th May 2011

List of spawnings Trial 2

A) Treatment Tanks : Tank 1
: 3 fish spawned (total volume = 50ml of viable eggs) on post hCG @ Wednesday @ 18/5/2011.
: 2 fish spawned (total volume = 5ml of mixed egg maturation) on post Ovaprim® @ Thursday @ 19/5/2011.

: Tank 2
: 5 fish spawned (total volume = 90ml of viable eggs) on post hCG @ Wednesday @ 18/5/2011.
: 3 fish spawned (total volume = 30ml [20ml of whitish immature eggs + 10ml viable eggs] of mixed egg maturation) on post Ovaprim® @ Thursday @ 19/5/2011.

B) Control Tanks : Tank 3 and 4
: none of the fish spawn in both tanks.

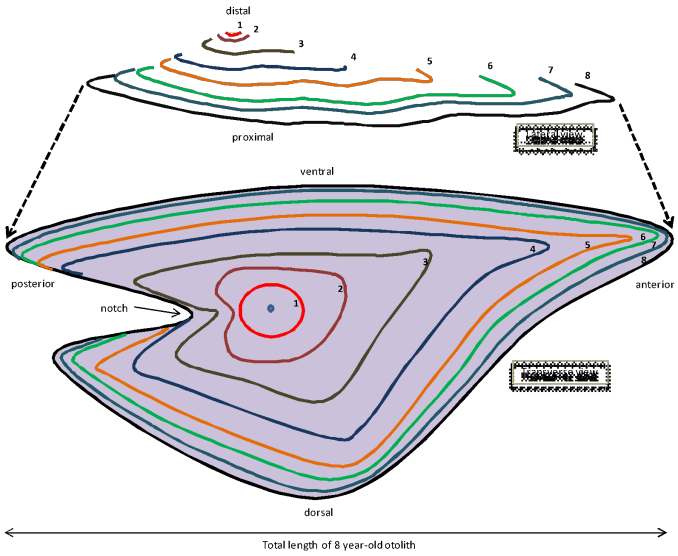
2nd egg batch record: Fertilized on 18th May 2011 and hatched on 24rd May 2011

Appendix 5 (Chapter four)

Feeding trial results from 0-112 dph comparing overall means of Diet A (n=136) and Diet B (n=136) in spawning batch I and Diet A (n=182) and Diet B (n=182) in spawning batch II of *Labrus bergylta*. Specific growth rate (SGR) is calculated for each growth parameter and expressed as a percentage of body mass increment per day. Standard length, myotome height, dry weight and wet weight are displayed in terms of mean, standard deviation (SD), standard error (SE), coefficient of variation (CV), minimum and maximum values.

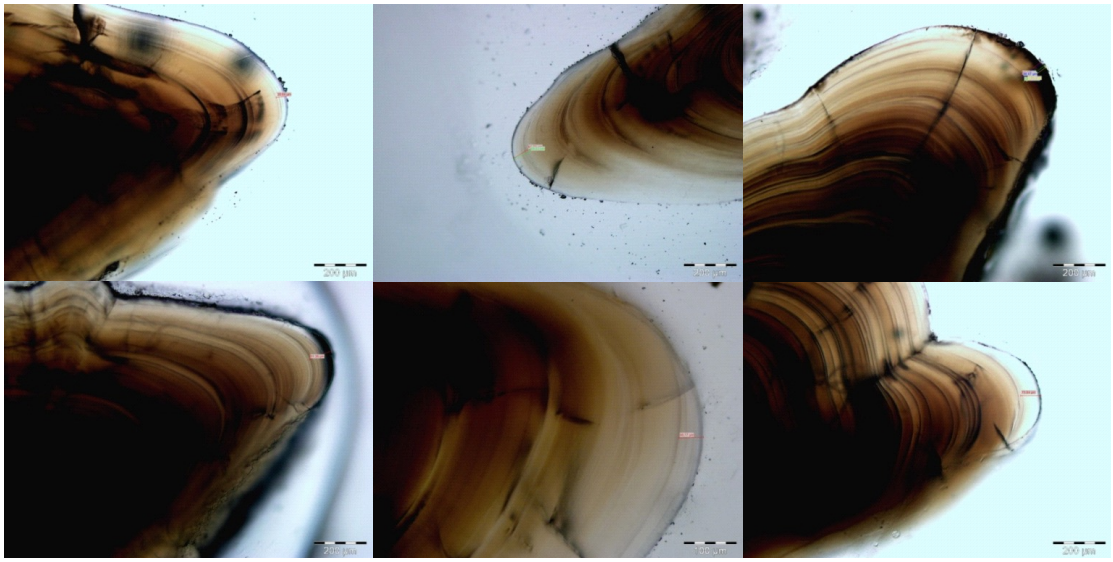
Variable	Spawning batch	Diet	Mean	SD	SE	CV	Min	Max	F	R^2	Result on diets (P)	Overall SGR ± S.E (%)	Result (SGR) on diets (P)
Standard length (mm)	I	A	10.522	7.141	0.612	0.679	4.20	34.00	0.43	0.96	0.52	1.575±0.636	IA = IB
		B	11.129	8.176	0.701	0.735	4.10	36.50		0.96		1.666±0.469	
	II	A	11.846	9.434	0.699	0.796	3.40	40.40	0.91	0.98	0.34	1.473±0.240	IIA = IIB
		B	12.842	10.464	0.776	0.815	3.40	41.10		0.98		1.451±0.175	
Myotome height (mm)	I	A	2.085	2.128	0.182	1.021	0.20	9.80	1.02	0.97	0.31	1.818±0.625	IA = IB
		B	2.377	2.630	0.226	1.106	0.20	11.00		0.96		2.062±0.633	
	II	A	2.524	2.832	0.210	1.122	0.20	11.20	1.27	0.97	0.26	1.713±0.321	IIA = IIB
		B	2.881	3.196	0.237	1.110	0.20	11.50		0.97		1.721±0.224	
Dry weight (g)	I	A	0.007	0.012	0.001	1.676	-0.0003	0.0555	2.05	0.91	0.15	3.507±0.736	IA = IB
		B	0.009	0.017	0.001	1.787	-0.0004	0.0784		0.93		3.554±0.542	
	II	A	0.012	0.018	0.001	1.457	-0.0005	0.0738	2.40	0.99	0.12	3.111±0.750	IIA = IIB
		B	0.016	0.024	0.002	1.501	-0.0004	0.0884		0.99		3.113±0.814	
Wet weight (g)	I	A	0.237	0.231	0.040	0.973	0.0235	0.9422	2.00	0.99	0.16	5.267±0.472	IA = IB
		B	0.331	0.301	0.052	0.909	0.0408	1.0578		0.99		5.404±0.497	
	II	A	0.333	0.267	0.032	0.803	0.0373	0.9400	6.88	0.93	0.01	4.769±2.463	IIA < IIB
		B	0.493	0.434	0.052	0.881	0.0487	1.8930		0.95		5.256±2.239	

Appendix 6 (Chapter five)



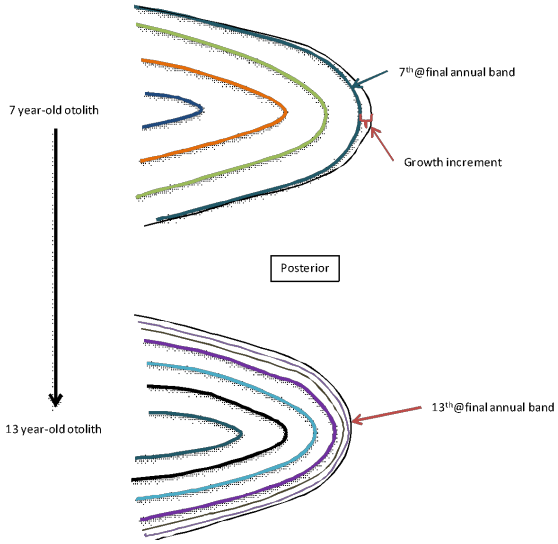
An illustration of lateral and transverse view of different coloured contour lines and the respected number of the annual rings which encircled an otolith representing of 8 years old *Labrus bergylta.*

Appendix 7 (Chapter five)



Growth increments measured from the inside edge of the last opaque band and the edge of the otoliths at the tip of anterior apex. Scale bar = 200 µm.

Appendix 8 (Chapter five)



Differences of observable growth increment between 7 and 13-year old otolith.

Appendix 9 (Chapter five)

Correlation matrices (Pearson correlation) for fish weight, fish length, otolith weight, otolith length and number of annual rings of *Labrus bergylta.*

	Fish weight	Fish length	Otolith weight	Otolith length	Number of annual rings
Fish weight	1	0.921**	0.851**	0.815**	0.786**
Fish length	0.921**	1	0.857**	0.913**	0.798**
Otolith weight	0.851**	0.857**	1	0.876**	0.901**
Otolith length	0.815**	0.913**	0.876**	1	0.797**
Number of annual ring	0.786**	0.798**	0.901**	0.797**	1

**($p<0.01$) highly significant