Analysis of size and shape in early larval stages of European seabass (*Dicentrarchus labrax*): effects of egg disinfection, axenity and bacterial load

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Thesis submitted in fulfilment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences

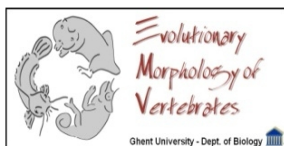

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Illustration on the cover page: photo of a European seabass larva of day after hatching 5 by Spyros Nikolakakis

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"It is a good life. Though you will not know how good
until you come to the end.
The destination cannot be described;
you will know very little until you get there;
you will journey blind.
But the way leads towards possession
of what you had sought for in the wrong place".

T. S. Eliot, The Cocktail Party

Obtaining my PhD was a long journey. There were many times I knew little of where I was going, or where it was leading. But there were many people who believed in me, supported me immensely, and helped me reach its end. Their faith fuelled mine, and kept me going. I would like to dedicate this thesis to them; especially to my mother, my sisters Garyfallia and Greta, my brother-in-law Bernd, and my beloved father who passed away, but I know that he is feeling now more proud and happy than any words could ever describe.

LIST OF ABBREVIATIONS AND UNITS

°C	Degree Celsius
DAH	Day after hatching
l	Litre
m	Meter
g	Gram
k	kilo (10^3)
c	centi (10^{-2})
m	milli (10^{-3})
µ	micro (10^{-6})
n	nano (10^{-9})
h	Hour
min	Minute
s	Second
cd sr m^{-2}	Candela steradian per square meter
CFU	Colony forming unit
p	Probability value of statistical hypothesis
pH	Measure of the acidity of solution
F	Formalin
E	Ethanol
G	Glutaraldehyde
DX	Disinfected and Xenic
DA	Disinfected and Axenic
NX	Non-disinfected and Xenic
A	Antibiotics
NA	No Antibiotics
F	F-test statistic
SD	Standard deviation
PCA	Principal Component Analysis
DA/CVA	Discriminant/Canonical Variate Analysis
ANOVA	Analysis Of Variance
ANCOVA	Analysis of Covariance
MANOVA	Multivariate Analysis of Variance
PERMANOVA	Permutation Multivariate Analysis of Variance

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Chapter 1

Introduction and thesis outline

1.1. The value of aquaculture today

Starting from ancient cultures flourishing well before 1000 BC, such as the Chinese and the Sumerians in Mesopotamia, aquaculture slowly signalled the move from the unstable supply of wild-capture fish to live storing and feeding them in ponds for ornamental or husbandry purposes, and finally to the intensive farming of fish and shellfish (Nash, 2011). It is particularly important today, as it plays a crucial role in the provision of food of highly nutritious value to the world.

The importance and necessity of a constant and sustainable means of the production of aquatic organisms, especially fish, through aquaculture is underlined from the fact that they are a key component to human health. Fish are a major component of our diet as a high source of protein and key fatty acids (Beveridge et al., 2013), and today about one billion people depend on fish to meet their basic requirement of animal protein, which comes in a form that is readily usable by the human organism (Nash, 2011). However, this global demand for protein is constantly increasing. Concerning this, on one hand some developments seem encouraging: in the past five decades, growth in the supply of fish for human consumption has outpaced population growth, resulting in an average per capita availability that has been increasing at an annual rate of 3.2% from 1961 until 2013. On the other hand, there are different trends that can appear ominous: in this aforementioned time span of five decades, world fish consumption has also increased from 9.9 kg per capita per year in the 1960s to 19.7 kg in 2013 (FAO, 2016). Using an average figure of only 15 grams of animal protein per person per day, which is far below that required by an active human body, this translates to a daily demand for 135 thousand tonnes of animal protein. Judging from the United Nations prediction that nine billion people will require feeding by the year 2050, it is calculated that about the equivalent of the current total annual aquaculture production of the United Kingdom will be needed to feed the world's population for just one day at that time (Nash, 2011).

In this context, it is difficult for the natural resources to keep up with the global demand, and it is likely that this situation will become more problematic in the future: since the end of the Second World War, their contribution to the world's annual fisheries production rose quickly from 18 to 85 million tonnes, but from approximately 1995 onwards reached a plateau

(Fig. 1.1). Therefore, it is up to aquaculture to provide crucial support in order for these increasing demands to be met.

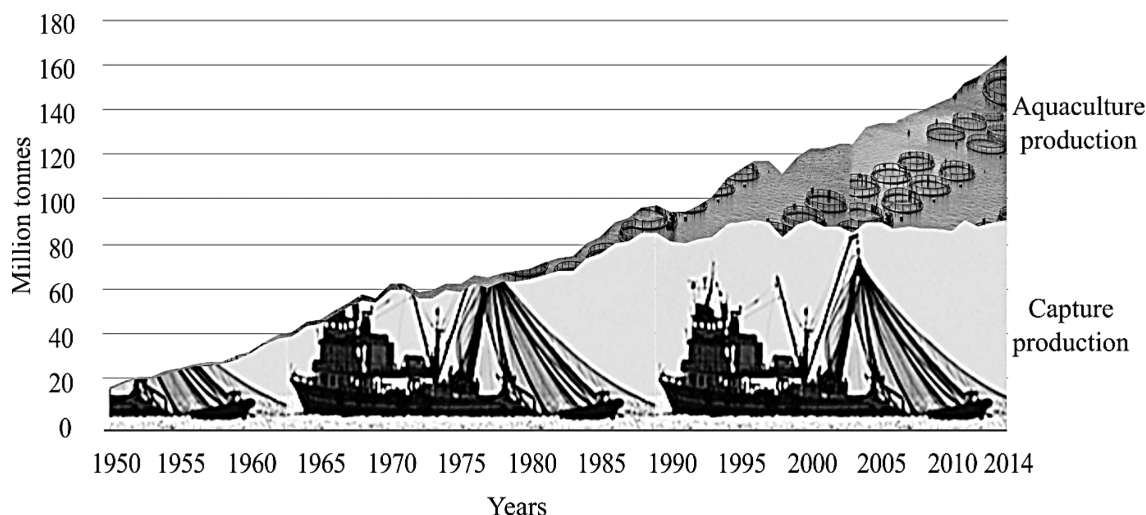

Fig. 1.1: World production of fisheries from capture and aquaculture between 1950 and 2014 (adapted from FAO, 2016).

Thankfully, it has so far been very successful in that role: the constantly increasing production of aquatic animals from aquaculture reached 73.8 million tonnes in 2014, with an estimated first-sale value of 160.2 billion US$ (Fig. 2). Additionally, on that year the contribution of aquaculture to the supply of fish for human consumption overtook that of wild-caught fish for the first time, a fact that reflects its current position as the fastest growing animal production sector (FAO, 2016), and one in line with the prediction that aquaculture will provide close to two thirds of global food fish consumption by 2030 (The World Bank, 2013). It is therefore clear that it has a very strong potential to keep supporting these growing demands - at least for the foreseeable future - in a sustainable way by alleviating the pressure on wild fish stocks, which are endangered due to the pressure originating from the constant need of supplying aquatic organisms for human consumption, and for other uses as well, among which are the production of direct aquaculture and livestock feeds, fishmeal, fish oil, and raw material for use in the pharmaceutical industry. Furthermore, it plays this role while remaining a global source of income of great financial importance and magnitude.

Over the past two decades, finfish have been the major species group in today's aquaculture production in terms of value (Fig. 1.2), and in terms of species number: in 2014, 362 finfish

species were cultured, followed by 104 molluscs, 62 crustaceans, 37 aquatic plants, 9 invertebrates, and 6 species of frogs and reptiles. In 2013, the finfish with the largest value were salmon (farmed Atlantic *Salmo salar* and the wild Pacific of the *Oncorhynchus* genus), trout, tuna, wild whitefish such as cod, hake and pollock, and farmed fish such as the whitefish cod, tilapia and *Pangasius*, and finally European seabass *Dicentrarchus labrax* and gilthead seabream *Sparus aurata*. The whitefish species originating from the wild used to dominate the world fisheries market, but their production is now met with strong competition from aquaculture (FAO, 2016).

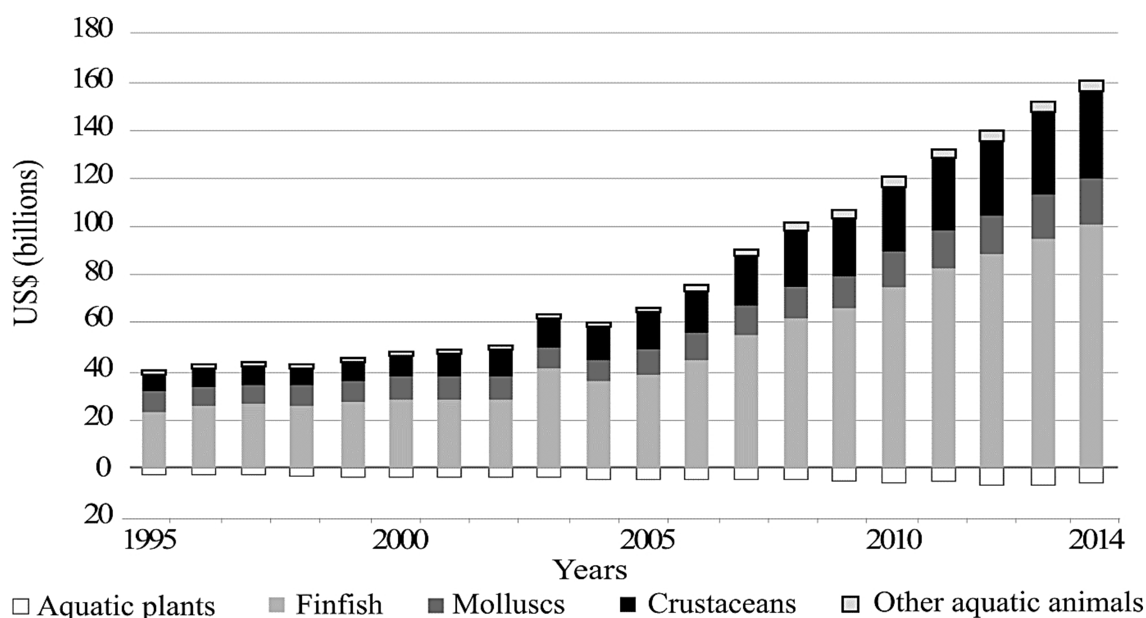

Fig. 1.2: World aquaculture production value of aquatic organisms between 1995 and 2014, with the exclusion of non-food products such as seashells and pearls (adapted from FAO, 2016).

1.2. Abnormalities and deformities: importance and financial losses

“Abnormality” is defined as an indication of a difference or deviation from the average or norm, “deformity” as an alteration in shape and/or in structure of a previously normally formed part, and “malformation” as a morphological defect of an organ or larger region of the body, resulting from an intrinsically abnormal developmental process. In many cases, these terms are synonymous (Boglione et al., 2013b and references therein).

Aquaculture increasingly focuses on upgrading larval fish quality, but its production nowadays is plagued by morphological abnormalities that produce a considerable economic loss. As an example, 1,057,629,000 seabass and seabream juveniles were produced in Europe and Turkey in 2015 (FEAP, 2016), and according to Kolios and Mazzora (2009) a 15% mean total deformities rate - a rather optimistic value since 20% is considered to be a good but rare result (Boglione et al., 2013a) - on an annual production of 1 billion seabass and seabream juveniles can raise the cost for the sector to 10-15 million € per year, or 18 million € according to Autin et al. (2012). What is more, the minimum annual economic impact of abnormalities of all European aquaculture species is setimated to be more than 50 million € per year (Boglione et al., 2013b), out of the estimated total value of 3.9 billion € from the European aquaculture production of fish, crustaceans, molluscs and other aquatic organisms in 2014 (Eurostat, 2017), with 2.4 billion € originating from fish alone in 2012 (STECF, 2014). In spite of the impressive progress that has been made over the past years in the rearing methods, they still pose a significant problem in aquaculture, as they affect the survival of the fish, their external morphology, growth rate, behaviour, and eventually the production cost and effectiveness of the hatcheries (Sfakianakis et al., 2006a). An added disadvantage of deformed fish is that they often present a food conversion ratio (FCR) that is higher than unaffected ones, and are thus more costly to produce.

Malformations can lead to fish that are not easily marketable, or even to mortalities at the larval stage or later. The early detection can be crucial in this prevention, because deformed specimens exhibit a slow growth rate, compete for food and space with healthy fish, are prone to disease, and have poor marketability (Moretti et al., 1999). There is a chance for recovery at later adult stages (Palaniappan et al., 2008; Kayim et al., 2010; Beraldo and Canavese, 2011; Hou et al., 2011; Amoroso et al., 2016) but it is not certain, and in the cases of sublethal abnormal development, the final product can be difficult or impossible to sell. As deformed fish are less acceptable to consumers, they must be either discarded or further processed - if possible - to save any of the meat. Additionally, even fish with lesser deformities may be difficult to fillet, resulting in lower fillet yields. Consequently, deformed fish translate into financial losses to fish farmers (Gjerde et al., 2005). This is especially important in markets where the sale of the whole, unfilleted fish is desired as a consumer habit, with the specific needs of these markets covered by a global production that is far larger than fish that are processed, including filleting: in 2014, 67 million tonnes of fisheries

were in live, fresh or chilled form, and 43 million in frozen, while only 19 million tonnes were in prepared or preserved form, and 17 million in cured form (FAO, 2016).

1.3. Scientific goals of the thesis

Judging from the above, the early detection of deformities is crucial in order to avoid mortalities or slow growth rates, and consequently the waste of time, funds, energy and effort. The main time point that the sorting and data recording of deformities takes place is when fingerlings are being sold in the hatcheries, or transferred to the fingerling growing tanks. The timing for the first overall deformities sampling is species-specific, but regarding European seabass and gilthead seabream it is usually around the 50th day after hatching (hereby referred to as DAH) at the earliest.

Common methods for the identification and quantification of deformities at this time point include visual examination, radiography, staining of cartilage and bone, and size analysis. These methods can provide information of high significance, but have also been known to present limitations: they are useful in the identification of deformed specimens, but can fail to find and objectively quantify more subtle abnormalities, especially in earlier stages, and fail to provide information on the shape of the specimens, which can be crucial for this purpose of identification and quantification. Furthermore, even scientific studies in literature have used these four methods with limitations, such as the frequently overlooked issue of specimen distortion originating from handling, anesthetisation, fixation and mounting.

Therefore, it is evident that there is a need to develop a size and shape quantification protocol for the crucial larval stages that come earlier than the aforementioned time points, while also being one that takes into account all of the aforementioned artefact-inducing and error-introducing limitations and minimises them to the best possible degree, thereby increasing the quality and reliability of the extracted information. Establishing such a protocol for the objective mathematical quantification of abnormalities in the earliest larval stages can be crucial in their prevention, and is the first scientific goal of this thesis.

In hatcheries, the minimisation of the occurrence of diseases, abnormalities and deformities is pivotal for the successful establishment of ideal conditions for the rearing and breeding of fish, and consequently for the maximisation of their growth. Another problem which has been known to play a very important role during these early stages is the often challenging management of the bacterial load. These are all essential areas of research, as they significantly affect the overall yield, efficiency, and health of the fish (Boglione et al., 2013b). The bacterial load and the application of disinfection agents during the egg incubation stage as a means for its control have documented adverse effects on larval hatching rates and survival, but largely unexplored size and shape effects.

In the context of the study of the effects of bacterial load and its communities, a technique that has received considerable attention in recent years is the rearing of gnotobiotic animals in axenic environments, and specifically of fish larvae in ichthyology and aquaculture research, in which a specific known microbial community is inserted as a means to study host-microbial interactions, to eliminate bacterial interference that tends to confound experimental results, and to study how microbial colonisation or microbial products influence host biological processes including gene expression, immunity, lifespan, physiology and development (Pham et al., 2008).

In the framework of investigating the morphological effects of the microbial load, studies on differences between xenic and axenic fish larvae have been focused mainly on the development and maturation of the gastrointestinal tract, and to a limited degree on differences in total length. However, detailed knowledge on a range of morphometric characters of size and the shape of the larvae is still lacking. This can be valuable, as both pathogenic and non-pathogenic bacteria have been thought to be associated with deformities, mostly in the adult stages, but with very scarce data for the larval stages. Furthermore, the use of disinfection agents and antibiotics has also occasionally been associated with the occurrence of abnormalities. The evidence in literature is presented in Chapter 2.

Consequently, as the aforementioned protocol can be a valuable tool in the quantification of the effects of any biotic or abiotic factor that has the potential to induce size and shape changes, it was used in the investigation of the potential size and shape effects of axenity, difference in bacterial load, and egg disinfection on fish larvae, with that investigation being the second scientific goal of the present thesis.

Therefore, the specific objectives and the outline of the present thesis are:

Chapter 2 (Literature review) provides an overview of the biology and the aquaculture techniques of European seabass, the importance of its early larval stages, the deformity conditions encountered today and the means for their identification and detection, their drawbacks, how a size and shape quantification protocol for their detection in these stages can also provide feedback on the potential morphological effects of axenity, bacterial load and the application of egg disinfection agents, and the current status of knowledge on their effects.

In **Chapter 3 (Protocol for quantitative shape analysis of deformities in early larval European seabass *Dicentrarchus labrax* L.)**, this protocol is investigated on European seabass larvae from DAH 2 until DAH 14, and includes the **examination of the effect of fixatives and mounting on their total length and body shape** (comparison between live and fixed specimens five months post fixation). The four fixation treatments chosen for comparison are: (1) 8% formalin, (2) 70% ethanol, (3) 8% formalin for 48 h and then to 70% ethanol, and (4) 3% phosphate-buffered glutaraldehyde. The study of the effects of shape is done using of geometric morphometrics. Furthermore, an effort is made to identify the **error introduced by anesthetisation and handling**, and minimise it to the best possible degree. The final protocol was then used to quantify size and shape effects, as reported in chapters **4** and **5**.

In Chapter 4 (Phenotypic effects of antibiotic-induced axenity and egg disinfection in early larval European seabass *Dicentrarchus labrax* L.), the hypothesis that **the difference in axenic conditions in the incubation and rearing environment of European seabass larvae induces size and shape effects on the specimens** is tested. This difference is studied between xenic and axenic seabass larvae of DAH 0, 5, 11 and 15, the latter incubated and reared with the use of the protocol by Dierckens et al. (2009). A size effect on seabass larvae has already been shown by Rekecki et al. (2009), with the xenic larvae exhibiting a smaller total length than axenic on DAH 6, 9, 12 and 15, but this is examined here in further detail, using linear and angular measurements (including lengths, depths, the yolk sac area and the pectoral angle) and outline based shape information of the specimens. The aforementioned axenic rearing protocol of Dierckens et al. (2009) also involves the use of glutaraldehyde

disinfection of the eggs in the lab, additionally to the primary iodine disinfection at the hatchery, and **the hypothesis that this secondary disinfection induces size and shape effects is also tested**. In order to accomplish this, three egg and larvae treatments are included: “DA” (Disinfected Axenic), “DX” (Disinfected Xenic”) and “NX” (Non-disinfected Xenic).

The study of Chapter 4 was performed on the egg stage as a starting point. However, the application of antibiotics and other disinfection agents has been known to produce adverse effects on the eggs, with the main ones being an impairment of their hatchability and of the larvae survival. Furthermore, it is also suspected to play a role in the occurrence of abnormalities. Hence, in order to avoid these adverse effects of the axenic process on the eggs, a new study was performed in **Chapter 5 (Effects of antibiotic-induced differences in bacterial load on growth and shape of early larval European seabass *Dicentrarchus labrax L.*** on specimens obtained as larvae of DAH 3 from the hatchery. In it, **the hypothesis under testing is that the difference in bacterial load, as induced by antibiotics, induces size and shape effects on European seabass larvae between DAH 3 and 14**. The treatments are the control, featuring xenic larvae reared with no antibiotics (“NA”), and the antibiotics one (“A”) reared with a mix of rifampicin, ampicillin, kanamycin, trimethoprim and gentamicin. Additionally, in contrast to the study of the previous chapter, the hypothesis of the existence of morphological differences is not based on the difference in axenic conditions, but on a difference in bacterial load while both treatments were xenic. This was done in order to examine the morphological differences with the gastrointestinal tracts of the larvae colonised and their immune activities stimulated by bacteria, whereas this has been known to happen to a much smaller degree in axenic and gnotobiotic larvae (Nayak, 2010).

Finally, in **Chapter 6 (General Discussion)**, the advantages and drawbacks of the size and shape quantification protocol are being evaluated. Its practical application in a commercial hatchery setting is discussed, along with the shortcomings presented by its time-consuming and labour-intensive nature, and the possibility of automation through the use of image recognition technology is argued. Furthermore, the significance of the manifested size and shape effects due to the presence of axenic conditions, the antibiotic-induced differences in bacterial load and the application of glutaraldehyde disinfection on the larvae is discussed, in the context of possible suggestions that can be made regarding the critical factors that need to be closely monitored in hatcheries. Additionally, the need to establish a direct link between

the manifestation of aquaculture abnormalities in the earliest larval stages and adult stages at the end of metamorphosis is highlighted. Finally, suggestions for further study are made regarding the need to investigate the existence of deformity-inducing bacterial mechanisms, and their possible causative link with metabolic changes induced by increased bacterial load, in hatchery conditions for a longer period of time than the first 15 days after hatching, as examined in the present thesis for European seabass.

1.4. The choice of European seabass as a case study

To investigate these effects, European seabass *Dicentrarchus labrax* was chosen as a target species. This was done due to its great importance as one of the most popular and successful species in today's aquaculture industry, especially in the Mediterranean: in 2015, 158,479 tonnes were produced in Europe, which comes as a record following the constant increase of its production since the early 1990s, while the fisheries catches remain around 10,000 tonnes per year (FEAP, 2016). In 2014, it was the fifth most important aquaculture species in Europe in terms of value after Atlantic salmon *Salmo salar*, rainbow trout *Oncorhynchus mykiss*, Pacific cupped oyster *Crassostrea gigas* and gilthead seabream *Sparus aurata* (Eurostat, 2016).

The main production of sea bass is located in Turkey (48.5%) and Greece (28.4%), although it is also cultured in many other Mediterranean countries, such as Spain, Italy, Egypt, Croatia, France, Tunisia, Cyprus, Portugal, etc. The other most popular aquaculture species in the Mediterranean, gilthead seabream, has been coming usually second in production, with 147,649 tonnes in the same year (FEAP, 2016). European seabass is considered to be the first marine fish species in the Mediterranean region to be successfully cultured from egg up to its commercial size (Filic et al., 1987). Due to its success and popularity, it has been the focus of a wide range of studies for its biology, rearing methods and its deformities. Therefore, this range of studies and its importance as an aquaculture species were the reasons for its choice as a case study.

Chapter 2

Literature review

2.1. European seabass - biology and aquaculture life stages

European seabass *Dicentrarchus labrax* (Linnaeus 1758) belongs to the Actinopterygii class of the ray-finned species of the Osteichthtyes superclass, and more specifically within the Moronidae family of the order of Perciformes, which is the largest order of vertebrates and the most diversified of all fish orders, with 62 families and 2,248 species (Nelson et al., 2016). Its *Dicentrarchus* name comes from ancient Greek, with the etymology of di (two), kentron (sting) and archos (anus), possibly referring to its two anal spines, although today they are normally three (Fishbase, 2017). Its full taxonomic hierarchy and distribution are displayed in Fig. 2.1.

It is a eurythermic and euryhaline marine species which spends most of its life in coastal lagoons and estuaries, although it has been occasionally observed in rivers. It inhabits waters ranging from brackish to hyperhaline, and shows oceanodromous behaviour. Its great tolerance to low and high temperatures, ranging from 5 to 32 ºC, and its ability to regulate osmotic stress allows it to present a wide geographic and depth range of distribution: it is situated in the Eastern Atlantic from Norway to Senegal, and in the Mediterranean and Black Sea, from shallow water depths (2–10 m) to more than 100 m. It can also be found in countries where it has been introduced for culture purposes such as Israel, Oman and the United Arab Emirates. It is a strong swimmer, voracious predator of small pelagic fish and a large number of invertebrates, and it reaches sexual maturity around the fourth year of its life, with reproductive migrations following it (Pérez-Ruzafa and Marcos, 2015 and references therein).

Sexual differentiation occurs late, with undifferentiated gonads appearing one year after hatching. The females differentiate their gonads earlier than males, and sexual dimorphism is observed, as in many marine species. The female reproductive effort is higher than that of males, and this determines that females are larger. In the natural environment, the sex ratio is biased towards females, increasing from 52.0% of females in the younger fish (<30 cm total length) to 69.5% of females in the older fish. However, in farmed populations, sex ratios are highly biased towards males (75% to 95%), which is problematic for aquaculture where maximised growth is constantly being aimed for. The level of stress during the larval period

and temperature are thought to be the major inductors of sex determination (Saillant et al., 2001; Koumoundouros et al., 2002a; Pérez-Ruzafa and Marcos, 2015 and references therein).

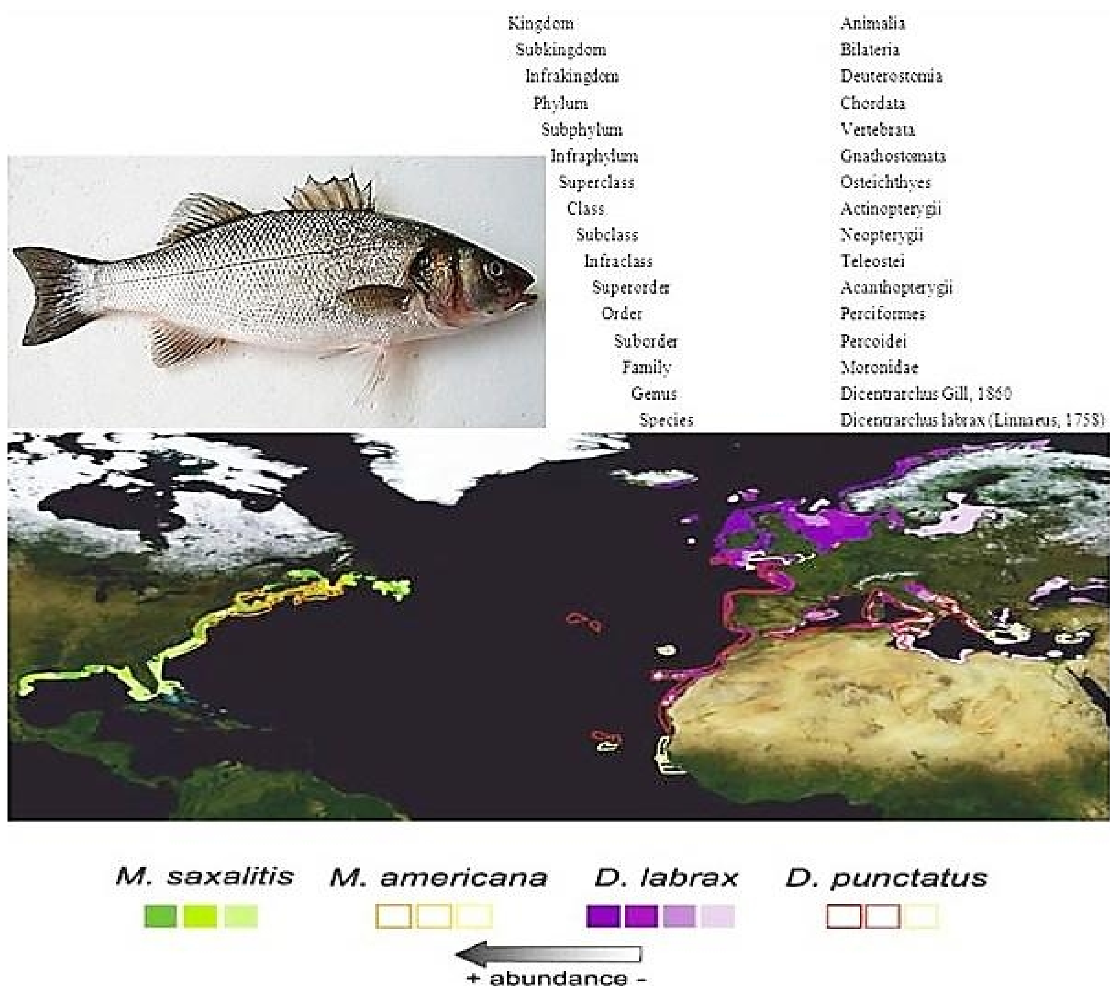

Fig. 2.1: Taxonomic hierarchy of European seabass *Dicentrarchus labrax* from the Integrated Taxonomy System (ITIS) online database (http://www.itis.gov) [retrieved on 24th January 2017], together with a photograph of an adult specimen from F. Crocetta (http://www.fishbase.org/summary/63) and distribution map of the Atlanto-Mediterranean species of sea bass (Pérez-Ruzafa and Marcos, 2015).

During the spawning season, each mature female produces small pelagic eggs, and their incubation period depends on the ambient temperature. Higher temperatures induce a faster development, and it lasts for about 50 h at 18°C and 36 h at 22°C. Newly hatched larvae have a total length of around 3.5-4 mm, have eyes that are not pigmented, the mouth is closed and the digestive tract is still incomplete. During this initial period of growth, the larvae survive

on the reserves of their yolk sac, and its volume is progressively reduced. Within the following three to four days their body pigmentation increases, the mouth opens and external feeding commences. The swimbladder starts inflating when the larvae yolk sac is almost completely reabsorbed, and with slight variations according to the rearing temperature. In European seabass and gilthead seabream, normal swim bladder development, from the initial vesicle, is characterized by two stages. The first stage, called “initial inflation”, occurs in 5mm sea bass larvae (around DAH 7) and in 4mm sea bream larvae (around DAH 5). Inflation coincides with oil globule resorption. When initial inflation fails, the swim bladder development is stopped at a stage resembling that prior to inflation and is non-functional. The second stage corresponds to an expansion phenomenon. In larvae greater than 12mm long, the swim bladder looks like an ellipsoidal vesicle which progressively stretches backwards during growth. It becomes stable in 40-50mm fish, reaching 20 to 30% of the total length (Chatain, 1986). When the primary swimbladder inflation is complete, the oil droplets of the yolk sac are almost completely reabsorbed, and the larvae start going through an ontogenetic period of metamorphosis from the post-larval to the juvenile (fry stage) that ends around DAH 45. Finally, the juvenile stage in seabass is approximately reached after 20mm, and the definitive morphology after approximately 30mm (Moretti et al., 1999; Pérez-Ruzafa and Marcos, 2015 and references therein).

According to the intensity of the culture conditions and the yield, aquaculture systems are divided into extensive, semi-intensive and intensive. European seabass is a species cultured in extensive and semi-intensive lagoon systems, but most commonly in intensive aquaculture facilities. The intensive system is based on a life cycle that is summarised in Fig. 2.2. Breeding individuals of various age groups, originating from either wild-caught or captive-bred broodstock, are transferred from their long-term tanks to spawning tanks, where fertilised eggs are produced under controlled conditions of temperature and photoperiod that are optimal for reproduction, and occasionally with the aid of hormonal treatment. The eggs are then collected, disinfected as a means to reduce the intense bacterial load, placed into cylindrical incubation tanks or directly into the rearing tanks, and left to hatch. Different definitions of a “larva” have been described in literature according to the development of the embryo, its hatching and its endogenous, mixed or exogenous feeding. However, in the majority of studies, a fish developing inside an egg is called an embryo, whereas for a hatched fish the term “larva” is used (Kamler, 1992 and references therein). This terminology will also be used in the present thesis.

After mouth opening, the yolk sac larvae are fed initially with live food such as brine shrimp *Artemia* or rotifers, and gradually weaned into artificial. They are reared in the hatchery until they reach their juvenile form, undergoing the aforementioned process of metamorphosis, which refers to the postembryonic developmental stage of an animal that is characterised by intense changes at the morphological, physiological, biochemical, behavioural and ecological levels, marking the transition from a larva to a juvenile that is morphologically distinct (Paris and Laudet, 2008; Pittman et al., 2013). After the weaning of the larvae from live feed into artificial is achieved and the fry stage is reached (species-specific in weight, but approximately 2 g concerning European seabass), they are transferred from the hatchery into the fattening facilities, being either pre-growing tanks or floating cages, in which they reach their commercial size. This is roughly 350-400 g in the case of European seabass, and is reached in about 18-24 months. Intensive culture facilities usually have their own broodstock units, hatcheries and ongrowing cages, as shown in the life cycle in Fig. 2.2. There are however many aquaculture companies only involved with the ongrowing phase in sea cages, and in these cases juveniles are bought from hatcheries and stocked at a size of approximately 1.5 to 2.5 g. Exceptionally, in a few cases this phase can occur in inland tank facilities using water recirculation systems, in which high stocking densities of 20-35 kg/m³ are used, under very closely monitored conditions for the necessary control of water quality (FAO, 2005).

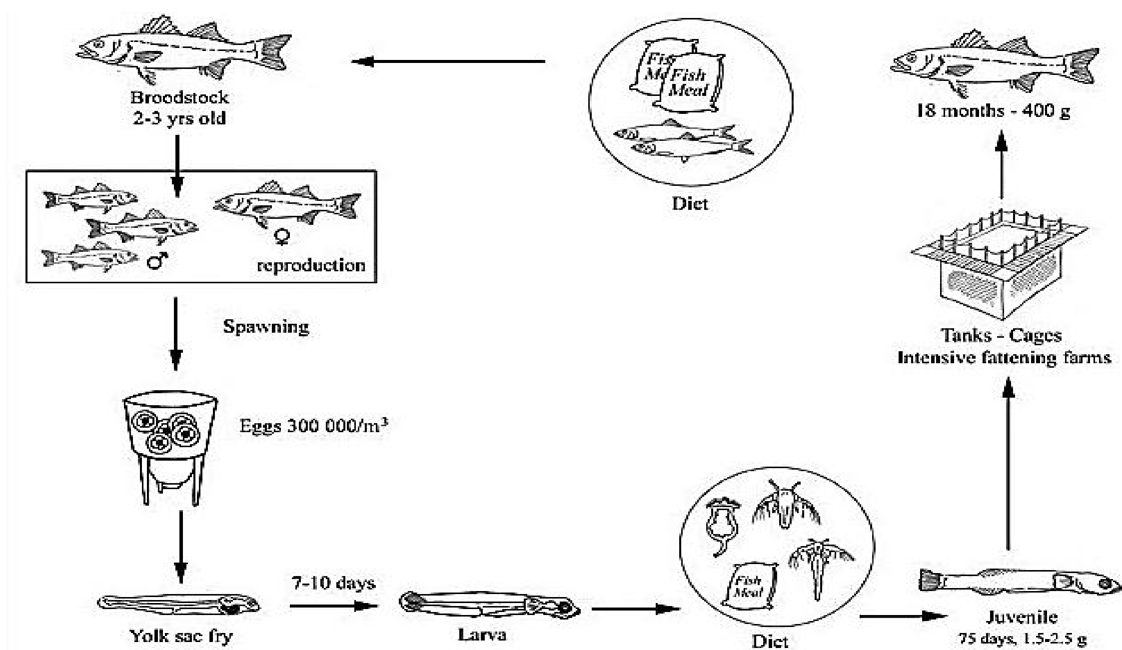

Fig. 2.2: Life cycle of the intensive system aquaculture production of European seabass *Dicentrarchus labrax* (FAO, 2005).

2.2. The importance of the early larval stages

During this life cycle, the early life stages are thought to be the most crucial and sensitive. This is largely due to the fact that fish larvae have a greater susceptibility to pathogens than adults, since they possess very limited innate defences in their earliest life stages, and rely on immunological material deposited in the egg by their mother. They must therefore be reared in an environment of optimal quality without significant challenges by pathogenic microorganisms, until they reach a point near the end of their metamorphosis where their immune system is fully functional (Bowden and Bricknell, 2013). Additionally, there are individual critical time points throughout this long period, such as the time of the transition from endogenous to external feeding, which is characterised by high vulnerability to external factors and increased mortality. These external factors include (a) biotic factors such as disease and parasitism, and (b) abiotic factors such as low oxygen content, extremes of temperature, pH or salinity, toxic substances etc. (Kamler, 1992), but with these extremes mainly encountered in nature. In spite of considerable scientific progress over the past years in breeding and husbandry methods of aquaculture species, and in the development of new methodologies designed to optimise rearing conditions, ensuring this optimal environmental quality while also stimulating the innate immune defences of the larvae still remains a difficult and very delicate task (Darias et al., 2008).

For European seabass, two different commercial larval protocols exist: the first takes place in a lighted environment with rotifers as first feeding, and the second, referred to as the “French technique”, in a dark environment during the first days after hatching, with the use of small newly hatched *Artemia* nauplii as first food. Additionally, there is another protocol which is mostly used for experimental purposes, but is also recommended for small and resource limited producers: the "mesocosm". It is an indoor or semi-outdoor hatchery technique with low densities (2–8 larvae/l) in relatively large (30–100 m^3) and deep (1.5–2.5 m) tanks with a long photoperiod, and *Chlorella* or *Tetraselmis suecica* plankton blooms prior to the introduction of the larvae, which feed on daily provided *Artemia* and rotifers. By largely resembling the natural environment and by keeping the densities low, it has been shown to yield juveniles of high quality, with very small or no occurrence of deformities (Gisbert et al., 2015).

Another reason for the importance of early larval stages is the fact that most of the deformity types encountered in aquaculture today have been shown to have their first manifestation or origins during that period. In the next section, their types, occurrence and causative factors will be analysed.

2.3. Abnormalities, deformities and malformations

2.3.1. Definition and types

In literature, there are a few different terms used to refer to the occurrence of morphological anomalies during the development of an organism, with abnormality, deformity, and malformation being the most common. Based on the terminology for human pathology, “abnormality” is defined as an indication of a difference or deviation from the average or norm, “deformity” as an alteration in shape and/or in structure of a previously normally formed part, and “malformation” as a morphological defect of an organ or larger region of the body, resulting from an intrinsically abnormal developmental process, sometimes also being a localised error of morphogenesis. In many cases, they are synonymous and clear distinctions in their use cannot be made, because actual, clear knowledge on the development or aetiology of the different disorders observed in fish is still lacking (Boglione et al., 2013b and references therein).

Based on data from Web of Science (https://webofknowledge.com), annual publications for fish deformities slowly started appearing after 1990 with approximately 9 per year, and over the course of a decade they were more than tripled, reaching 29 in 2001. Nowadays there is a considerable research focus, with 82 publications in 2016. The most prevalent types of malformations and abnormalities in aquaculture today include skeletal deformities such as in the skull (for example deformities in the operculum and the jaw) and the vertebral column (lordosis, kyphosis, scoliosis and vertebrae compression), dorsal and caudal fin anomalies, the latter originating at least partly from notochord deformities encountered in the yolk sac stage or earlier, lack of scales on the lateral line or throughout the whole body, problematic body pigmentation, and the development of urinary calculi as calcium phosphate crystals in

the urethra or urinary bladder (Moretti et al., 1999; Koumoundouros, 2010). As far as the two most commonly cultivated species in Mediterranean aquaculture are concerned, i.e. gilthead seabream *Sparus aurata* and European seabass, the usual deformity conditions involve snout/mouth dysplasia, operculum deformities, the lack of dorsal fin, hypoplasia of the caudal fin, and malformations such as lordosis and kyphosis.

Opercular deformities mainly begin during the larval period and continue occurring until juvenile or adult sizes (Boglione et al., 2001). In *Sparus aurata*, the opercle skin fold is seen at DAH 7 when there is no bone present, but it ossifies as a very thin opercle at DAH 14. It can be expected that at this stage, an unsupported opercular skin fold is more susceptible to altered external influences, especially mechanical ones such as an excessive water flow in rearing tanks (Thuong et al., 2017). Opercular deformities can be unilateral or bilateral, with a folding of the opercle or subopercle inside or outside the gill cavity during the early larval stage (Koumoundouros et al., 1997a; Galeotti et al., 2000; Beraldo et al., 2003; Verhaegen et al., 2007). As the function of the opercular complex is not only for protection of the gill but also for respiration, opercular deformities might indirectly cause gill diseases by a lowered resistance to environmental stress (Paperna et al., 1980). Exposed gills can decrease respiratory efficiency, as well as reduce market value (Divanach et al., 1996; Beraldo & Canavese, 2011).

Haemal vertebrae are characterised by the haemal arch in the ventral side of the vertebra of the caudal region, and are divided into the categories of pre-haemal (for gilthead seabream: 3rd-10th), haemal (11th–21st) and caudal (22nd–24th) (Boglione et al., 2001). Lordosis is a V-shaped vertebral curvature, and can affect the pre-haemal or the haemal part of the vertebral column (Sfakianakis et al., 2006a). Kyphosis refers to a Λ-shaped vertebral curvature, and pre-haemal kyphosis (mainly affecting vertebrae 5–6) in Dicentrarchus labrax develops during the larval stage (10–17 mm in total length), while in the next stage of metamorphosis, it is lethal for affected fish (Koumoundouros et al., 2002b).

As seen in Fig. 2.3, many of these conditions have been known or strongly suspected to have their first manifestation and origins in the crucial earliest life stages (Koumoundouros, 2010; Boglione et al., 2013b). They are known to occur mostly during larval development and osteogenesis, which is a process starting in larval fish with the formation of cartilage prior to ossification (Karahan et al., 2013), along with membranous bones that start developing

directly without replacing cartilages. Deformities are usually associated with osteogenesis but are not limited to skeletal malformations, since some have been shown to occur at the beginning of the ossification process, or even before it. Examples include twisted spinal cord and jaw abnormalities in European seabass immediately after hatching (Fig. 2.4), with a maximum occurrence of 32% up to DAH 9 (Barahona-Fernandes, 1982), plus fin anomalies as early as 9.4 mm of standard length (Marino et al., 1993) around DAH 23, fin anomalies in sole *Solea senegalensis* on DAH 24 (Gavaia et al., 2002), and notochord anomalies in gilthead seabream *Sparus aurata* of 3.4 – 3.9 mm total length on DAH 2 (Koumoundouros et al., 1997b). Typical examples of the development of early conditions into skeletal deformities in later stages are haemal and pre-haemal lordosis (Fig. 2.5), pre-haemal kyphosis and saddleback syndrome (Fig. 2.6).

If the percentage of deformities such as body twisting in newly hatched larvae is above 10%, they are often lethal, and the discard of the entire batch may be recommended (Moretti et al., 1999). Malformations are also fully manifested near the end of metamorphosis or later, and a frequency of about 20% of severely deformed fish at the end of the hatchery phase is considered to be a good, but quite rare, result (Boglione et al., 2013a), since they can occasionally affect 100% of the production (Koumoundouros, 2010). The percentage of lethal deformities increases considerably the already very high total mortality losses from the moment of egg stocking until the moment of fry sale at the hatchery. Data for these losses are scarce, but they have been known to reach typical values ranging from 72% up to 88% in seabream *Sparus aurata* (Theodorou et al., 2016). Additionally, deformity conditions have been shown to generate considerable losses in the juvenile stage, with an example of 30-80% of the total Norwegian production of Atlantic cod *Gadus morhua* juveniles in 2006 being affected (Lein et al., 2007 in Saele et al., 2009).

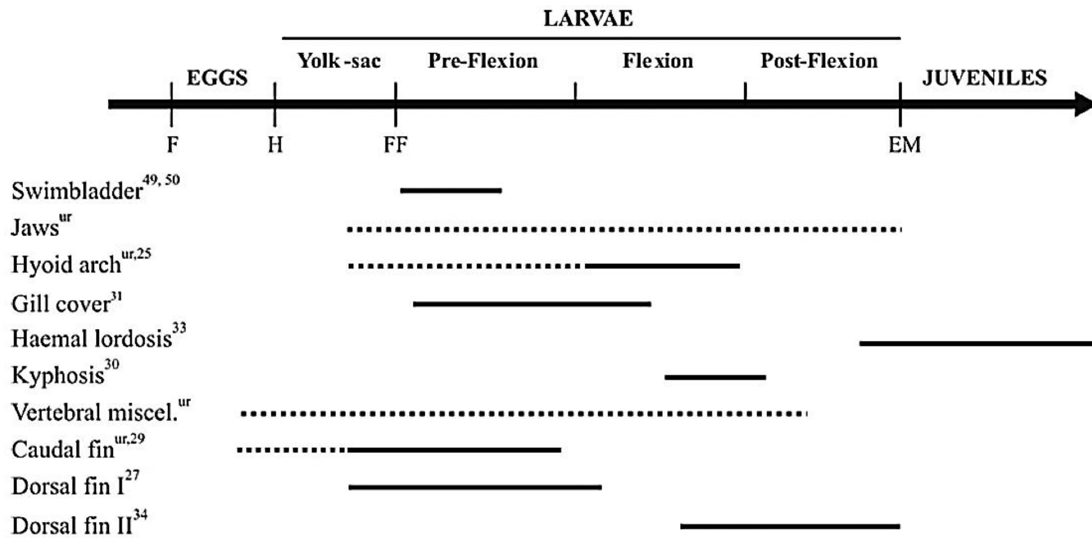

Fig. 2.3: Ontogenetic periods of the occurrence of abnormalities of different anatomical parts in aquaculture. EM: end of metamorphosis, F: fertilization, FF: first feeding, H: hatching. Unpublished data are being represented with dashed lines (Koumoundouros, 2010).

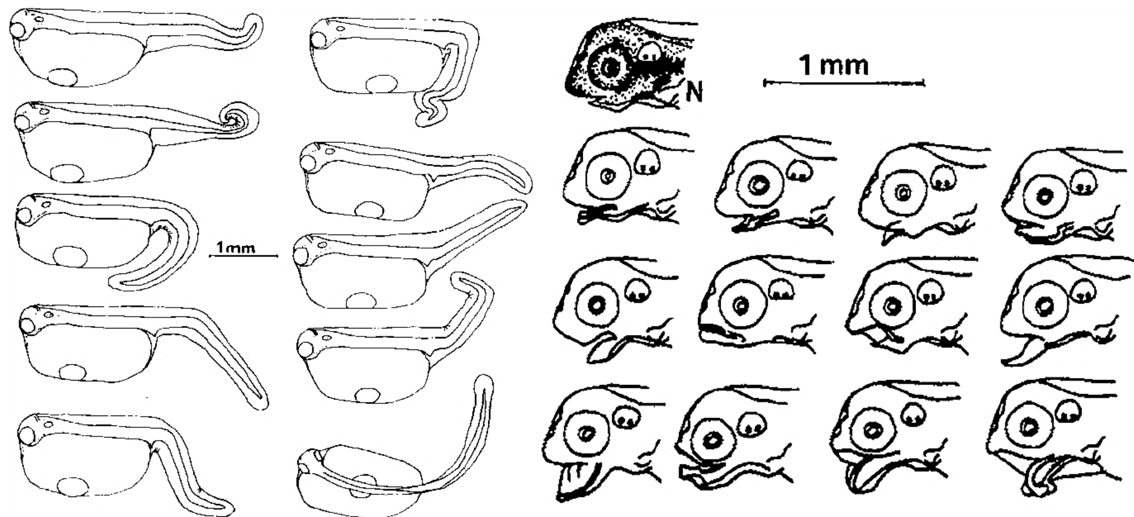

Fig. 2.4: Spinal cord abnormalities in newly hatched European seabass larvae on the left, and jaw abnormalities in larvae of DAH 6, with a normal larva marked with "N" (Barahona - Fernandes, 1982).

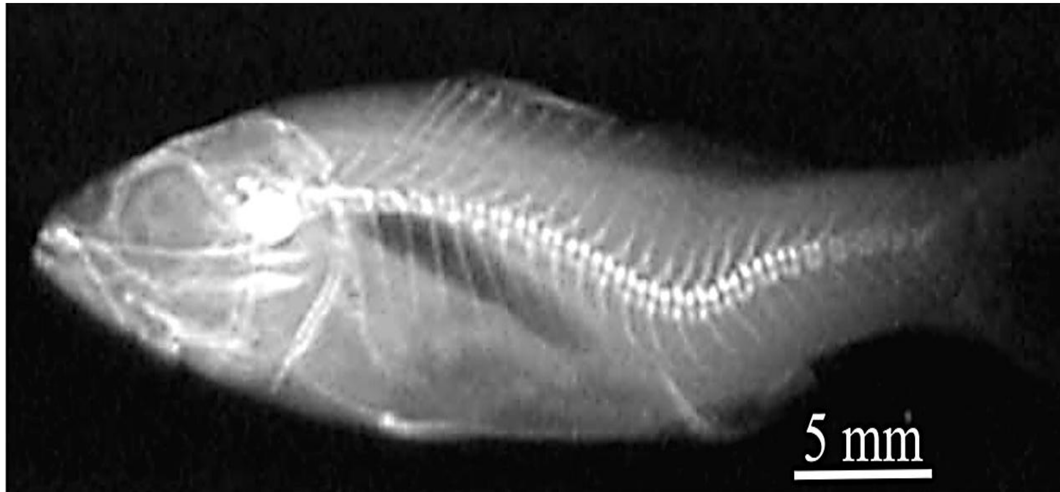

Fig. 2.5: Identification of haemal lordosis in European seabass of approximately DAH 75, visible as a curvature in the vertebral column, through the application of radiography (Sfakianakis et al., 2006a).

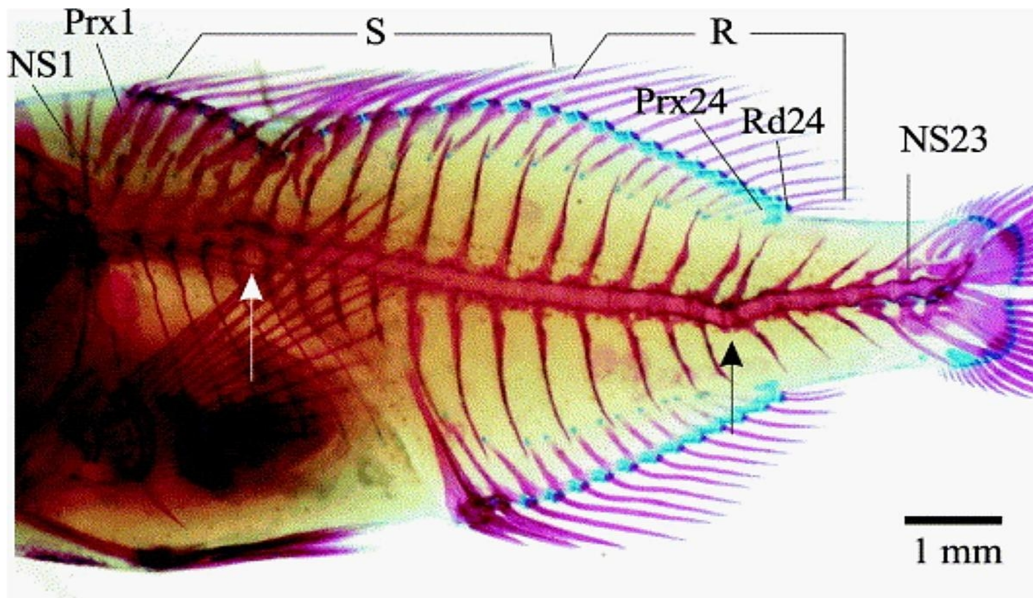

Fig. 2.6: Identification of saddleback syndrome on a white seabream *Diplodus sargus* specimen of approximately DAH 35 through the application of cartilage and bone staining. NS marks the neural processes, Prx the proximal pterygiophores, S the spines, R the lepidotrichia, and Rd the distal radials. Blue colour indicates cartilage, red indicates ossification, and the white arrow shows kyphosis, while the black shows lordosis at the haemal part of the spine (Sfakianakis et al., 2003).

2.3.2. Causative factors

Causes for most skeletal deformities are still not well known, although they are mainly attributed to environmental, nutritional and genetic factors. Environmental factors include, but are not limited to, temperature, salinity, pH, O_2 concentration, water quality, water currents, pollutants and handling stress. The nutritional factors that have been shown to be responsible are unfavourable levels of vitamins, such as excess of vitamin D or deficiency in vitamin C due to its high water solubility from the provided feed, abnormal concentration of proteins, lipids, minerals etc. (Moretti et al., 1999; Koumoundouros, 2010). As an example, phosphorus deficiency has been known to induce bone deformities in several fish species, such as Atlantic salmon *Salmo salar*, common carp *Cyprinus carpio* and haddock *Melanogrammus aeglefinus* (Saele et al., 2009 and references therein). As far as the genetic origin, heritability and triploidy have been known to play a role (Koumoundouros, 2010).

A problem which makes the identification and control of these factors difficult and unpredictable is that they are expressed in a complicated and non-standardised way: the same factor can induce different anomalies in different fish species, the sensitivity to a factor may change dramatically during ontogeny, and the same factor may provoke a high incidence of anomalies in some skeletal elements, but not in others with the same bone type and ossification, in the same individual (Boglione et al., 2013a and references therein).

Some of the factors heavily involved in the occurrence of many malformations have been identified, and to a satisfactory degree dealt with. A good example is swimbladder inflation, which is a crucial early ontogenetic event. Inflated swimbladder ensures neutral buoyancy of the fish in the water, and fish sink without the presence of a functional swimbladder. In physoclistous fish, its normal development starts with its first inflation with air through the pneumatic duct, at which moment the larva needs unobstructed access to the air-water interface to perform the subsequent gulp of air (Koumoundouros, 2010). In European seabass, this usually begins at DAH 5-7 and is completed around DAH 16. In gilthead seabream, the primary swimbladder inflation is occurring even sooner, starting around DAH 5 and finishing at DAH 15 (Moretti et al., 1999). If this inflation becomes problematic, mainly by the existence of lipid films on the water surface, skeletal deformities such as

lordosis are then caused by the intense swimming effort against the water currents in the rearing tanks. By using water surface skimmers, the water surface is kept free of these lipid films, and the associated deformities are then greatly reduced (Koumoundouros, 2010 and references therein). However, even in this example of an identified factor, it is not certain that the associated problems are successfully solved, or even fully recognised. In this case, the problem still persists in hatcheries today, with a typical occurrence value of 5-10% (Boglione et al. (2013b), and approximately 9-11% in seabream hatcheries (Theodorou et al., 2016). A factor thought to play a role in the over-inflation of the swim bladder is gas super-saturation, which can be easily present in rearing tanks due to various reasons, with the most common being the increase in water temperature (an increase of 1°C represents a 2% increase in gas saturation), or by air trapped in pipes under pressure (Helvik et al., 2009). Moreover, this over-inflation can result in the more serious problem of Swimbladder Stress Syndrome, due to environmental stress from disturbances of abiotic factors, or from handling and transportation, or even originating from a genetic basis (Koumoundouros, 2010).

Deformities of the jaw and the notochord, such as the ones pictured in Fig. 2.4, have been attributed to many different factors in different species. The jaw conditions are usually associated with abnormal values of temperature and salinity (Koumoundouros, 2010), but other factors have also been known to play a part, such as light exposure. These values and their effects are different from species to species, but as an example, light exposure during the early yolk sac stage has been shown to induce jaw deformity in Atlantic halibut *Hippoglossus hippoglossus* (Bolla and Holmefjord, 1988). For the notochord, the aforementioned abnormal temperature and salinity values plus pH and O_2 concentration have been suspected to play a significant role (Koumoundouros, 2010). For example, O_2 deficiency during the somitogenesis of the embryos of red seabream *Pagrus major* has been shown to induce vertebral deformities (Hattori et al., 2004).

Kyphosis is a common vertebral deformity in different fish species, which is usually attributed to Myxosporean parasites, dietary deficiencies, pollutants, or to non-identified factors (Koumoundouros et al., 2002b). Lordosis (Fig. 2.5) starts to manifest itself at the post-flexion stage and towards the end of the metamorphosis (Koumoundouros, 2010) mostly due to abnormal temperature and the existence of strong water currents: if it affects the pre-haemal part of the vertebral column, it is induced by the non-inflation of the swimbladder, and is believed to occur due to a compensation for the lack of buoyancy by an abnormal

oblique swimming orientation. If it affects the haemal part as seen in Fig. 2.6, it can occur in rates up to 70%, and is developing in fish with inflated swimbladder under conditions of intense swimming effort, especially when the velocity of water current in the weaning tanks is high (Koumoundouros, 2010). In general, the existence of strong water currents, and the turbulence generated from the use of air bubbling and near the water inlets have been shown to be causative factors, due to the generation of shear forces that provide drag on the delicate larval body in different directions (Helvik et al., 2009).

Saddleback syndrome (Fig. 2.6) is linked to severe abnormalities of the dorsal fin, and to a depression of the dorsal body profile. It is manifested in seabass starting from the early larval stage as an incomplete formation of the dorsal fin, affecting its shape and number of the dorsal proximal pterygiophores, but has largely unknown causative factors (Koumoundouros, 2010).

The aforementioned sensitivity and significance of the early stages that have been known or strongly suspected to play a crucial role in the manifestation of deformities mostly originates from the intensity of the culture conditions. These have been known to negatively affect the fitness and survival rates of hatchery-reared juveniles compared to wild ones, and induce morphological differences, as documented in a range of fish species. Although malformations have been initially and repeatedly documented in wild populations due to environmental disturbances or pollution, their incidence is significantly smaller than in reared fish (Koumoundouros, 2010). Anatomical differences include increased abnormalities in body shape, pigmentation, lateral line development, and opercular, fin ray, branchiostegal membrane and skeletal deformities compared to juveniles reared in the wild (Le Vay et al., 2007 and references therein). A causative factor is the aforementioned existence of turbulence in culture tanks, which creates shear forces that affect the delicate larval body: wind and tidal waves also create turbulent conditions in the open sea, but fish larvae can to some extent avoid exposure by evacuating regions of strong turbulence, while this is less possible in a rearing tank (Helvik et al., 2009).

2.4. Methods for identification of deformities, and their limitations

2.4.1. Morphometric size analysis, radiography and staining

In aquaculture, the most common method for identification of abnormalities is visual examination of their body and swimming behaviour. Other methods include the use of traditional morphometric analysis on meristic characters of size, radiography, and whole mount staining of cartilage and bone.

Morphometric analysis of size (Fig. 2.7) involves the measurement of metric characters of whole or parts of the fish, and is the most widely used technique in fisheries biology studies. Total length is the character most frequently chosen due to the ease of measurement, which is then linked to others such as weight, age and maturity (Holden and Raitt, 1974). These metric characters are predominantly linear such as total length, occasionally volumetric such as yolk sac volume (Blaxter and Hempel, 1963), and rarely angular such as pectoral angle (Ehrlich et al., 1976) and lordotic angle (Sfakianakis et al., 2006a). Size analysis is not used by itself for the identification and quantification of deformities, but in the context of biometric/morphometric analysis as a biometric (in contrast to geometric) estimate of shape, by comparing one size character to multiple others. It is also used indirectly through the association of the presence of a deformity condition to the developmental state and size of fish (a) at a certain point in time, and (b) as a correlation to a size change (or lack thereof). For example: (1) as mentioned in Cataudella et al. (2011b) in Boglione et al. (2013b), all anomalies in ribs, neural and haemal arches tend to be augmented with size of fish both in European seabass and gilthead seabream. (2) Senegalese sole individuals of different sizes have been shown to present different response to loads and strain on deformed vertebrae according to their size (Cardeira et al. 2012 in Boglione et al. (2013b). (3) in Atlantic halibut, accelerated organogenesis while the body size remains normal results in hypertrophic vertebrae, contributes to vertebral column bending, and finally results in prehaemal lordosis and scoliosis (Lewis-McCrea and Lall (2004) in Boglione et al. (2013b).

Radiography (Fig. 2.5) is the use of X-rays, which have enough energy to penetrate soft tissues but not bone and other hard substances, for the creation of a negative image of the skeletal structures of the fish, thus allowing the evaluation of the development and

identification of pathology in the bones (Hjelde and Baeverfjord, 2009). It is a method occasionally followed in large hatcheries when fingerlings are being sold (for seabass and seabream around DAH 70-90), as proof of their quality.

Staining of cartilage and bone (Fig. 2.6) involves the use of dyes in order to visualise cartilage and bony tissues, in the process of investigating their presence and form. An example of these dyes is the use of alcian blue for cartilage, and alizarin red for bone (Dingerkus and Uhler, 1977). This can be useful in the determination and localisation of possible disruptions during the ontogenetic process that could constitute the origin or manifestation of skeletal deformities (Darias et al., 2010).

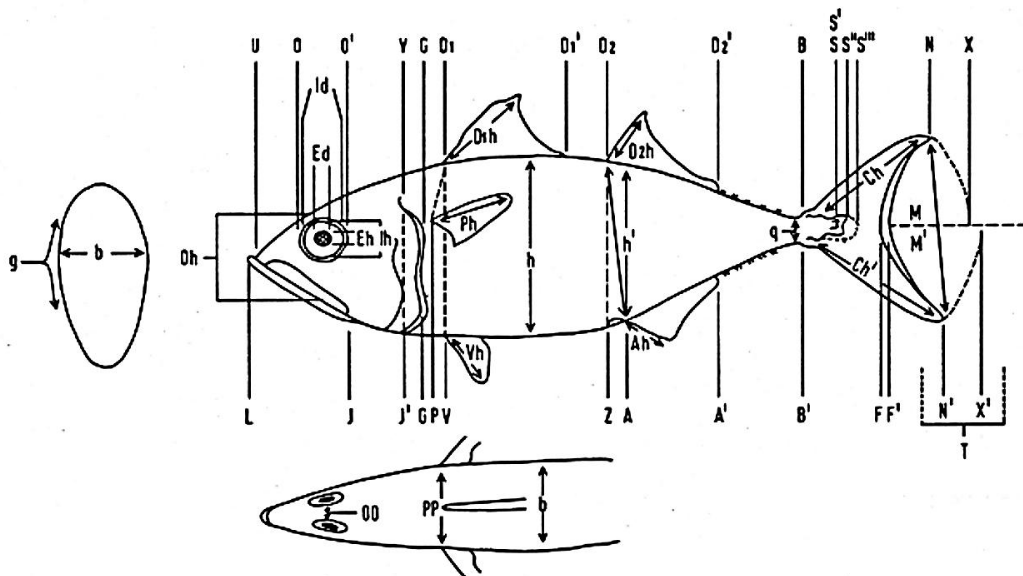

Fig. 2.7: Body measurements on fish, taken for morphometric analysis (Holden and Raitt, 1974).

These methods can provide valuable information for the identification of deformities, but they have been known to present limitations. One of them is that their discriminative power can be problematic when dealing with early life stages of fishes, especially when skeletal development is still lacking, or very basic. As mentioned in Chapter 1, the main time point that the sorting and data recording of deformities takes place is when fingerlings are being sold in the hatcheries, or transferred to the fingerling growing tanks. Occasionally, it also happens earlier, together with the size sorting of specimens with functional swim bladder that

have been separated from the ones without it via the buoyancy test (in hypersaline water, anesthetized specimens with functional swim bladder float, while the others sink to the bottom) (Moretti et al., 1999). However, a major drawback is that skeletal deformities will fully develop to be visually identifiable only when fish reach a certain stage which is species-specific, with the example of European seabass and gilthead seabream being at least 0.5 g in size (Moretti et al., 1999), which is normally reached around DAH 40-50 (Kayim et al., 2010).

Another limitation is their subjective nature when simple visual identification of deformed specimens is involved. For example, in many deformity studies so far, the evaluation of malformations has been based on the estimation of erratic skeletal development by categorizing specimens as being normal, mildly distorted or severely distorted based on the number of deformed or fused vertebrae. The methods that rely on the subjective estimation of deformities by individual researchers or quality control personnel in aquaculture facilities may be adequate in the cases of easily detectable abnormal development, but bias might be introduced, especially when more than one researcher is scoring the deformities (Karahan et al., 2013).

Furthermore, these methods can fail to detect subtle size or shape effects, which may be very important as precursory signs of sublethal or lethal pathologies, or as objects of studies that aim to quantify the impact of any factor that is known or suspected to play a role in the occurrence of abnormalities. In this context, the subjective estimation of deformities can be problematic when a detailed quantification of these effects is needed, especially regarding shape: traditional morphometric analysis provides useful information on changes in metric characteristics, but is strongly limited in describing subtle variation in shape (Rohlf and Marcus, 1993). Methods of traditional morphometrics rely on fish measurements that are taken primarily along the horizontal and secondarily the vertical axis of the body, with the aforementioned rare exceptions of angle or volume data, but thus fail to capture other aspects of shape (Bookstein et al., 1985).

2.4.2. Geometric morphometrics: the landmark and outline methods

An answer to these shortcomings can be provided through the quantification of shape. Early shape analysis cannot play the role of replacing the double staining method for bone and cartilage for the quality control of larval populations, but can complement them at a time point before their earliest application at ontogenetic stages where most of the skeleton is not formed yet (e.g. before the flexion stage). In this context, it can potentially be used for the purpose of prognosis as a helpful predictor of quality, assuming that research establishes that these early phenotypic traits are indeed precursory signs of later deformities. For example, specific shapes can provide a focus on a specific part of the body which can be judged as abnormal, and then staining can illustrate the erratic internal skeletal development. This is discussed further in Chapter 6 (section 6.2.4).

Shape data are obtained through the use of geometric morphometrics, which is an accurate and objective method that permits a mathematical evaluation of shape variation (Adams et al., 2004). Two data types are generally used to describe shape in morphometrics: landmarks and outlines. The former (Fig. 2.8) involves the collection of two or three dimensional coordinates of biologically definable landmarks, preferably being homologous anatomical points (Smith, 1990), whereas the latter (Fig. 2.9) involves the tracing of the body outline. Both approaches then involve the mathematic removal of the non-shape variation and the use of shape variables to statistically compare samples, while also providing a graphical representation of this comparison.

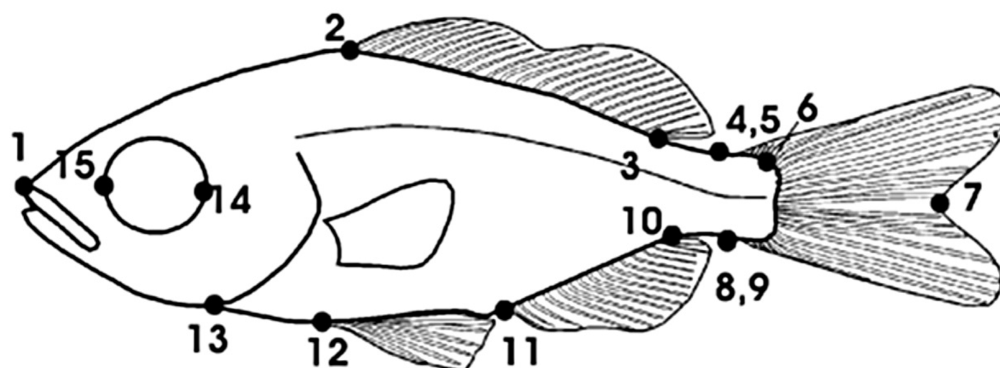

Fig. 2.8: Application of landmarks in sharpsnout seabream *Diplodus puntazzo* (Kouttouki et al., 2006).

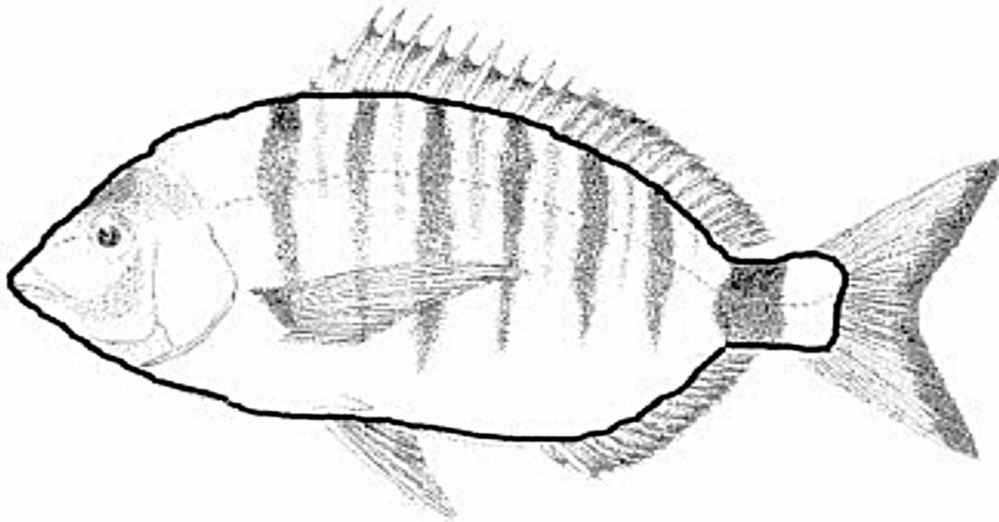

Fig. 2.9: Application of outline analysis in sharpsnout seabream *Diplodus puntazzo* (Loy et al., 2000b).

With respect to fish development, the landmark-based method has been applied on studies of deformities (Loy et al., 2000a; Sfakianakis et al., 2006b; Verhaegen et al., 2007; Georgakopoulou et al., 2010 etc.), on normal ontogeny (Adriaens and Verraes, 2002; Koumoundouros et al., 2005; Kouttouki et al., 2006), as well as on the study of the effects of stocking densities (Ambrosio et al., 2008), and on the genetic and environmental influences on shape variation (Costa et al., 2010). Methodologically, the effect of artifacts such as arching of the body shape and the correction of the landmark data after the process of their capture have also been studied (Valentin et al., 2008). Recent studies on deformities of cultured fish have almost exclusively focused on the use of the landmark method, but in cases of very early larval stages it cannot be applied safely due to the small numbers or absence of discrete and homologous features to which landmarks can be assigned. As a result, these cases usually involve a rather subjective prediction of the homology of landmarks. Another disadvantage of the landmark method is the loss of useful information on shape variation in regions between landmarks (O'Higgins, 1997).

A procedure that overcomes these problems, and the one that is used in this study in terms of geometric morphometrics, is the outline-based method, with Elliptic Fourier coefficients being the most commonly used shape descriptors. They can delineate any type of shape with a closed two-dimensional contour, and the procedure to obtain them involves the decomposition of the shape contour into a sum of harmonically related ellipses (Kuhl and

Giardina, 1982). According to this method, a mathematical approximation of the shape outline of the object is performed, with the first and most basic given by one harmonic ellipse. A second and slightly better approximation is obtained by using two, with further increase in quality when more harmonic ellipses are being used (Fig. 2.10). The total number of harmonics that are necessary to produce a good approximation is small with simple shapes, but increases when the shape contours are more complicated. According to Iwata and Ukai (2002), the use of 20 harmonics produces good results in most shapes, especially in the case of simple and rounded shapes such as fish bodies. The coefficients of the harmonics are then normalised to be invariant with respect to the size, rotation, and starting tracing point of each ellipse. Finally, due to their large number, there is a need to summarise their shape variation information, and are subsequently undergoing a principal component analysis (PCA), with the obtained principal component scores used directly as shape variables (Iwata and Ukai, 2002).

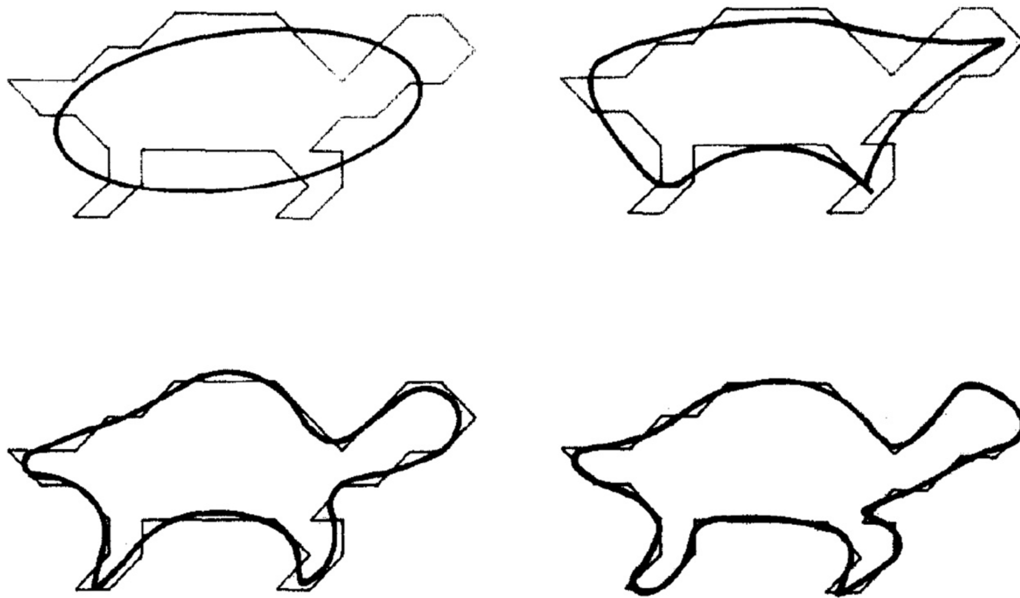

Fig. 2.10: Approximation of a shape outline with the use of 1 harmonic (upper left), 2 (upper right), 4 (bottom left) and 8 (bottom right). The original image is superimposed with its Fourier approximations, showcasing their increasing success by incorporating increasingly higher harmonic content (Kuhl and Giardina, 1982).

Since the outline analysis is a curve-fitting approach, it is particularly useful for describing global form characteristics that are not dependent on homologous points (Lestrel, 1997). The

outline-based method has been used for stock discrimination based on otoliths (for example in Begg and Brown, 2000; Burke et al., 2008) or body shapes of bivalves (Palmer et al., 2004; Krapivka et al., 2007), as well as in the study of the relationship between otolith asymmetry and temporal patterns in the occurrence of late-stage larvae on a tropical reef, in the context of studying the association of larval stress with the deviation of otoliths from their normal shape (Lemberget and McCormick, 2009). It has however been very uncommon in aquaculture research, with a rare example of application in adult specimens of European seabass (Costa et al., 2013).

Comparisons between the results of the landmark and the outline methods have been drawn in studies by Loy et al. (2000b), Russo et al. (2007) and Marquez et al. (2010). In these, the outline method presents the advantage of evaluating information on shape variability from the whole outline, and possibly including axes of variation not shown by the landmark method (Loy et al., 2000b). Furthermore, it is particularly powerful when curvilinear shapes with few or no landmarks are examined. Examples of the preference in applying the outline method when few landmarks exist are the studies of shape variation of roots in Iwata et al. (1998) and Lootens et al. (2007). However, a disadvantage of the outline method is that it cannot reflect ontogenetic changes manifested by the positioning of the landmarks in the inner areas. Therefore, in some cases these two methods have been shown to complement each other, such as in the shell of the striped clam *Ameghinomya antiqua*, where outline analysis focuses only on hard structures, due to the fact that it studies only the outer shell shape, and landmark analysis provides information on the shell scars of the soft structures (Marquez et al., 2010).

2.4.3. Limitations originating from fixation, mounting, handling and anesthetisation

Even when using morphometrics, which is the suggested method of objective size and shape quantification, there are additional drawbacks that are frequently overlooked in quantitative size and shape analyses, in the form of artifacts induced by the necessary processes of fixation, mounting, anesthetisation and specimen manipulation.

After sampling, two crucial steps in the preservation of specimens are performed: fixation and mounting. Fixation is the process of preservation of the configuration of cells and tissues

in the living state (McClung, 1950), thereby preventing post-mortem activities of decay, bacterial and enzyme attack to occur (Carson and Hladik, 2009). For morphology studies, it also increases the visibility and contrast between different tissue elements, and in most cases also enhances staining (Carson and Hladik, 2009). Fixation in shape analyses is crucial due to their labour intensive nature, preventing them from being applied on small fish, especially in larval stages, in their natural state. Mounting is the process of long-term enclosure of the prepared specimens, or their sections, in a medium which will preserve them under suitable conditions for microscopic observation (McClung, 1950). It is performed primarily in order to protect the specimen and its staining from physical damage, and to help improve the clarity and contrast of the image during microscopy (Renshaw, 2007).

Fixatives have been known to be able to influence the geometric morphometric analysis of fishes (Martinez et al., 2013). Their induced morphometric changes on preserved fish have been well documented, but are difficult to predict because of several factors that can be involved. These can be the type and concentration of the preservative, solution osmolality, the duration of preservation, skeletal rigidity, time between death and fixation, the origin of species (marine or freshwater fish), the species concerned, their life stage, the size of the specimens and others (Tucker and Chester, 1984; Sagnes, 1997). Shrinkage is usually observed, as are changes in total length, mass, external appearance of the larval samples and axial curvatures (Fowler and Smith, 1983; Oozeki and Hirano, 1988; Jennings, 1991). This has important implications for studies on subtle shape changes in larval fishes, as various fixation methods have been known to cause shrinkage differences that could be interpreted as morphological differences (Theilacker, 1980). Shrinkage is an unpredictable and confounding factor that is part of the random measurement error introduced in morphometric studies, which poses a considerable obstacle due to the inflation of the amount of artificial variance without biological meaning, thereby increasing the level of this noise to the point of obscuring the biological signal (Fruciano et al., 2016). Algorithms for live-length corrections for larval shrinkage have been developed (for example in Theilacker, 1980; Hjorleifsson and Klein-MacPhee, 1992; Fox, 1985; Santos et al., 2009), although they are by definition limited in providing an approximation of the original measurement, and with varying degrees of success.

Larval fish have also been known to be sensitive to handling, with shape changes introduced due to the fact they are soft-bodied and fragile (Tucker and Chester, 1984). Furthermore, in

later stages an inherent variability in the distribution of fish larvae measurements has been observed, due to individual investigator handling and measuring bias (Pepin et al., 1998). Additionally, anesthetisation is a factor that should also be taken into account, as it has been suggested by Parker (1963) and Theilacker (1980) that it can contribute to the shrinkage of live larvae, due to the interference of common anesthetisation agents such as MS-222 with osmoregulation.

These limitations can compromise the efficiency of the detection of information, and therefore their effect is investigated in detail in Chapter 3, and minimised in the context of a size and shape quantification protocol that can be used to study the effects of axenity, the application of disinfection agents at the egg stage, and bacterial load.

2.5. Bacterial load, disinfection and axenic studies

2.5.1. Effects of bacterial load and disinfection in aquaculture

To meet the constant demand for a high volume larval production, intensive techniques are being used, but as mentioned the intensity of the hatchery conditions, especially the high stocking density, is a factor that has been known to frequently create problems, with one of the most significant being bacterial overgrowth during the culture of egg and larvae, leading to poor hatching rates and high larval mortality (Moretti et al., 1999; Darias et al., 2008; Scarano et al., 2014). In particular, marine fish eggs are very sensitive to this bacterial load, due to the fact that their external surface is a good substratum for the adhesion of bacterial strains (Hansen and Olafsen, 1999). These tend to multiply rapidly under confined environments such as the incubation facilities in hatcheries, which are rich in nutrients (Douillet and Holt, 1994). Infection routes include not only the surface of the egg, but maternal transfer, penetration of the skin, absorption through the gills, ingestion of water for purposes of osmoregulation, and ingestion of feed. In particular, prey items such as the live food *Artemia* and copepods are often heavily contaminated with bacteria, both pathogenic and non-pathogenic, as they are grown in non-sterile nutrient rich environments, which are suitable for rapid bacterial replication (Bowden and Bricknell, 2013 and references therein).

Today's intensive culture methods tend to increase the carrying capacity for microbes of aquaculture systems, in the form of increase in bacterial load, but it is important to note that the identity of the microbes that are present and dominating is often considered more important than their quantity. In these hatchery conditions, the addition of high loads of bacteria and organic matter to the system together with the feed, and the presence of high levels of organic matter as dead larvae and as faeces from live feed and larvae, have been known to induce large oscillations in this bacterial load. Such oscillating conditions select for opportunistic species that can multiply rapidly, such as pathogens and also non-pathogenic strains (Vadstein et al., 2013). These non-pathogenic strains in large numbers in the water can consume excessive amounts of oxygen (Hansen and Olafsen, 1999), or release products toxic to fish (Kawai et al., 1964).

In order to minimise these adverse effects, a disinfection process is applied on the eggs, due to its high importance as the first effective barrier against the transmission of fish diseases. However, antibiotics or antimicrobial agents have been known to produce adverse effects, such as a negative impact on the hatchability and reduced stress tolerance (De Swaef et al., 2015 and references therein). Additionally, they can also have depressive effects on the fish immune response (Alderman, 1988), leave unwanted residues in fish tissues (Steffenak et al., 1991; Samuelsen et al., 1992), pose a threat to human health by exchange of antibiotic-resistant genes to human pathogens through the food chain and in the environment, and their extensive use has led to the acquisition of antibiotic resistance in aquaculture environment bacteria (Scarano et al., 2014).

2.5.2. Bacterial control with microbial maturation

An optimal approach to bacterial control would be to prevent the invasion and proliferation of pathogens in the aquatic environment. To obtain this, techniques for continuous water disinfection include ozonation and ultraviolet irradiation. Ozone is thought to impart water quality improvements by oxidizing larger and relatively complex organic molecules into smaller and more biodegradable ones, while UV irradiation is used to destroy ozone residuals (catalyzing the conversion of O_3 to O_2) and to denature the DNA of microorganisms, causing the microorganisms to die or lose their function (Summerfelt, 2003). However, a problem associated with these techniques is that they do not specifically target the pathogens, but all

bacteria present. Thus, their application disturbs the natural microbial balance in the water. Furthermore, such an environment with a low number of bacterial species, rich in nutrients and with frequent perturbations, especially in periods of live feed provision, promotes the selection for opportunistic microorganisms with a relatively high growth rate, and with a high capacity to exploit nutrients and increase in population size. These are called *r strategists* (De Schryver and Vadstein, 2014 and references therein). Several of these fast-growing opportunists can be classified as facultative pathogens, and even though they are not always pathogenic (as dictated by the expression of virulence factors), their interactions with fish and shrimp larvae can have detrimental results, especially under stress conditions or due to their poorly developed immune system (De Schryver et al., 2012). The virulence of micro-organisms that infect aquaculture systems is thought to be closely related to the expression and release of virulence factors and the formation of biofilms, both of which are regulated by quorum sensing (QS), which is a type of cell-to-cell communication in bacteria (Zhao et al., 2015).

Instead of their elimination, an alternative method to prevent the negative effects of the opportunistic microorganisms is through the promotion of their competition. This can be achieved by making a selection in the water for slow-growing non-opportunistic competition specialists, known as *K strategists*. These create high interspecific competition among crowded bacterial populations, and are able to grow in a stable microbial environment where there are scarce resources per cell (De Schryver and Vadstein, 2014). Through their proliferation, a highly diverse but also stable microbial community, relatively insensitive to perturbations in the organic matter concentration in the water, can be obtained. Additionally, the opportunities for an invader to find a niche in a highly diverse ecosystem are thus considerably decreased. This is the idea behind the concept of microbial maturation, where the conditions promoting the growth of K strategists are achieved through the filtration of the water for the removal of organic material and bacteria, and subsequently through its passage over a biofilter containing a microbial community that has been allowed to mature, and thus is mainly composed of K strategists (De Schryver et al., 2012 and references therein). The concept has been successfully applied in recirculation systems, where K-selected systems have been known to present a more stable microbial community composition in the incoming water compared to a conventional flow-through system (Attramadal et al., 2014). In that study, the selection for K strategists was also met with a 65–70% increase in survival of cultured Atlantic cod *Gadus morhua* larvae.

2.5.3. Axenic and gnotobiotic studies

Today, our understanding of host-microbial interactions and the specific contribution of microbial communities to the biology and pathobiology of the host is being obtained, to a notable degree, from studies using animals raised under gnotobiotic conditions. The word gnotobiotic originates from the Greek words 'gnosis' (knowledge) and 'bios' (life), and refers to an experimental environment in which all microorganisms are defined in the best possible degree, or in the case of axenic rearing, excluded. Gnotobiotic conditions are accomplished by raising germ-free animals in the absence of any microorganisms, and in these axenic conditions specific known microbial species or more complex consortia are then inserted (Pham et al., 2008).

It is a concept originally attributed to Nuttal and Thierfelder in 1896, who in response to a hypothesis by Louis Pasteur in 1885 that animal life would be impossible in the absence of microorganisms, used aseptic Caesarean section to produce the first germ-free animals, guinea pigs, and raised them for up to 13 days. This was soon followed by successful production of germ-free chickens, goats, birds, amphibians etc. (Pham et al., 2008). In fish larvae, examples of such gnotobiotic rearing systems include the use of zebrafish *Danio rerio* (Rawls et al., 2004), European seabass *Dicentrarchus labrax* (Dierckens et al., 2009; Schaeck et al., 2016), cod *Gadus morhua* (Forberg et al., 2011) and tilapia *Oreochromis niloticus* (Situmorang et al., 2014).

Rearing of fish larvae in the presence of known bacteria provides a means of eliminating bacterial interference that tends to confound experimental results. They also are an excellent platform to study microbe interactions within the physiological context of a living host. This platform is also used in the study of how microbial colonisation or microbial products affect crucial biological processes of the host such as gene expression, immunity, lifespan, physiology and development, and in the selection of bacteria that restrict the growth of harmful opportunistic pathogens (Munro et al., 1995; Pham et al., 2008).

2.5.4. Associations with deformities

The presence of bacteria has been associated with abnormalities and deformities, but data have been very scarce, and not linked to any effects originating from metabolic changes, but mostly to pathological causes. Narrow tail fins were witnessed by Bergh et al. (1989), Pittman et al. (1990) and Morrison and MacDonald (1995) during the larval development of Atlantic halibut *Hippoglossus hippoglossus*, and were hypothesized to originate from the colonisation of tail fin wounds by filamentous or rod-shaped bacteria that were not necessarily pathogenic, with these wounds possibly caused by strong tank water currents. Harboe et al. (1994) also hypothesized that bacterial infections might induce jaw deformities in the same species in the yolk sac stage. According to Morrison and MacDonald (1995), the witnessed bacterial invasion of the larval head tissues was associated with the anterior parts of the jaw cartilages being pushed apart, and a disintegration of the thin oral membrane, leading to a development of a gaping jaw condition. Furthermore, in adult fish malformations have been associated with pathogenic bacteria, such as *Mycobacteria neoaurum* and *Aeromonas salmonicida* in Atlantic salmon (the former upon spread of the infection to the skeleton, and the latter upon contraction of the collagen of the scar tissue), *Arcobacter cryaerophilus* inducing upper jaw deformations (Austin and Austin, 2007; Plumb and Hanson, 2011).

Apart from bacteria, viruses and parasites have also been known to induce malformations. Examples include (a) the infectious hematopoietic necrosis virus (HNV), which is known to be responsible for malformed heads, scoliosis, or lordosis in 5–60% of survivors in rainbow trout *Oncorhynchus mykiss*, chinook *Oncorhynchus tshawytscha*, sockeye *Oncorhynchus nerka* and chum salmon *Oncorhynchus keta*, (b) nervous necrosis virus (NNV) inducing enlarged swim bladders and vertebrae deformities in many species, (c) the myxosporean parasite *Myxobolus cerebralis* inducing head deformities and/or curvature of the spine, which are signs of the whirling disease in salmonids (Plumb and Hanson, 2011).

Apart from the above associations of bacteria, and some pathogens in general, with abnormalities, to the best of our knowledge there have been no reports or studies identifying and quantifying the potential size and shape effects of bacterial load on fish larvae. The only

source of relevant information exists on the morphological effects of the differences between xenic and axenic fish larvae. However, even they have not provided detailed knowledge on any size and shape effects, but have been focused mainly on the development and maturation of the gastrointestinal tract on a cellular level such as in zebrafish *Danio rerio* (Rawls et al., 2004; Bates et al., 2006) and seabass (Rekecki et al., 2009), and to a limited degree in differences in total length: Forberg et al. (2011) did not witness a difference in the total length between gnotobiotic and xenic cod *Gadus morhua* larvae, while Rekecki et al. (2009) found a reduced total length growth of xenic seabass larvae versus germ-free on DAH 6, 9, 12 and 15.

Regarding the use of disinfection agents and antibiotics, the administration of antibiotics such as penicillin procaine, dihydrostreptomycin sulfate and oxytetracycline-HCl has been shown to induce teratogenic malformations in the progeny of adult spring chinook salmon *Oncorhynchus tshawytscha* that are manifested from the fry up to the yearling stages (De Cew, 1972). Another example is the use of kanamycin and chloramphenicol, which has been shown to be teratogenic to zebrafish larvae of DAH 2-3 upon exposure at the embryonic stage (Song et al., 2010). The use of antibiotics has been hypothesized to be able to cause adverse effects on the eggs, as unwanted and unknown interactions with the target organism may occur (Marques et al., 2006). Furthermore, they can be considered an extra stress factor detrimental to eggs that come from a batch of suboptimal quality (Schaeck et al., 2016). Reported disinfection deformities from ozone in larval gilthead seabream include enlarged head and a curved spine at mid-body, tail and at the base of the head, which caused it to be angled dorsally (Ben-Atia et al., 2007). Egg disinfectants have been hypothesized to affect the caudal fin development in the embryos of Atlantic halibut *Hippoglossus hippoglossus* (Bergh et al., 1996), but have largely not been associated with visible deformities (De Swaef et al., 2016).

Due to the lack of information on any specific size and shape effects of the aforementioned factors, an effort is made in Chapters 4 and 5 to quantify them, discuss their impact in the ontogeny of European seabass, and attempt to link them to any witnessed abnormalities in literature, manifested in its very early life stages.

Chapter 3

Protocol for quantitative shape analysis of deformities in early larval European seabass *Dicentrarchus labrax* L.

This chapter is based on:

Spyridon Nikolakakis[1,2], Peter Bossier[1], Grigorios Kanlis[3], Kristof Dierckens[1] and Dominique Adriaens[2] (2014). Journal of Fish Biology (2014) **84**, 206 – 224

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Abstract

This study established an optimized protocol for quantifying the shape variation of newly hatched larvae of European seabass *Dicentrarchus labrax*, focusing on the effect of fixatives and mounting on body shape from hatching until 14 days post hatching, while also minimizing the error introduced by handling. This assessment was performed based on both biometric and geometric shape data, with the latter relying on outline based elliptic Fourier analysis. The fixation and mounting effect on the total length and shape of newly hatched larvae of *Dicentrarchus labrax* was tested for four fixation treatments: (1) 8% formalin, (2) 70% ethanol, (3) 8% formalin for 48 h and then to 70% ethanol and (4) 3% phosphate-buffered glutaraldehyde. The analyses showed that no significant size and shape effect was observed on anaesthetized specimens 5 months post-fixation in glutaraldehyde, and that the glycerol mounting process of specimens fixed in glutaraldehyde provided the best results for further ontogenetic qualitative and quantitative analysis. The protocol proved to be sufficiently sensitive to even quantify and visualize subtle pre-fixation shape differences originating from a different egg batch.

3.1. Introduction

Aquaculture increasingly focuses on improving larval fish quality, but in spite of the continuing progress in rearing methods, morphological abnormalities still pose a significant challenge in Mediterranean finfish aquaculture. Deformities, including reduction of the gill cover, pre-haemal lordosis and kyphosis, pugheadness, jaw deformities and swimbladder abnormalities, often appear in early stages of larval development (Koumoundouros, 2010) and affect the phenotype of the fish, their survival, growth rate, behaviour and finally the production cost and effectiveness of the hatcheries (Sfakianakis et al., 2006). They can lead to fish that are not easily marketable, or even to mortalities at the larval stage or later. The first malformations in European seabass *Dicentrarchus labrax* (L. 1758), such as a twisted spinal cord and jaw abnormalities, can appear immediately after hatching (Barahona-Fernandes, 1982), and if they affect 10% or more of the newly hatched population, the discard of the entire batch is recommended (Moretti et al., 1999).

In hatcheries, detection of the early manifestations of abnormalities is crucial, as these can be signals of more pronounced deformities in later stages. Early embryonic and larval stages, however, generally pose few characteristics showing distinguishable variation, and therefore a powerful tool is required for its detection and quantification. Identification of deformities has mainly been done through the use of traditional biometric analysis, radiography and whole mount staining, most often based on estimations of erratic skeletal development by categorizing specimens as being normal, mildly distorted or severely distorted (e.g. based on the number of deformed or fused vertebrae). The limited discriminative power of these methods, however, becomes clear when dealing with early life stages of fishes that lack mineralized elements. Furthermore, traditional biometric analysis is not well suited in describing subtle variation in shape (Rohlf and Marcus, 1993). Methods of traditional morphometrics in fishes rely on measurements that are taken along the longest axis of the body, and thus fail to capture other aspects of shape (Bookstein et al., 1985). In this study, metric data are complemented with shape data obtained from geometric morphometrics, which is a more accurate and objective method to evaluate shape variation (Adams et al., 2004).

Two data types are generally used to describe shape in geometric morphometrics: landmarks and outlines. The former involves the collection of two or three-dimensional co-ordinates of biologically definable landmarks, preferably anatomically homologous points (Smith, 1990), whereas the latter involves digitized points along an outline. Both approaches then involve a standardization to remove all non-shape variation, yielding a set of shape variables that allow a statistical testing of hypotheses, as well as a graphical representation of their variation. Studies on deformities of cultivated fishes have mainly focused on the use of the landmark method but in very early larval stages it cannot be applied safely due to the absence of discrete and homologous features to which landmarks can be assigned. Another disadvantage of the landmark method is the loss of useful information on shape variation in regions between landmarks (O'Higgins, 1997).

A procedure that overcomes these problems is the outline-based method, with elliptic Fourier descriptors being the most commonly used shape descriptors. It involves the decomposition of a shape contour into a sum of ellipses (Kuhl and Giardina, 1982), and can delineate any type of shape with a closed contour without the need for homologous points (Lestrel, 1997). This is particularly useful in very early larval stages similar to the ones of this study, where their position changes rapidly or they might be too few or even absent. Comparisons of the landmark and outline methods highlight the cases where the study of shape variability from the whole outline is advantageous (Loy et al., 2000b; Russo et al., 2007; Marquez et al., 2010).

So far, the outline method has been uncommon in aquaculture research, with rare applications in adult specimens of *Dicentrarchus labrax* (Costa et al., 2013). Considering the rounded form of larval fishes, having their finfolds undifferentiated until the onset of the juvenile period (Balon, 1975), this approach can be sufficiently powerful to detect the early onset of deformities in cultured fishes, even before the first signs of skeletal differentiations have occurred. To achieve this, the first goal of this study was to establish a protocol for performing a detailed shape analysis on larval fish using outline-based geometric morphometrics, from the image acquisition up to the actual shape analysis.

The successful use of a detailed analysis of shape in any ichthyological study dealing with morphological changes or differences depends on the optimal reduction of possible artefacts caused in the preparatory steps: fixation and mounting. Fixation in shape analyses is crucial

due to their labour-intensive nature which prevents them from being applied on live fishes. Consequently, the second goal of this study was to provide an optimal protocol for fixing the specimens, and mounting them for purposes of improved preservation and handling. The effects of different fixatives and preservatives on fishes are difficult to predict due to the involvement of several factors (Tucker and Chester, 1984; Sagnes, 1997), but they have been known to be able to influence the geometric morphometric analysis of fishes (Martinez et al., 2013). Shrinkage is usually observed, as are changes in total length, mass, external appearance of the larval samples and axial curvatures (Fowler and Smith, 1983; Oozeki and Hirano, 1988; Jennings, 1991), and has important implications for studies on subtle shape changes in larval fishes. Another factor that should be taken into account is handling: there is an inherent variability in the distribution of fish larvae measurements due to individual investigator handling (Pepin et al., 1998), and the method involved causes shrinkage differences that could be interpreted as morphological differences (Theilacker, 1980). Shape changes might therefore be introduced, especially due to the fragility of the specimens. Furthermore, anaesthetization is a factor that should be examined as well, as it has been suggested by Parker (1963) and Theilacker (1980) that it contributes to the shrinkage of live larvae because MS-222 interferes with osmoregulation.

As far as is known, a detailed morphometric analysis on the effect of handling, anaesthetization, fixation and mounting on the shape of fish larvae is still lacking. This study tests the length and shape effects of four common fixation treatments (using formalin, ethanol, formalin followed by ethanol and glutaraldehyde), and tests a mounting technique on the specimens, analysing larvae of *Dicentrarchus labrax* from hatching until 14 days after hatching (DAH). These procedures are tested and critically evaluated, and a protocol is suggested, yielding the lowest amount of artificially induced deformations. With this, it is hoped to provide a tool for future morphometric analyses during the early larval stages by establishing a protocol that can allow an accurate mathematical quantification of shape variation, while taking into account the detrimental shape effects of the aforementioned procedures, an issue which is frequently overlooked in morphometric studies.

3.2. Materials and Methods

3.2.1. Fixation treatments, procedure and sample selection criteria

The shrinkage effect of four different fixation techniques was tested in two experiments where fertilized eggs of *Dicentrarchus labrax* obtained from the Ecloserie Marine de Gravelines hatchery in France were kept at a constant temperature of 16°C, range ±1°C and continuous blue light with an intensity of 50 cd sr m^{-2}. The eggs were acclimatized immediately after arrival for 4 h in UV-treated natural sea water, with a salinity of 36 g l^{-1} and continuous aeration.

The larvae were stocked in 40 ml screw cap vials with filtered autoclaved sea water, at a density of 12 larvae per vial. They were reared until DAH 14, and fed with 35 axenic *Artemia franciscana* per vial once every 2 days from DAH 7 onwards. Axenic *A. franciscana* were prepared according to the method of Marques et al. (2004). The larvae of DAH 7 and 14 were not fed on their sampling day. The remaining uneaten *A. franciscana* were not removed from the vials, nor was there any water exchange during the whole culture period. This method was chosen as a general protocol that can also be used in future studies of axenic eggs and larvae of *Dicentrarchus labrax*, in accordance with the procedure described by Dierckens et al. (2009). It was also used to permit sampling until DAH 14, which might have been hindered by mortalities if normal xenic water and xenic *A. franciscana* were used.

In the first experiment, the larvae were divided into three groups: the larvae of the formalin group (group F) were fixed in 8% unbuffered formalin in fresh water (pH 3.7), those of the ethanol group (group E) in 70% ethanol (pH 7.6) and those of group FE in 8% formalin for 48 h and then transferred to 70% ethanol. In the second experiment, larvae of the glutaraldehyde group (group G) were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), prepared according to Glauert (1987), frozen immediately after preparation and kept at -20°C until the time of application. The fixation time was 5 months. The specimens could be sampled at any time during this period, but this choice was made in order to allow ample time for the time-consuming process of photographing

the larvae. For clarity, treatment groups are coded according to the fixative applied (F, E, FE and G) and DAH 2, 7 and 14. In the case of treatment FE, specimens in formalin before their transfer to ethanol were coded with "48 h". In the case of treatment G, three groups were fixed without prior anaesthetization (code "na") to test the effect of anesthetic on shape. Details of the groups are given in Table 3.1.

Group code	Fixative	Anaesthetisation	DAH 2	DAH 7	DAH 14
F	8% Formalin	Yes	F2	F7	F14
E	70% Ethanol	Yes	E2	E7	E14
FE	8% Formalin for 48 h	Yes	FE2.48 h	FE7.48 h	FE14.48 h
	70% Ethanol		FE2	FE7	FE14
G	3% Phosphate-buffered glutaraldehyde	Yes	G2	G7	G14
		No	G2.na	G7.na	G14.na

Table 3.1: Codes used for the experimental units according to fixation of *Dicentrachus labrax* larvae, DAH (days after hatching), time of fixation (5 months for all groups, 48 h in formalin for specimens of treatment FE before their transfer to ethanol for 5 months) and application of anesthetic (all groups except G.na).

In both experiments, at DAH 2, 7 and 14, a minimum of 30 specimens per fixative group were collected from their vials, anaesthetized with MS-222 and photographed with an Olympus Altra 20 digital camera mounted on an Olympus SZX9 microscope via AnalySIS GetIT software (www.olympus.com). Larvae were placed horizontally on glass slides facing to the right in a 100 µl seawater droplet, with their left and right palatoquadrate cartilages vertically aligned (Fig. 3.1). After taking their photograph, each larva was placed in an individually marked Eppendorf vial filled with 1.2 ml of fixative at room temperature (20°C), and photographed again after 5 months, with the same volume of 100 µl of fixative on top of each specimen. Group FE larvae were also photographed 48 h after fixation, immediately before their transfer to ethanol. The use of individually marked Eppendorf vials allowed evaluation of length and shape changes of each individual specimen in each step of the process.

For anesthesia, 1.8-4.2 ml of an MS-222 solution of 1.2 g l^{-1} was added to each stocking vial, resulting in a slow stopping of activity within 10 min, after which heartbeat was observed to stop. Sudden overdoses were prevented, as preliminary tests showed that they may react violently to it, thus introducing deformation artefacts not related to the effect of the fixatives. Furthermore, in these preliminary tests, larvae had visibly unaffected body shapes when not anaesthetized prior to fixation. Therefore, the effect of the anesthetic needed to be included in the analysis as a factor potentially influencing larval body shape. As such, in the second experiment, an extra group of a minimum of 35 larvae was fixed in phosphate-buffered glutaraldehyde on 2, 7 and 14 DAH without prior anaesthetization. For these larvae, individual photographs were taken only post-fixation, but were sampled from the same vials of the glutaraldehyde treatment, thus allowing a comparison between these two groups. The decision not to test the effect of anaesthetization with the other treatments was based on preliminary results where the best performance of glutaraldehyde with anaesthetisation was already apparent, and in order to avoid subjecting the larvae into any further potential risk of pain or distress by working with the other treatments, which did not appear to be promising.

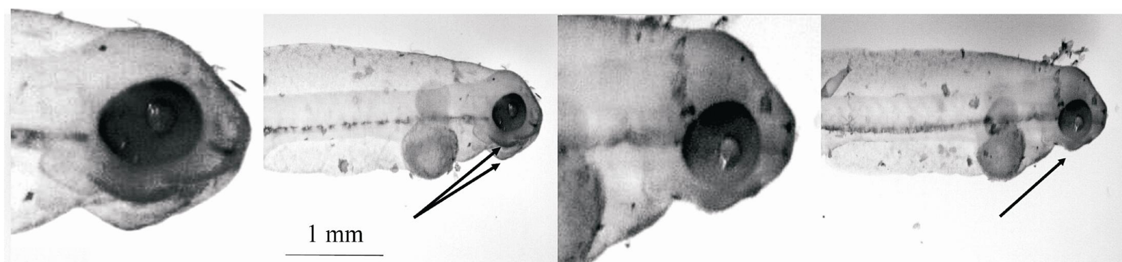

Fig. 3.1: Photographs of a *Dicentrarchus labrax* larva with its two palatoquadrates not vertically aligned (left, indicated with arrows) and in the proper vertical position (right).

As specimens were fragile, a standardized procedure was established for the selection of larvae for further shape analyses. In order to maximize their explanatory power for studying deformities induced directly by the fixative, the following specimens were excluded: the ones that were not sedated properly, that were not completely submerged in the fixative and those that were asphyxiating or developing visible signs of stress and tissue contraction. Due to this, some specimens were severely bent after the end of the fixation period, which did not allow their positioning in a completely flat position, and hence the reliable acquisition of size and shape data. Based on these selection criteria, they were excluded from the analysis, as this protocol aims to examine subtle shape variations. The percentages per group are

shown in Fig. 3.2. There was a highly significant effect of the fixative on the numbers of discarded specimens (ANOVA, p<0.001 at the nominal signifance level of alpha=0.05, which is also the critical significance value used throughout the thesis). The Tukey's post hoc pair-wise comparisons indicated that they did not differ significantly between treatments F and FE, but treatments G were significantly higher than those of F and FE (P<0.01 in both cases), and the non-anaesthetized specimens of treatments G were significantly fewer than those for all the other groups (p<0.001 against G, p<0.05 against F and FE). Group E larvae clearly showed a high degree of body and fin folding and bending, and were not included in further analyses.

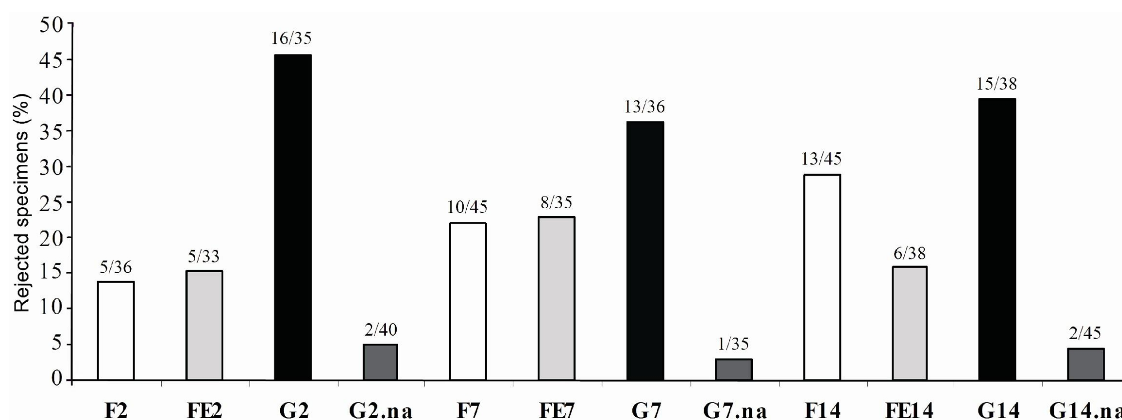

Fig. 3.2: *Dicentrarchus labrax* larvae that did not meet the analysis selection criteria (white bars for treatment F; light gray for treatment FE; black for treatment G; dark gray for G.na; see Table 3.1). The numbers of discarded specimens out of all sampled larvae are included on top of each bar.

3.2.2. Optical distortion testing

To avoid larvae becoming dehydrated, they were submerged in water while taking photographs. The 100 µl drop spread around each specimen was the minimum in order to avoid optical distortions as a result of the curved surface of the water droplet. Optical aberrations using the stereo microscope were avoided by using lenses corrected for spherical aberration, and a ×0.5 planar lens was chosen over ×1 and ×1.5 lenses due to its deeper focal range. The possibility of this droplet inducing an optical distortion was tested using photographs of a calibration glass slide with a 2 mm cross under the ×0.5

lens with magnifications of ×20, ×25 and ×28.5 (as used during the experiment). The cross was placed at five different positions within the photograph frame: bottom left, bottom right, centre, upper left and upper right. The photographs were taken with and without 100 µl of seawater, and the length of the horizontal and vertical lines of the cross was measured in each photograph. There was no significant effect of the application of the water droplet on the lengths of the cross lines in any magnification (for ×20: paired t-test, $p>0.05$, $SD_{water_drop}=0.011$ mm; for ×25: paired t-test, $p>0.05$, $SD_{water_drop}=0.006$ mm; for ×28.5: paired t-test, $p>0.05$, $SD_{water_drop}=0.008$ mm), and therefore no optical distortion effect was induced. As such, all further analyses were based on data obtained with the application of the droplet.

3.2.3. Staining and mounting

In order to establish a complete deformation study protocol that includes long-term storage of the larvae in a compact form for further qualitative and quantitative analyses, a number of different staining and mounting techniques were applied in order to determine whether they induce artefacts. Mounting media can be classified into two categories: organic and aqueous (Renshaw, 2007). For this study, an aqueous mounting medium (glycerol) and three organic media (DPX by Sigma Aldrich, www.sigmaaldrich.com; toluene-based mounting medium by Richard Allan Scientific, www.thermoscientific.com; and transparent nail polish) were used. To avoid fungal contamination, a few crystals of thymol were added to the glycerol solution. Specimens to be mounted in aqueous media, such as glycerol, can be mounted straight from the aqueous phase with no dehydration or clearing (Renshaw, 2007). In the case of organic media, however, the specimens were placed for 10 s in 80% ethanol, and twice quickly in 96% ethanol prior to staining and mounting, following the method of Gurr (1962). This prevented the presence of a whitish colour that reduced the clarity of the specimens.

After 5 months' fixation, the specimens were washed with distilled water, and stained with toluidine blue and haematoxylin-eosin to increase the contrast between the larvae and the background, in order to improve the outline tracing for the shape analyses. The specimens were then mounted by being placed on a glass slide inside a pool of mounting medium within a paraffin ring, and sealed with a cover glass on top. For this, a modified version of the nematode mounting method of Maeseneer and d'Herde (Hooper, 1986) was followed. One of

the factors that was clearly introducing deformations in this method was the placement of the slide with the mounted larvae on top of a heated metal strip at 65° C for a few minutes, in order to melt the paraffin ring for attaching the cover glass. These distortion effects were also present when the temperature of the metal strip was raised gradually over a few minutes. Consequently, the use of the heated metal strip was omitted. Furthermore, the immediate addition of pure glycerol produced specimen distortion, and a series of consecutive glycerol baths was applied instead.

The modified procedure was then as follows. A 5.7 cm × 1.7 cm orthogonal metal frame was dipped into liquid paraffin kept at 68° C, and used to apply a wax ring on a glass slide. The paraffin was allowed to cool and dry for a few minutes. In this way, four consecutive wax rings were applied on top of each other in order to increase the height of the wax formation and hence to prevent the squashing of the larvae under the cover glass. The next steps were performed quickly in order to avoid any dehydration of the specimens caused by their prolonged exposure to air. A total of 3 ml of distilled water was added on top of each specimen in order to remove traces of fixative and excess water was removed by drying it very gently with the tip of an absorbent paper towel. This was repeated three times. Staining was then performed with a drop of toluidine blue or haematoxylin – eosin on top of each specimen (both staining methods gave equally good results).

In the case of glycerol, two different methods were applied: (1) specimens were placed in a series of four 30 min baths (distilled water, 25% glycerol, 50% glycerol and 75% glycerol). A maximum of three specimens per slide were picked up with a 400 μl micropipette, and placed parallel on the prepared slide. In order to avoid distorting the larvae, pipette tips with a cut edge were used. Finally, a few drops of pure glycerol were added on top. (2) a few drops of pure glycerol were applied on top of a very thin dried layer of transparent nail polish over each of the three specimens. In the cases of DPX and Richard Allan Scientific mounting medium, these were applied directly on the three specimens without any intermediate baths. Finally, a fresh wax ring was applied to all preparations, and a cover glass was very gently placed on top of it. After a few minutes, the paraffin solidified and enclosed the specimens in a pool of the mounting medium. Finally, nail polish was applied around the wax ring to seal the slide.

3.2.4. Size analyses

The total length of each larva was measured using ImageJ v1.46 (Abramoff *et al.*, 2004), with an accuracy of 0.001 mm. The shrinkage ratio was calculated as total length at pre-fixation minus that at post-fixation, divided by pre-fixation total length. Differences in total length and shrinkage ratios between the different treatments were examined using PAST 3.16 (Hammer et al., 2001). The normality of the distribution of the total length and shrinkage ratios of the larvae was tested with a Shapiro–Wilk's test, and the homogeneity of group variances with Levene's test. In the cases where the conditions of normality and homogeneity of variances were not met, the non-parametric Kruskal-Wallis test was performed, with Bonferroni-corrected P-levels for post hoc Mann-Whitney pair-wise comparisons. When the conditions were met, the one-way ANOVA test was applied to determine whether there were significant differences, followed by a Tukey's post hoc test for pair-wise comparisons.

As the larvae of treatment G came from a different egg batch than those of F and FE, a test was performed for difference in pre-fixation larval total length at the onset of the treatments. There was a highly significant effect of the egg batch origin of the specimen groups on *L*T at all three ages (for DAH 2: ANOVA, $p<0.001$; for DAH 7: Kruskal-Wallis, $p<0.001$; for DAH 14: Kruskal-Wallis, $p<0.001$). Post hoc pair- wise comparisons indicated that the total length of the specimens at all three ages was not significantly different between groups F and FE, but was highly significantly different between the G and both the F and FE groups ($p<0.001$ in all cases). Consequently, the pre-fixation total length had to be taken into account in the next steps of the analysis, and for this purpose the individual shrinkage ratios of each specimen were used.

3.2.5. Shape analyses

To perform an outline-based morphometric analysis, the outlines of the larvae were traced manually on the images using Corel Draw 12 (Corel Corp.; www.corel.com). Specimens with widely opened mouths were traced according to the best approximation of their shape corresponding to their mouths closed, relying on the orientation of the upper jaw and their palatoquadrates. Outlines of larval profiles were traced in black on a white background and analysed using Shape 1.3 (Iwata and Ukai, 2002). A total of 20 harmonics was used to

describe the observed shape, with a normalization based on the first harmonic, involving a standardization for position, size and orientation of the specimens. According to Iwata and Ukai (2002), the use of 20 harmonics produces good results in most shapes, and is hence suitable to describe the simple shape of the larvae of *Dicentrarchus labrax*.

To reduce the number of variables, a principal component (PC) analysis (PCA) was performed on the elliptic Fourier coefficients using Shape. All further statistical analyses were performed using PAST 3.16 (Hammer et al., 2001). The analysis of between-group variation was done through a canonical variate analysis (CVA) and a multivariate analysis of variance (MANOVA) on the scores of all effective PCs, which Shape defines as the PCs with percentages of explained total variation >1/77, with 77 being the total number of analysed coefficients. This allowed a reduction in the dimensionality of the dataset from 77 components to between six and nine, depending on the groups being pooled. Consequently, without losing relevant information, between 93.0 and 94.5% of the total variation was included in the datasets for further analyses.

Regarding the assumptions for MANOVA, multivariate normality was checked with Mardia's multivariate skewness and kurtosis test, and the equivalence of covariance matrices with Box's M test. In the cases that were not met, a one-way non-parametric multivariate analysis of variance (PERMANOVA) was performed with 10000 permutations. When significant differences were detected, a post hoc test was applied. In the case of MANOVA, Bonferroni-corrected Hotelling's p-values were used. In the case of PERMANOVA, the Bonferroni-corrected p-values were calculated by permutation tests on distances, with these being the point-to-point Mahalanobis distances between pairs of individual multivariate observations (Anderson, 2001).

To test the significance of shape changes before and after fixation, and to determine whether each fixative has a different effect on shape, MANOVA or PERMANOVA tests were performed on the relevant PC scores of each separate age group, with prior anaesthetization. Similarly, in order to examine whether the fixatives induce different shape changes depending on the age of the specimens, the same tests were performed per fixative treatment. This included all 5 month fixation groups, and also the groups of 48 h fixation in the case of treatment FE, and the groups that were not anaesthetized in the case of treatment G. When significant differences were detected, the Mahalanobis distances between the centroids of the

corresponding groups were calculated to examine which groups exhibited the largest differences.

In order to provide a percentage of specimens that were affected by the fixation, a PCA was performed on the Fourier coefficients of each fixation group per age class, followed by a CVA linear classification. The post-fixation specimens classified to belong in the pre-fixation group were identified as not affected by fixation. In the cases where some specimens of the pre-fixation group were classified as post-fixation, they were discounted, as they cannot be considered to represent an effect of the treatment.

Due to the fact that larvae for the glutaraldehyde treatment came from a different egg batch, a test similar to the one performed for the total lengths of the specimens had to be performed in order to check whether the groups before fixation had significant differences in shape. There was a highly significant effect of the egg batch origin of the specimen groups on shape at all three ages (in all cases: PERMANOVA, $p<0.001$). Post hoc pairwise comparisons indicated that there were no significant differences between the F and FE groups at DAH 2 and 7 ($p>0.05$), but at both ages there were highly significant differences between G and both F and FE ($p<0.001$ at both ages). On DAH 14, there were significant differences between all groups ($P<0.05$ between F and FE and also between F and G, $p<0.001$ between FE and G). Therefore, due to the fact that there were significant pre-fixation shape differences between the specimen groups, they had to be taken into account in the next steps of the analysis. They are visualized in Fig. 3.3 with a scatterplot of CVA on the PC shape scores of all age groups. On DAH 2, these differences are evident, as the clusters of treatments F and FE clearly occupy a different morphospace than the cluster of G.

3.3. Results

3.3.1. Size effects

For treatment G, the pairwise comparisons indicate that there was no significant shrinkage effect apart from the non-anaesthetized specimens of DAH 2 (Kruskal-Wallis, $p<0.001$; for DAH 2: $p_{na}<0.05$). For treatments F (formalin) and FE (formalin to ethanol), according to the pair-wise comparisons in every age class a highly significant shrinkage effect was observed,

including treatment FE at 48 h (in both cases: Kruskal-Wallis, $p<0.001$). As far as the shrinkage ratios are concerned, a highly significant effect of the fixative was observed (Kruskal-Wallis, $p<0.001$), and according to the pairwise comparisons the ones of treatment F were always highly significantly lower ($p<0.001$ in all cases) than the ones of treatment FE of the corresponding DAH (Fig. 3.4). No pattern could be detected for age-dependent shrinkage in any fixative treatment.

Regarding the two fixation stages of treatment FE, there was a highly significant effect on shrinkage (Kruskal-Wallis: $p<0.001$). The pairwise comparisons indicated that in every age class the 5 month storage clearly induced a larger shrinkage than the initial 48 h fixation ($p<0.001$ in all cases). This means that in addition to the initial shrinkage induced by the formalin during the first 48 h, a significantly additional shrinkage occurs during the 5 month storage in ethanol, with shrinkage levels almost doubling.

The comparison of the shrinkage ratios of group FE's 48 h formalin fixation with group F's 5 month formalin fixation shows that there was a highly significant effect of the duration of formalin fixation on shrinkage (Kruskal-Wallis: $p<0.001$). According to the pair-wise comparisons, the shrinkages after 5 months were significantly higher than after 48 h, both on DAH 7 and 14 ($p<0.001$), but not in DAH 2 ($p>0.05$). In all three cases, however, the major formalin shrinkage had already occurred at 48 h: 6.6% after 48 h versus 7.09% after 5 months in the case of DAH 2, 5.89% versus 8.25% on DAH 7 and 5.62% versus 8.3% on DAH 14.

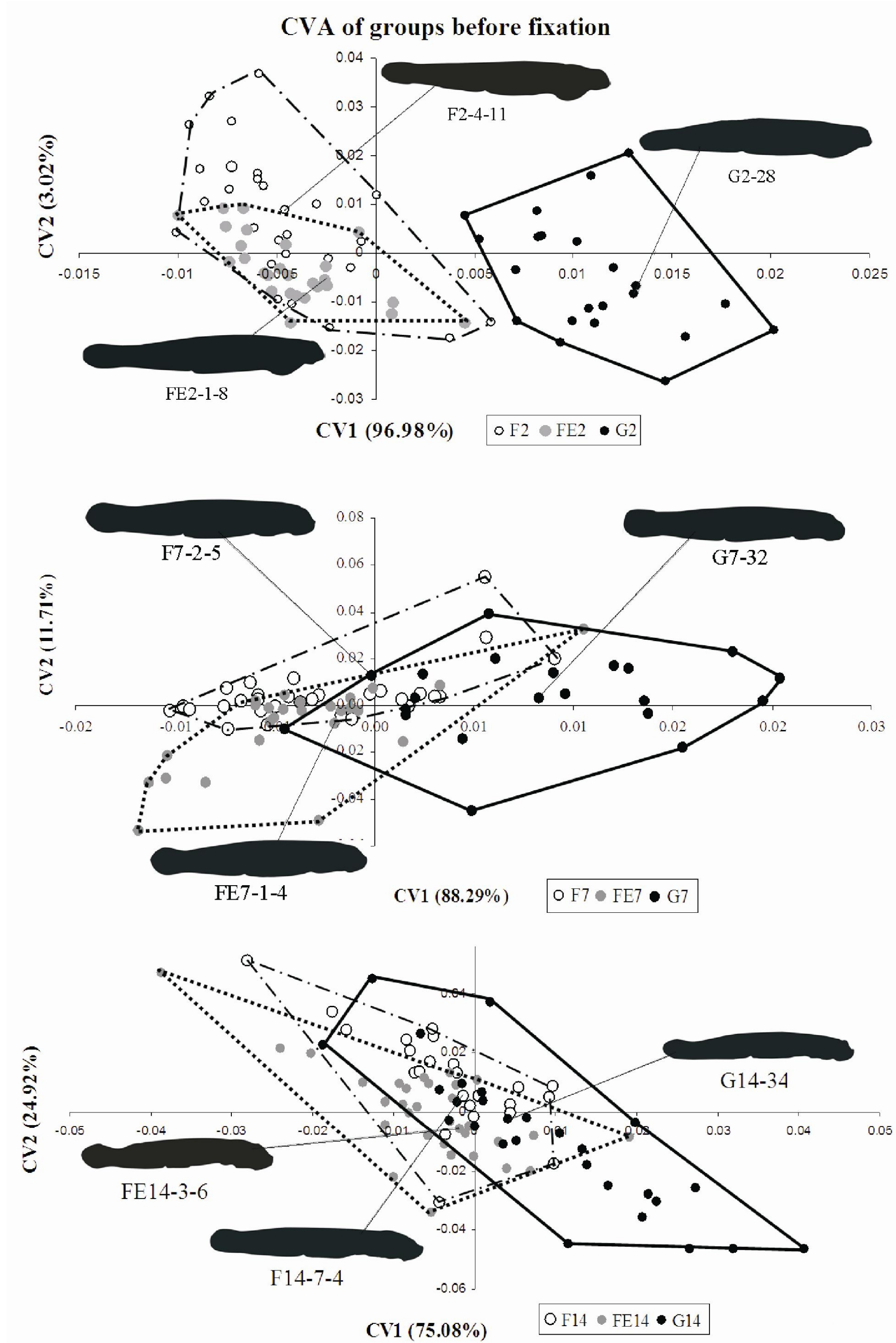

Fig. 3.3: Canonical variate analysis (CVA) scatterplot and group clusters of DAH 2, 7 and 14 groups before fixation. The *Dicentrarchus labrax* larval shapes correspond to specimens that lie close to the group means, as indicated by the connecting line, with the label of each individual specimen also noted [._._._._ , formalin group (F); …..., formalin and ethanol group (FE); _____ , glutaraldehyde group (G); see Table 3.1].

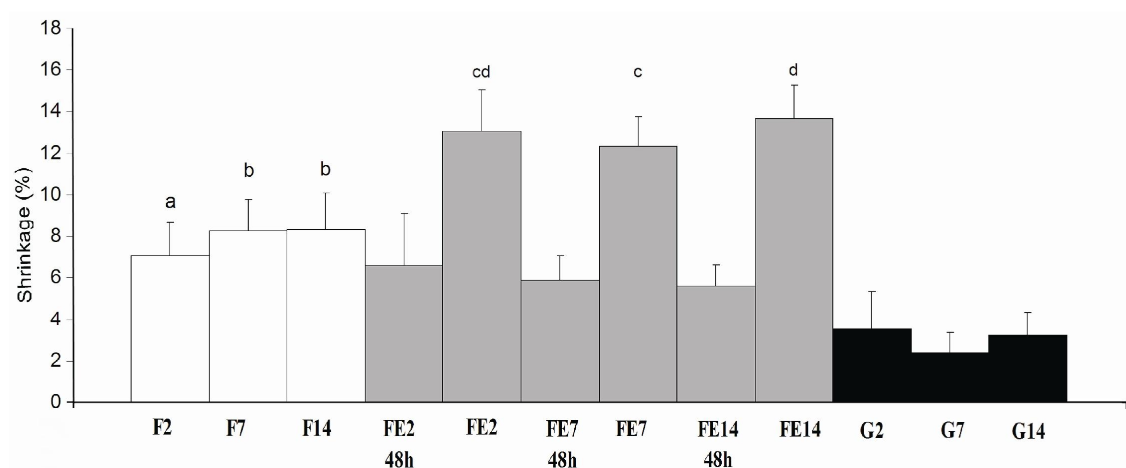

Fig. 3.4: Mean + SD shrinkage ratios of all anaesthetized specimen groups of *Dicentrarchus labrax* on DAH 2, 7 and 14 after a 5 month fixation in formalin (group F), in formalin for 48 h followed by ethanol (group FE) and in glutaraldehyde (group G). The shrinkage ratios of the FE specimens after their initial 48 h fixation in ethanol are also provided. Mean 5 month shrinkages with the same letter are not significantly different (P>0.05). The shrinkages of the glutaraldehyde groups were not significant.

3.3.2. Shape effects

There was a highly significant effect of the fixative on shape at all three ages (for all DAH: PERMANOVA, p<0.001). Post-hoc pair-wise comparisons indicated that glutaraldehyde did not induce significant shape differences after the 5 month fixation of anaesthetized specimens in every age class, unlike the formalin and formalin to ethanol groups where shape changes were highly significant (p<0.01 in every case). Additionally, in every age class, glutaraldehyde presented the lowest squared Mahalanobis distances between the corresponding groups before and after fixation, indicating that it exhibited the smallest shape difference (squared Mahalanobis distances of G2: 1.53, F2: 5.29, FE2: 7.23; G7: 2.15, F7: 3.42, FE7: 11.83; G14: 1.15, FE14: 2.95, F14: 3.44). Glutaraldehyde also gave the lowest percentage of specimens classified as affected by fixation in all age classes (G2: 66.67%, FE2: 84%, F2: 88.46%; G7: 64.71%, F7: 85.71%, FE7: 96%; G14: 61.11%, F14: 76.19%, FE14: 86.67%).

On DAH 2, PC1 explained 38.55% of the total shape variation and PC2 26%, on DAH 7 PC1 61.12% and PC2 13.84%, and on DAH 14 PC1 explained 65.36% and PC2 12.92% of the total shape variance. A pattern of an increase in the post-fixation variation represented by PC1 is evident in the fixed specimens of all treatments (Fig. 3.5), but to a lesser degree in the formalin to ethanol treatment (FE) of DAH 7 and 14. As far as changes in PC2 scores are concerned, there is no indication of an increase in post-fixation variation. Deformations are mostly present at the head and tail with positions that deviate from the horizontal axis of the body, as well as at the mid-body region. Furthermore, based on the squared Mahalanobis distances of the groups before and after fixation, there was no overall pattern of age-dependent shape changes in any treatment.

There was a highly significant effect of formalin fixation time on shape (PERMANOVA, $p<0.001$). According to the post hoc pair-wise comparisons, on DAH 2 and 14, the shape changes after 48 h were not significant. This suggests that the first fixation stage of 48 h in formalin does not induce a significant shape effect to the specimens. This was not the case, however, for DAH 7 ($p<0.01$), unlike glutaraldehyde that exhibited the best results in every age class.

There was a highly significant effect of anesthesia on shape (PERMANOVA: $p<0.001$). The post hoc pair-wise comparisons indicated that the non-anaesthetized specimens fixed in glutaraldehyde on DAH 2 and 7 presented highly significant shape differences ($p<0.01$ in both cases). This suggests that omitting anaesthetization is detrimental. Such degree of shape change was not present in any group of properly anaesthetized specimens of the glutaraldehyde treatment.

In qualitative terms, glutaraldehyde also gave the best results regarding specimen condition; it did not induce visible tissue degrading or heavy folding at the margin of the finfolds. This was not the case for the ethanol fixation where these effects were frequently visible, and to a lesser degree in treatments F and FE. Fixed specimens of treatment FE had slightly better clarity than those of treatment F, which were more opaque. When the formalin fixation time was shorter, these effects were less evident; after 48 h in formalin and right before their transfer to ethanol, the majority of the specimens were in good shape with no apparent fin folding or tissue degradation.

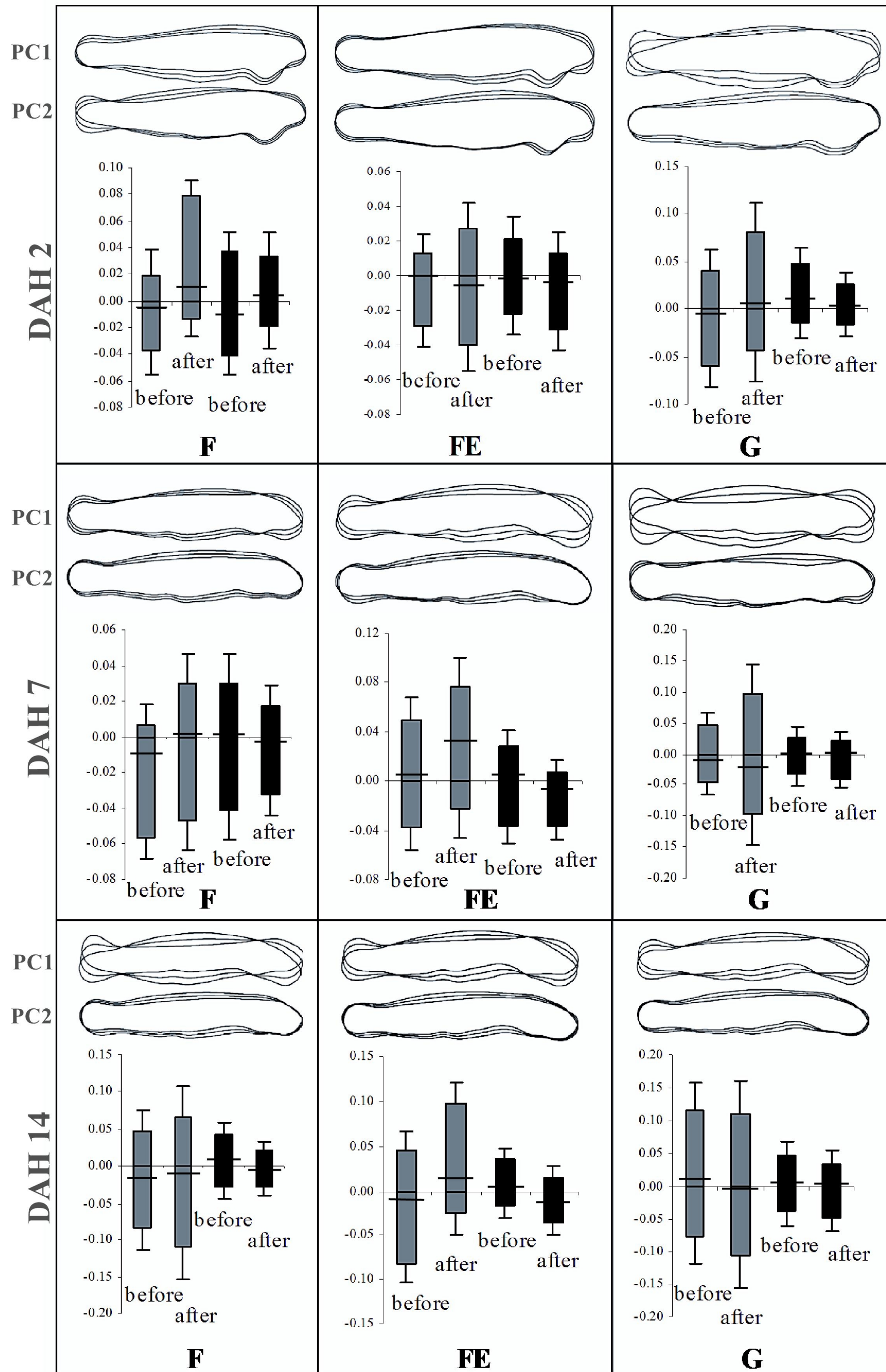

Fig. 3.5: Principal component 1 (PC1) and PC2 score ranges with means ± SD for *Dicentrarchus labrax* larval groups of DAH 2, 7 and 14 before and after their 5 month fixation in formalin (F), formalin for 48 h followed by ethanol (FE) and glutaraldehyde (G) (grey bars for PC1; black for PC2). The superimposed PC contours include the outlines of the mean and ±2 SD larvae shapes of each age group, reflecting the individual deformation effects of each group per day.

3.3.3. *Mounting*

Deformation artefacts were induced in specimens from all treatment groups after the application of all mounting media. The mounted larvae remained fit for qualitative analysis, but unsuitable for further quantitative size and shape analyses. Dehydration of the fins and the head region, body folding, body damage and bending were sometimes visible (Fig. 3.6). This was not always the case, as demonstrated in Fig. 3.7. The use of DPX on specimens fixed in phosphate-buffered glutaraldehyde, and especially glycerol used according to the above procedure, yielded very good results. These were not, however, consistent and a few specimens still exhibited small deformities or fin foldings.

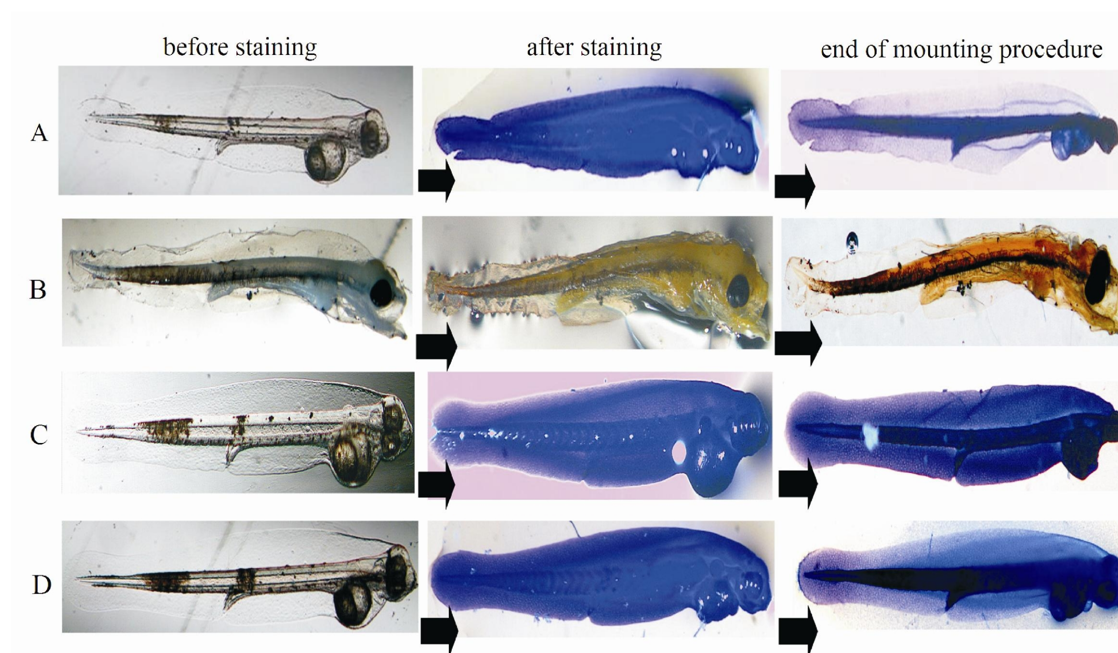

Fig. 3.6: Examples of the different mounting and staining methods applied. (a) FE2 *Dicentrarchus labrax* larva stained with toluidine blue and mounted with DPX, (b) F14 larva stained with haematoxylin – eosin and mounted with glycerol over dried nail polish, (c) F2 larva stained with toluidine blue and mounted with toluene-based mounting medium by Richard Allan Scientific and (d) F2 larva treated in exactly the same way with (c), but mounted successfully with minimal changes in its condition (see Table 3.1).

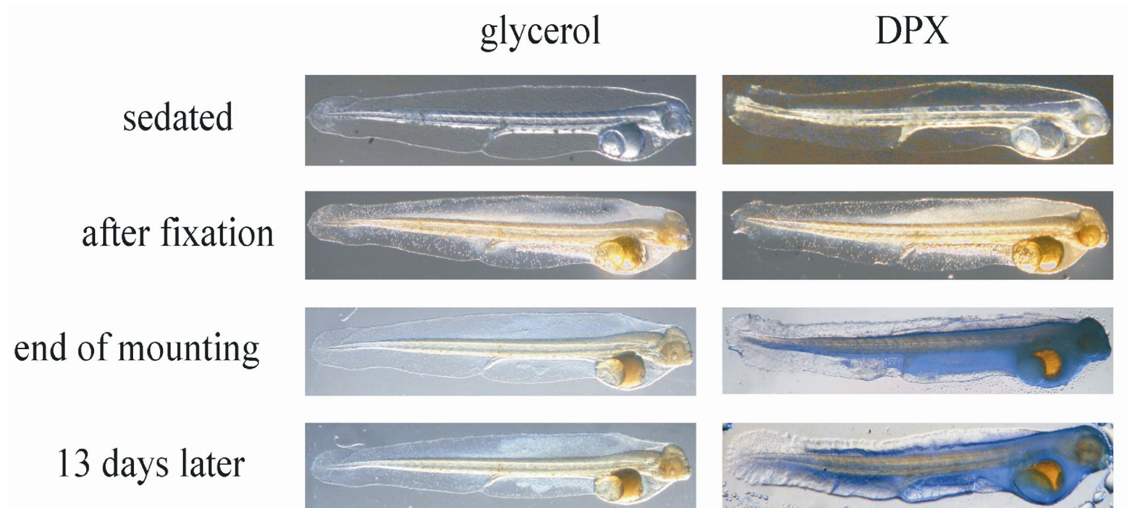

Fig. 3.7: Two examples of *Dicentrarchus labrax* larvae fixed in phosphate-buffered glutaraldehyde (group G2; see Table 3.1) and mounted with glycerol and DPX. Neither of the specimens was stained.

3.4. Discussion

As shown by the results of ANOVA, PERMANOVA and the squared Mahalanobis distances, the 5 month glutaraldehyde treatment of anaesthetized specimens performed best for every age class, without inducing any significant size or shape changes. This treatment was followed in effectiveness by formalin and formalin for 48 h to ethanol, except for DAH 14 where the latter induced smaller shape changes than the former. As opposed to glutaraldehyde, both of these treatments also demonstrated significant specimen shrinkage. One possible cause of this effect is the differences in osmolarity between the specimens and these two fixatives, which causes larvae to lose fluids passively through gills and other surfaces and shrink (Theilacker, 1980; Jennings, 1991). The formalin used in this study had an osmolarity of 1.0 Osm l^{-1}, and ethanol 1.52 Osm l^{-1}, which are high compared to blood osmolarity values between 0.305 and 0.376 Osm l^{-1} reported by authors for larger specimens of *Dicentrarchus labrax* of 24-100 g (Marino et al., 2001), and osmolalities between 0.4 and 0.55 Osm kg^{-1} for larvae between DAH 0 and 27 at a salinity of 39.5 (Varsamos et al., 2001), which was close to 36 of the seawater used in this study. These values are similar, however, to the osmolarity of the phosphate-buffered glutaraldehyde fixative of treatment G (0.32 Osm l^{-1}), which may well explain its better performance.

Glutaraldehyde is a common fixative used for electron microscopy. It is usually more suitable than formalin due to the fact that it gives lower osmolarities while providing more available reactive groups for fixation (Fox et al., 1985). It has twice the potential of formalin to cross-link proteins, and acts more rapidly (Renshaw, 2007). Among aldehydes, it also presents the best preservation of tissue ultrastructure (Carson and Hladik, 2009). It did not induce significant size and shape changes after 5 months, which should be adequate for the time-consuming morphological analyses. Similarly to the findings of this study, it performed the best in terms of specimen preservation and shrinkage of yolk-sac larvae of the common snook *Centropomus undecimalis* (Bloch 1792), without any significant changes in mean notochord length after 3 months (De Leon et al., 1991). Oozeki and Hirano (1988) also reported that glutaraldehyde did not produce a significant difference on the total length of larvae of the red seabream *Pagrus major* (Temminck and Schlegel 1843) on DAH 3, 12, 18 and 25 over a 6 month fixation period, with the fixed specimens presenting a more natural appearance. This again concurs with the observations of this study: glutaraldehyde retained very well the clarity of the transparent bodies of the larvae, which was not the case for the other fixatives.

Standalone ethanol performed the worst, which might be explained by the fact that it does not form cross-links between proteins in tissues and has a poor tissue penetration (Renshaw, 2007). In some cases of long-term storage, ethanol may cause excessive tissue shrinkage due to dehydration effects (Carson and Hladik, 2009). This was also noticeable in the specimens of this study that were fixed for 5 months in ethanol, and also to a lesser degree where a fixation of 48 h in formalin was followed by a 5 month fixation in ethanol. In marine species such as the Northern anchovy *Engraulis mordax* Girard 1854, however, immediate fixation of larvae in ethanol has been shown to minimize the shrinkage effect (Methot and Kramer, 1979). Ethanol solutions of 70% (as used in this study) or 95% are sometimes chosen as preservatives in bone development studies in fishes ranging from the larval to the adult stage, due to the fact that they do not decalcify these structures. They also present the advantage of not restricting the use of tissues in subsequent genetic analysis, as formalin does (Giannella et al., 1997). Unfortunately, due to the inability to stabilize protein constituents, they frequently degrade fish larvae tissues (Gagliano et al., 2006).

Formalin induced significant shrinkage in larvae over both 48 h and 5 months. Oozeki and Hirano (1988) report that formalin fixation of fish larvae usually results in acute tissue shrinkage. The formalin solution used in this study was unbuffered, instead of the common use of 4-10% formalin buffered to pH 7 with 0.1 M phosphate buffer in fish larvae research. Normally, formalin and glutaraldehyde demineralize skeletal structures, which can be avoided by buffering the solution to slow down the decalcification process (Hay, 1981). Buffer salts, however, increase the osmolarity of the fixative solution, with the danger of raising it to extreme values (Fox et al., 1985). Long-term storage in a buffered acid fixative can also be problematic, as buffers may damage larvae or ultimately allow the solution to become acidic (Lavenberg et al., 1984; Leis and McGrouther, 1994). Furthermore, buffered formalin as a preservative destroys the stain uptake in cartilage, thus making it impossible to be stained (Pothoff, 1984). It is for these reasons that unbuffered formalin was used. This solution also becomes acidic (Carson and Hladik, 2009), as reflected in its pH which had a value of 3.7, but can produce larvae shrinkage not significantly different than buffered (Hay, 1982) or even less (Tucker and Chester, 1984). Another way to deal with the problems presented by buffered formalin is to transfer fixed specimens for long-term preservation in 70-95% ethanol, as tested in this study (treatment FE). The usage of ethanol after formalin fixation is also useful in cases where immunohistochemical stains are scheduled, as it stops cross-linking of tissue proteins, and is applied commonly in the post-fixation process to enhance the staining reaction (Carson and Hladik, 2009). It produces significant shape changes, however, in the adult silver moharra *Eucinostomus argenteus* Baird & Girard 1855 and the roughneck grunt *Haemulopsis corvinaeformis* (Steindachner 1868) (Martinez et al., 2013).

In larvae of *Dicentrarchus labrax*, only the first signs of ossification are starting to appear at the age of DAH 14 (Rahman, 2008; Darias et al., 2010). At later stages, the more ossified specimens become less fragile, limiting shrinkage and deformations during fixation and mounting (Radtke and Waiwood, 1980; Tucker and Chester, 1984; Jennings, 1991; Mabee et al., 1998). Additionally, these effects may be limited at later stages due to the higher water content of smaller specimens, as reported for the Atlantic herring *Clupea harengus* L. 1758 and the European plaice *Pleuronectes platessa* L. 1758 (Ehrlich, 1974a, b), which would explain the higher shrinkage due to greater water loss during fixation (Hay, 1982; Mabee et al., 1998).

In the glutaraldehyde treatment without prior anaesthetization, a highly significant lower number of specimens had to be discarded compared to treatments with anaesthetization. According to the Guidelines for the Use of Animals in Behavioural Research and Teaching (Anonymous, 2006), anaesthetics should be applied for ethical reasons, but in order to examine whether they hinder the size or shape analysis, their effect had to be studied and considered. As noted above, it has been suggested by Parker (1963) and Theilacker (1980) that MS-222 interferes with osmoregulation, and therefore can contribute to the shrinkage of live larvae. Additionally, in Methot and Kramer (1979), Theilacker (1980) and other comparative studies on the effect of fixatives such as by De Leon et al. (1991), it was not used. In this case, non- anaesthetized larvae fixed in glutaraldehyde displayed almost no visible changes in body posture. According to the protocol of this study, in the level of subtle distortions, this lack of anaesthetization did not produce the expected advantages as it introduced significant shape changes on DAH 2 and 7, unlike the anaesthetized glutaraldehyde groups where no significant shape changes were present. Therefore, anaesthetization is recommended.

One of the problems encountered in this study was finding the correct dosage of MS-222 for larvae of *Dicentrarchus labrax* at these very early stages. Poorly sedated larvae or larvae that distort due to an overdose can limit the usefulness for further analysis. Gradually applying the dosage can serve as a starting point, avoiding the shock effect of a quick death by overdose. There were high levels of discarded larvae in treatment G under anaesthetization (Fig. 3.2), and they might be attributed to a variety of reasons, such as insufficient anaesthetization during the experiment, additional larval stress induced by transportation and handling (as witnessed in this study) or to an effect of the fixative itself. This study does not allow discriminating between these random effects. When the specimen selection criteria are met, however, glutaraldehyde remains the recommended fixative for studies focusing on subtle shape differences in early stages of *Dicentrarchus labrax*.

The protocol proved to be sufficiently sensitive to even quantify and visualize shape differences originating from a different egg batch, as indicated by the lack of significant pre-fixation differences on DAH 2 and 7 between groups F and FE that came from the same egg batch, and the presence of highly significant differences between G and both F and FE (Fig.

3.3). Furthermore, as indicated by the gradually increasing overlap of clusters of the different groups, the common shapes that start to appear as larvae grow suggest that observed differences during the early stages might gradually decrease. Similar ontogenetic convergences of shape as fish continue to develop into later juvenile stages have been demonstrated in *Dicentrarchus labrax* (Georgakopoulou et al., 2007).

Finally, the mounting procedure did induce some deformation artefacts, rendering it suitable for qualitative morphological studies, but not for morphometrics. Regarding the former, very good results were obtained with specimens fixed in phosphate-buffered glutaraldehyde that were mounted with glycerol using the described modified method of Maeseneer and d'Herde. The reason for the better results yielded by the consecutive glycerol baths instead of its immediate application can be the fact that glycerol is a hygroscopic agent that absorbs and replaces water in the specimens, penetrating the cells to the same extent as water, but at a slower rate than water release (Alemohammad and Knowles, 1974). Thus, it is assumed that the series of baths ensured a more gradual glycerol and water exchange.

The present analysis allowed the visualization and quantification of normal shape changes during the early life stages in *Dicentrarchus labrax*, and especially deformities resulting from common manipulations such as fixation and mounting. The broader applicability of this protocol is that it can be used to quantify deformities occurring in hatcheries at very early stages, thus allowing decisions and improvements to be made at this time point. Furthermore, it can prove useful not only in deformity studies of commercial fish species, but also for studying patterns of natural variation during their early life history, and in studies that aim to demonstrate an adverse effect attributable to any factor that has the potential to induce shape changes. As a final point, due to a certain degree of morphological similarity in the earliest fish developmental stages and the suitability of the outline analysis in cases of curved specimens where landmarks can- not be applied safely, it is highly likely that this protocol might be applicable to early stages of other marine or freshwater fish species, although this will have to be tested.

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Appendix

In the following chapters, instead of Mahalanobis distances, Euclidean distances are being used in the PERMANOVA tests, as well as between the shape centroids of the treatment groups. This was done due to new arguments presented recently in literature, after the publication of the manuscript. These point to the fact that in a similar fashion to the associated canonical variate/discriminant analysis (CVA/DA), Mahalanobis distances are calculated from a process that transforms the original data, maximizing between group variation. CVA/DA is then decomposing the total among-group variance with respect to within-group variance, and provides an ordination of the groups. However, DA maximises group separation (Viscosi and Cardini, 2011), tends to bias upward ordinations of group differences, and is sensitive to unequal sample sizes across groups and small samples, as these are likely to increase over-fit and reduce generalisability of results (Kovarovic et al, 2011).

Euclidean distances are considered more neutral than Mahalanobis distances in the transformation or distortion of the data in their original morphospace, since no such process is involved (Brereton and Lloyd, 2016). For this purpose, they are nowadays used more in studies of morphological variation (for example in Gabelaia et al., 2017; Sánchez-González and Nicieza, 2017 etc.), as they are more conservative in the determination of differences between groups.

It is noted that this change does not alter the results presented in this article. All PERMANOVA tests were performed again, and in every case, the significance results were exactly the same.

Chapter 4

Phenotypic effects of antibiotic-induced axenity and egg disinfection in early larval European seabass (*Dicentrarchus labrax* L.)

This chapter is based on:

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Abstract

In the present study, the hypothesis that the difference in axenic conditions in the incubation and rearing environment of European seabass larvae induces size and shape effects on the specimens is tested. This difference is studied between xenic and axenic seabass larvae of DAH 0, 5, 11 and 15. The axenic rearing protocol involves a secondary egg disinfection with glutaraldehyde after the primary one with iodine in the hatchery, and the hypothesis that it induces size and shape effects is also tested. In order to accomplish this, three egg and larvae treatments are included: "DA" (Disinfected Axenic), "DX" (Disinfected Xenic") and "NX" (Non-disinfected Xenic). Regarding the effect of axenity, DA exhibited larger bodies than both DX and NX on DAH 5 and 11. They also had a smaller yolk sac than DX at hatching, but consumed it slower, probably due to reduced energy demands from lack of bacterial colonisation of the digestive tract, and reduced energy expenditures to combat or assimilate bacteria. Towards the end of the experiment, DA larvae were thicker, but slightly more curved than DX and NX, which may be an abnormal shape, or a slightly more advanced ontogenetic stage. As far as egg disinfection, it had significant but very moderate shape effects on DAH 5 and 11, and disinfected larvae consumed their yolk sac faster. An adverse effect was that it induced smaller anal finfolds on DAH 0 and 11, which might be associated with increased energy expenditures, possibly explaining the increased yolk sac depletion rate.

4.1. Introduction

One of the most significant problems that aquaculture is facing today is bacterial overgrowth during the culture of egg and larvae, leading to poor hatching percentages and high larval mortality (Moretti et al., 1999). In particular, marine fish eggs are very sensitive to this increased bacterial load, due to the fact that their external surface is a good substratum for the adhesion of bacterial strains (Hansen & Olafsen, 1999). These tend to multiply rapidly under confined environments such as the incubation facilities in hatcheries, which are rich in nutrients (Douillet & Holt, 1994). Fish larvae are usually kept in incubators with hatching eggs and debris, which can result in a 1000-fold increase in bacterial counts of the ambient water through hatching (Hansen & Olafsen, 1989).

In the context of the study of the effects of bacterial load and its communities, recently there has been an increased research focus on the study of gnotobiotic fish larvae reared in axenic environments, in which a known microbial community is inserted as a means to study host-microbial interactions, to eliminate bacterial interference that tends to confound experimental results. Examples of such gnotobiotic model systems of fish larvae include the use of zebrafish *Danio rerio* (Rawls et al., 2004), European seabass *Dicentrarchus labrax* (Dierckens et al., 2009) and Atlantic cod *Gadus morhua* (Forberg et al., 2011). The protocol of Dierckens et al. (2009) involves the use of antibiotics (rifampicin and ampicillin) for the egg incubation, subsequent to an egg disinfection with glutaraldehyde. Hatched larvae are then stocked in filtered autoclaved seawater with rifampicin, and monitored until DAH 15 when increased mortalities usually occur.

These model systems are also used to study how microbial colonisation or microbial products influence the physiology and development of the host (Pham et al., 2008). Studies have shown morphological differences between xenic and axenic larvae, but they have focused almost exclusively on the development and maturation of the gastrointestinal tract, such as in zebrafish (Rawls et al., 2004; Bates et al., 2006) and European seabass (Rekecki et al., 2009). As far as size is concerned, Forberg et al. (2011) did not witness a difference in the total length between xenic and axenic Atlantic cod larvae, while Rekecki et al. (2009) found a reduced total length growth of xenic European seabass larvae versus germ-free on days after

hatching (DAH) 6, 9, 12 and 15. However, beyond these differences in total length, detailed knowledge of the phenotypic effects of antibiotic-induced axenic conditions on the larvae is absent. Consequently, the first aim of this study was to test the hypothesis that the difference in axenic conditions in the incubation and larval rearing environments induces size and shape differences on the specimens, as a proxy of their effects on fish performance during their rearing, and to describe them. This hypothesis is tested between xenic and axenic European seabass *Dicentrarchus labrax* larvae of DAH 0, 5, 11 and 15, the latter incubated and reared with the use of the protocol by Dierckens et al. (2009), by comparing a series of morphometric characters of the specimens that represent body parts with important functional roles during ontogeny, including lengths, depths, the yolk sac area and the pectoral angle, and also the shape of the specimens.

In axenic protocols for fish larvae, disinfection agents have been used for egg surface disinfection before hatching, as a preliminary step to obtain bacteria-free larvae (Munro et al., 1995; Pham et al., 2008; Forberg et al., 2011; Schaeck et al., 2016). They are also used in hatchery systems on the fertilised eggs in order to minimise the aforementioned adverse effects of intense bacterial load, as the necessary first effective barrier against the transmission of fish diseases, and to improve the survival of fish larvae. Their effect on the eggs and the hatched larvae is quite variable, as it depends on many factors, such as the species on which they are applied, the specific developmental stage, the physiological state of the broodstock, the concentration and exposure time. However, they can be considered an extra stress factor detrimental to eggs that come from a suboptimal batch quality (Schaeck et al., 2016), and they have been known to produce adverse effects, such as reduced stress tolerance and larval malformations (De Swaef et al., 2015 and references therein).

Glutaraldehyde is a common disinfection agent commonly used for its bactericidal, virucidal and fungicidal properties. In axenic models such as the ones from Dierckens et al. (2009), Forberg et al. (2011) and Situmorang et al. (2014), the application of glutaraldehyde did not induce easily detectable teratogenic effects such as gross eye and cephalic deformations (Von Westernhagen, 1988), but its subtle phenotypic effect is still unexplored. Therefore, the second aim of this study is to test the hypothesis that glutaraldehyde used in the disinfection process in the protocol of Dierckens et al. (2009), induces size and shape effects on the newly hatched larvae.

European seabass *Dicentrarchus labrax* was chosen as a case study due to being a very popular and successful species in Mediterranean aquaculture, and also due to the considerable study focus on its ontogeny and morphological abnormalities, that many have been known to appear or strongly suspected to have their origins in the first life stages (Koumoundouros, 2010; Boglione et al., 2013b). An additional reason for the selection of the species was the existence of the necessary protocols for larval axenic rearing (Dierckens et al., 2009) and size and shape quantification (Nikolakakis et al., 2014). The latter is a procedure that is able to quantify subtle size and shape effects, taking into account and minimising the impact of handling, anesthetisation and fixation, and shown not to induce any significant size or shape effect between the fixed and live specimens up to five months after fixation.

4.2. Materials and methods

4.2.1. Rearing and sampling

Seabass eggs were obtained from the Ecloserie Marine de Gravelines hatchery in France. They were disinfected with 20 ml l^{-1} 0.5% active iodine for 10 min 24 h before the start of the experiment. Upon arrival at the laboratory, they were transported to the hatching room, where after acclimatization in UV-sterilised seawater they were split in three treatment groups. (1) The eggs of the disinfected-axenic group ('DA' group) underwent a secondary disinfection with glutaraldehyde (200 mg l^{-1}), and were subsequently added for incubation into axenic filtered autoclaved seawater with 10 mg l^{-1} ampicillin and 10 mg l^{-1} rifampicin for three days. After hatching, they were reared in filtered autoclaved seawater, with the addition of 10 mg l^{-1} rifampicin, but without the further use of ampicillin, as rifampicin alone was shown to be effective in establishing axenic conditions (Dierckens et al., 2009). (2) The eggs of the disinfected-xenic group ('DX' group) were disinfected in the same way and dosage with treatment DA, but added into xenic seawater for incubation and rearing. (3) Eggs of the control group (not disinfected-xenic 'NX' group) were not disinfected with glutaraldehyde, and put directly into xenic seawater for incubation and rearing. This experimental design allowed to discriminate between the effects due to axenity (DA vs DX), secondary disinfection (DX vs NX) and their interaction (DA vs NX).

In all treatments, the incubation took place in pre-autoclaved 1l glass bottles with gentle aeration, and randomly selected hatched larvae were stocked in 40 UV-irradiated 50 ml vials per treatment, with a density of 12 larvae per vial. The glutaraldehyde disinfection, axenic incubation, axenic rearing and axenity tests were performed according to Dierckens et al. (2009). The experiment took place until 16 days after hatching (hereby referred to as DAH), in a temperature controlled room at 16±0.5°C under constant dim light of 10 cd sr m^{-2}, and the salinity of the seawater was 37 g l^{-1}. The larvae were fed with 30 axenic *Artemia franciscana* nauplii per vial on DAH 7, 9, 11, 13 and 15, prepared according to the protocol of Marques et al. (2004).

Specimen samplings for the distance, angle-based and outline-based shape analyses took place on DAH 0, 5, 11 and 15 randomly from all available vials, with the larvae anesthetised with MS222 and added in a fixative of 3% phosphate buffered glutaraldehyde. In order to minimise any effects from the handling and fixation procedure, the protocol of Nikolakakis et al. (2014) was used, as it has been shown to induce no size or shape changes between the live and fixed specimens until 5 months after fixation. The larvae were photographed within this time frame. The present study was conducted by personnel certified in animal experiments by the Belgian State, in compliance with the EU Directive 2010/63/EU.

4.2.2. Hatching percentages

For the calculation of the hatching percentages, two 96-well plates per treatment were used. The recorded percentages were of (a) the hatched larvae, (b) the larvae that upon examination under a stereoscope were seen to have been developed in the eggs but unhatched, and (c) the eggs that contained undeveloped larvae. This allowed the hatching status to be taken into account. In order to determine whether there was a significant effect from the treatments, the hatching status or their interaction, a two-way analysis of variance (ANOVA) test was performed. In order to meet the assumptions of the test, the data were arcsin-square root transformed.

4.2.3. Mortalities

Dead larvae were counted and removed on DAH 1, 2, 3, 5, 7, 9, 11, 13, 15 and 16 from all replicate vials. On DAH 16, all remaining vials were sampled. In order to determine whether the treatments had an effect on the mortality, a one-way ANOVA test was performed on each individual sampling point, followed by Tukey's post-hoc tests in the cases where the ANOVA test assumptions of normality of distribution and equality of group variances were met, and Kruskal-Wallis tests followed by Mann Whitney post-hoc pairwise comparisons when they were not. The normality of distribution was tested with Shapiro Wilk's test, and the equality of group variances with Levene's test.

4.2.4. Analysis of distance & angle morphometry

4.2.4.1. Measurements

A minimum of 25 specimens per group were collected per treatment on DAH 0, 5, 11 and 15. The distance and angle analysis was conducted according to the protocol of Nikolakakis et al. (2014). The measurements were performed using ImageJ 1.51j (Abramoff et al., 2004) with an accuracy of 0.001 mm on pictures of the larvae, which were photographed with an Olympus Altra 20 digital camera mounted on an Olympus SZX9 microscope via AnalySIS GetIT software (www.olympus.com). In terms of the longitudinal aspect of size, their total length, standard length, head length, predorsal finfold length, dorsal finfold length, yolk sac length, gut length, anal finfold length and preanal finfold length were measured. Regarding their growth in overall body depth, measurements of the eye diameter, head depth, pectoral body depth, yolk sac depth, gut diameter, anal body depth, dorsal finfold depth, notochord diameter, anal finfold depth were also taken.

Total length **(TL)** was measured from the tip of the upper jaw to the distal margin of the caudal fin fold. All other longitudinal measurements were taken parallel to the direction of the TL measurement, and were defined as follows (Fig. 4.1): standard length **(SL)** from the tip of the upper jaw to the caudal tip of the notochord; head length (HL) from the tip of the upper jaw to the right edge of the post-otic vesicle; predorsal fin fold length **(PDFFL)** from

the tip of the upper jaw to the start of the dorsal fin fold at the upper end of the cranium; dorsal finfold length **(DFFL)** from the start of the dorsal finfold up to the distal margin of the caudal fin fold, and calculated as DFFL=TL–PDFFL; gut length **(GL)** from the tip of the upper jaw up to the level of the anus; anal finfold length **(AFFL)** from the anus up to the distal margin of the caudal fin fold, and calculated as AFFL=TL–GL; pre-anal finfold length **(PAFFL)** from the rostral point of the pre-anal fin fold up to the anus; yolk sac length **(YSL)** as the longest horizontal distance between yolk sac walls.

The vertical measurements were taken perpendicular to TL, and defined as follows: eye diameter **(ED)** as the largest vertical distance of the eye; head depth **(HD)** taken as an extension of ED between the lower and the upper part of the cranium, as used by Yin and Blaxter (1986); pectoral body depth **(PBD)** taken vertically between the uppermost and lowermost points of the body at the level of the right edge of the post-otic vesicle (**HL**); yolk sac depth **(YSD)** as the longest perpendicular distance between yolk sac walls; gut diameter **(GD)** the vertical distance between the two gut walls, taken at the midgut; anal body depth **(ABD)** at the level of the anus, following the "posterior dorsal depth" of Holden and Raitt (1974) and the "anal body depth" of Theilacker (1978) and McGurk (1985); dorsal fin fold depth **(DFFD)** the depth of the dorsal fin fold, taken at the same level as the anal body depth, measured between the upper point of the notochord and the dorsal finfold margin; notochord diameter **(ND)** the distance between the lower and the upper points of the notochord, measured at the same level as the anal body depth; anal finfold depth **(AFFD)** the depth of the anal fin fold across anal body depth between the lower point of the notochord up to the anal finfold margin, and calculated as AFFD=ABD-DFFD-ND.

4.2.4.2. Total length

To determine whether the treatments induced a significant effect on the TL (total length), one-way analysis of variance (ANOVA) tests were performed on the datasets of each DAH when the assumptions of the test were met, and Kruskal-Wallis tests when they were not. In case the tests determined there was a significant effect, ANOVA was followed by Tukey's post-hoc tests, and Kruskal Wallis with post-hoc Bonferroni-corrected Mann-Whitney U.

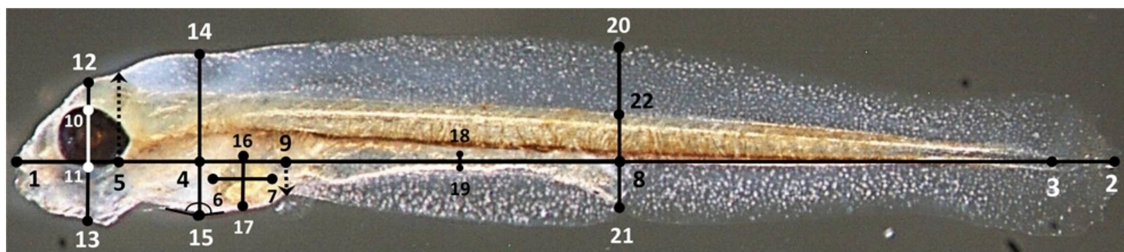

Fig. 4.1: Representation of the distance and angle measurements taken on a body of a larva between individual numbered points, with the corresponding measurements being: total length (TL) between points 1-2, standard length (SL) 1-3, head length (HL) 1-4, predorsal finfold length (PDFFL) 1-5, dorsal finfold length (DFFL) 5-2, gut length (GL) 8-1, anal finfold length (AFFL) 8-2, pre-anal finfold length (PAFFL) 9-8, yolk sac length (YSL) 6-7, eye diameter (ED) 10-11, head depth (HD) 12-13, pectoral body depth (PBD) 14-15, yolk sac depth (YSD) 16-17, gut diameter (GD) 18-19, anal body depth (ABD) 20-21, dorsal finfold depth (DFFD) 20-22, notochord diameter (ND) 8-22, anal finfold depth (AFFD) 8-21, and pectoral angle (PA) at point 15.

This only provided information on the differences on individual age classes, but not on the total length growth throughout the whole experiment. Therefore, to obtain a more representative description of the development of the total lengths in relation to time, the relationship between total length and time in DAH was fitted with a Gompertz model, with the following equation: $TL = a\ e^{-be^{-ct}}$. The parameters are defined as follows: **TL** is the total body length in mm; ***a*** is the asymptote in mm, which is the maximum length that the growth is still being described by the model before changing to another allometry equation such as a new Gompertz curve (not a predetermined constant, but a parameter calculated for a specific treatment); ***c*** the instantaneous maximum growth rate in $mm.day^{-1}$ at the inflexion point of the curve; **t** the age in days and ***b*** a dimensionless parameter, with $b\ x\ c$ being the instantaneous growth rate when t=0 (Ricker, 1979; Polo et al, 1991; Pilar Olivar et al, 2000; Bartsch, 2002). This model was chosen due to the fact it has been shown in literature to describe very well the early total length development of fish larvae in relation to time, with a specific application on newly hatched larval European seabass *Dicentrarchus labrax* (Pilar Olivar et al., 2000 and references therein).

In order to determine whether there were any statistically significant differences between the treatment models and also between their parameters of a, b and c, they were compared with nonlinear regression in Graphpad Prism 7.01 (www.graphpad.com), using the Gompertz curve fit and based on an extra-sum-of-squares F test, as suggested by the software's manual. In the cases of statistically significant differences, the F test was followed by Tukey's post-hoc pairwise comparisons.

4.2.4.3. Yolk sac

Blaxter and Hempel (1963) calculated yolk sac volume (YSV) from YSV= $\pi/6$ YSL*YSD^2, where YSL is the yolk sac length and YSD the yolk sac depth (Fig.4.1). However, in order to increase accuracy, since the photos only give a two dimensional representation of the yolk sac without ensuring it is a perfectly homogeneous ellipsoid, a calculation was made for the yolk sac area **(YSA)** that was clearly visible by using the general formula for an ellipse: YSA= $\pi/4$ YSL*YSD.

In order to see whether the treatment factor induced a significant effect on the yolk sac areas, analysis of covariance (ANCOVA) tests were performed on each DAH, with a measure of larvae size as a covariate. Instead of choosing total length, a variable that represents a more complete description of size was needed. For this purpose, a disregard-group principal component analysis (PCA) was performed on all measurements, excluding yolk sac length, yolk sac depth and pectoral angle, from the pooled dataset of groups from all DAH. Pectoral angle was excluded because it was not correlated to total length (DA: Pearson correlation statistic=0.235, p_{uncorr}=0.079; DX: Pearson correlation statistic=0.056, p_{uncorr}=0.69; NX: Pearson correlation statistic=0.16, p_{uncorr}=0.233).

PC1 explained 96.82% of the total variation, with all distance variables presenting high positive loadings. Therefore, this suggested a simultaneous increase in all dimensions, and thus PC1 was interpreted as a size component. Consequently, these PC1 scores were used as a size variable in the yolk sac ANCOVA tests on each individual DAH, and the regression of the yolk sac area.

For ANCOVA, the assumption of equality of linear regression slopes was tested with an F test, the normality of distributions with Shapiro-Wilk's test, and homogeneity of error variances with Levene's test. Regarding the assumption of normality of distributions, in case there was a violation under the test of Shapiro-Wilk, the skewness and kurtosis values were checked in order to see whether they fell within the range of -2 and +2, since it is considered acceptable in terms of normal univariate distribution (George & Mallory, 2016).

In order to see whether the treatments had an effect on the depletion of the yolk sacs of the specimens, a regression of their yolk sac areas versus the aforementioned PC1 scores was performed on the data from all age classes. For the choice of the regression model explaining this relationship, the AIC (Akaike Information Criterion) was chosen as the decision criterion between the compared models, which were the linear, quadratic, exponential, logistic, von Bertalanffy and Gompertz. The linear model was selected, since it presented the lowest AIC values in all treatments. Their slopes represented the rate of yolk sac depletion, and were compared for statistically significant differences in Graphpad Prism 7.01 (www.graphpad.com), using the straight line fit and based on an extra-sum-of-squares F test, as suggested by the software's manual. Similarly to the total length analysis, in the cases of statistically significant differences the test was followed by Tukey's post hoc pairwise comparisons.

4.2.4.4. Analysis of pectoral angle

On DAH 11 and 15, the pectoral angle **(PA)** of each specimen was measured as the angle formed by the ventral body contour at the level where head length (HL) was measured (Fig.4.1), at the bottom of the pectoral girdle (Ehrlich et al., 1976). It was not measured on DAH 0 and 5, as it would only reflect the curvature of the yolk sac, something that would compromise the measurement. After DAH 11, the yolk sacs were placed inside the larval body, therefore their interference with the measurement was minimised. In order to see whether the treatments had a significant effect on the pectoral angles of specimens of DAH 11 and DAH 15, a Watson-Williams test for equal means of angular data was performed between the three treatments on the dataset of each DAH, after the assumption of the data following a von Mises distribution was checked with a Watson's U^2 goodness-of-fit test.

4.2.4.5. PCA on lengths and depths

Firstly, in order to test the hypothesis that the treatments induced a consistent morphological effect in all age classes, and also in order to visualise the ontogenetic changes throughout the duration of the experiment, a PCA was performed on all measured lengths and depths. Secondly, in order to explore the treatment effects on each individual age class, between-group principal component analyses (PCA) were performed on all measured lengths and depths on the datasets of each DAH. A biological meaning was given for PC1 and PC2, and their scores were compared between treatments with ANOVA with Tukey's post hoc tests, and their variances with Levene's test, with Bonferroni-corrected post-hoc F tests.

4.2.5. Outline-based shape analysis

The analysis of shape effects was performed on the same larvae that were used for the distance and angle analysis. It was conducted as described in detail in Nikolakakis et al. (2014). Briefly, the outlines of the larvae were traced manually on their images by using Corel Draw 12 (www.corel.com). To evaluate the effect of the different treatments on the larval shape, an elliptic Fourier analysis was performed on their body outlines, and shape variation was analysed by disregard-group PCAs on the Elliptic Fourier coefficients, using Shape 1.3 (Iwata and Ukai, 2002).

Ontogenetic shape changes throughout the duration of the experiment were analysed with a PCA conducted on the pooled dataset of the treatment groups from all DAH. To determine whether there was a shape effect from the treatments on each individual age class, a PCA was performed on the individual DAH datasets. Multivariate normality was checked with Mardia's multivariate skewness and kurtosis test, and the equivalence of covariance matrices with Box's M test. In the cases these conditions were met, a one-way multivariate analysis of variance (MANOVA) was performed on the scores of all effective PCs (Iwata and Ukai, 2002; Nikolakakis et al., 2014). When they were not, a one-way non-parametric multivariate analysis of variance (PERMANOVA) test was performed with 10000 permutations, with Euclidean distances as the distance measure. After each MANOVA or PERMANOVA test, in case there was a significant effect from the treatments, it was followed by Bonferroni-corrected post-hoc tests.

In order to quantify the magnitude of the shape effects of the different treatments across time, the Euclidean distances between the shape centroids of all pairwise comparisons between treatment groups on each DAH were calculated as a proxy for shape differences, with larger distance measures indicating a greater effect.

The significance level of all statistical tests was set at the nominal signifance level of alpha=0.05. The statistical analysis was performed with PAST 3.16 (Hammer et al., 2001), apart from the following tests which were not included in the software: the Euclidean distances between shape-group centroids were obtained by the PopTools 3.2 plugin (www.poptools.org) of Microsoft Excel 2010, the analysis of covariance (ANCOVA) with Bonferroni-corrected post-hoc tests by SPSS 24 (www.ibm.com), and the comparison of model parameters by GraphPad Prism 7.01 (www.graphpad.com).

4.3. Results

4.3.1. Hatching percentages

The DX treatment presented a hatching percentage with a mean and standard deviation of 86.98±6.63%, which was higher than both the other two treatments (NX: 78.65±6.63% and DA: 64.06±0.74%, Fig. 4.2), but not significantly (treatment factor: p=0.99, interaction of treatment and hatching status: p=0.055).

4.3.2. Mortalities

Despite the fact that the means and medians of the mortalities of treatment DA remained the lowest throughout the experiment, the difference was not significant. Until DAH 11, the mortalities of treatment DA were equal to zero while the other two treatments presented slightly higher percentages, apart from treatment NX at DAH 2 which was also equal to zero (Fig. 4.3). Again, treatment effects were not significant (DAH 1: p=0.162, DAH 2: p=0.869, DAH 3: p=0.058, DAH 5: p=0.061, DAH 7: p=0.052, DAH 9: p=0.225, DAH 11: p=0.7; Kruskal-Wallis). After DAH 13, the mortalities of treatment DA started to occur, but there was still no treatment effect on that day (p=0.244, Kruskal Wallis). On DAH 15, there was a treatment effect (p<0.01, ANOVA), with the “DA” mortality of 50.91±12.42% SD

significantly lower than the 79.82±20.89% of "DX". However, on DAH 16 the treatment effect disappeared again (p=0.06, ANOVA).

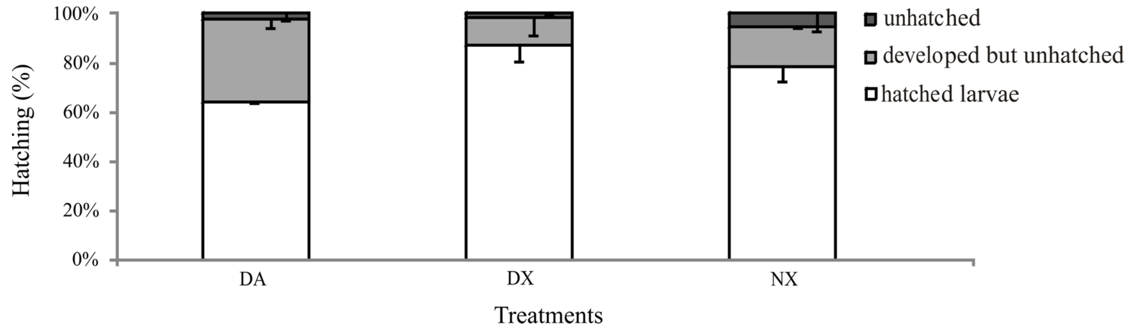

Fig. 4.2: Hatching performance of the three treatments in percentages as calculated from the two 96-well plates per treatment, with standard deviation bars, where for visual clarity, only the minus error bars are displayed. The bars of the "hatched" larvae are placed in the middle of the columns, the bars of the "developed but unhatched" slightly to the right, and the bars of the "unhatched" in the rightmost position. Differences are not significant.

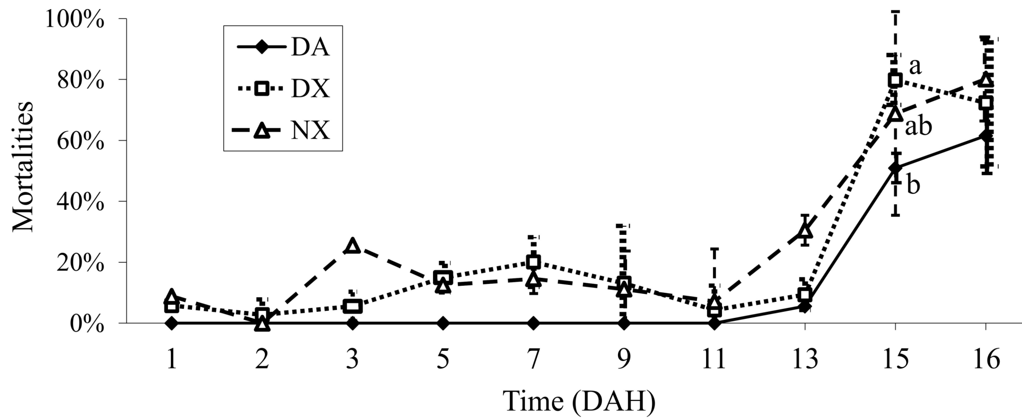

Fig. 4.3: Mortalities of treatments DA (disinfected axenic), DX (disinfected xenic) and NX (not disinfected xenic) versus time in days after hatching (DAH), with standard deviation bars. The treatment factor induced an effect on DAH 15, with different letters indicating significant differences.

4.3.3. Distance and angle analysis

4.3.3.1. Total length

On DAH 0, DAH 5 and DAH 11 there was a significant effect of the treatments on the total lengths (DAH 0: $p<0.05$, ANOVA; DAH 5: $p<0.01$, ANOVA; DAH 11: $p<0.01$, ANOVA). This is illustrated in Fig. 4.4. On DAH 0, “NX” larvae had significantly larger total lengths than “DA” ($p<0.05$, Tukey’s post-hoc test). On DAH 5, “DA” specimens were significantly larger than “DX” and “NX” (in both cases: $p<0.01$, Tukey’s post-hoc test). On DAH 11, “DA” larvae were larger in total length than “DX” ($p<0.01$, Tukey’s post-hoc test). However, on DAH 15 the treatments did not induce a significant effect ($p=0.73$, Kruskal Wallis).

On DAH 0, 5 and 11 the total length variances were not statistically different (Levene’s test for homogeneity of variance, $p=0.618$ for DAH 0, $p=0.482$ for DAH 5, $p=0.599$ for DAH 11) and consequently neither their standard deviations, as they are equal to the square root of the variation. On DAH 15, there was a treatment effect on the variances (Levene’s test: $p<0.01$), with the variances of treatment DA being significantly smaller than those for NX (Bonferroni-corrected Levene test: $p=0.009$). Consequently, even though there was no significant treatment effect on the total lengths of the larvae on DAH 15, the lengths of treatment DA were more uniform than NX. This difference was not met with a difference in mortalities between these two treatments, therefore it might be an indication of the combination of the egg disinfection and axenity factors creating conditions that favour a more standardized growth in total length.

The Gompertz models describing the total length of the three treatments in relation to time (Fig. 4.5) were significantly different (extra-sum-of-squares F test: $p<0.05$). The parameters with standard deviations were the following: for DA, a=5.4±0.3 mm, b=0.4±0.1, c=0.5±0.6 $mm.day^{-1}$; for DX, a=5.3±0.3 mm, b=0.4±0.1, c=0.5±0.5 $mm.day^{-1}$; for NX, a=5.4±0.3 mm, b=0.3±0.1, c=0.4±0.5 $mm.day^{-1}$. The parameters of a (asymptotic maximum length) and c (maximum growth rate) did not differ (extra-sum-of-squares F test, for a: $p=0.357$; for c: $p=0.339$). However, the treatments induced a significant effect on parameter b ($p=0.047$). Therefore, at the moment of hatching the growth rate of “DA” specimens (growth rate at hatching: b * c) was higher than that of “NX” ($b_{DA} > b_{NX}$; $p<0.05$, Tukey’s post-hoc test).

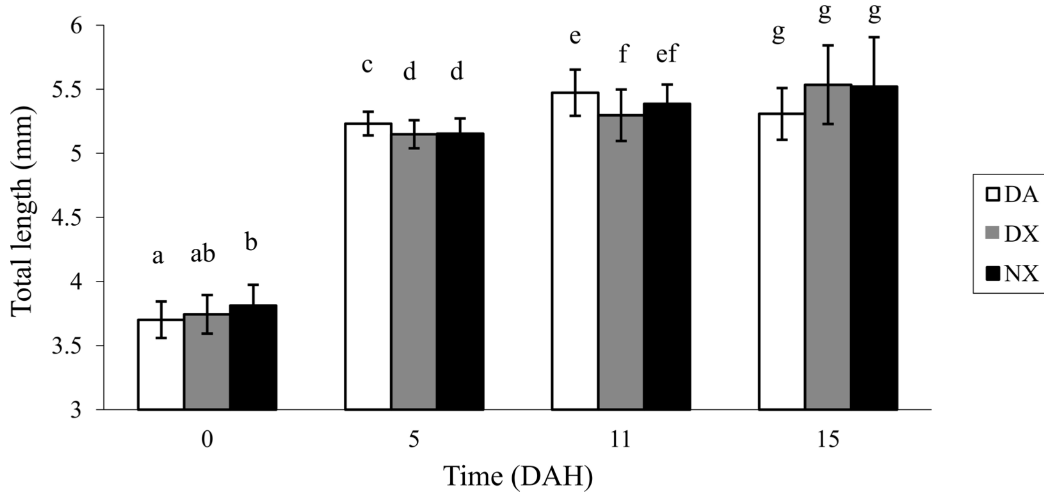

Fig. 4.4: Total lengths of larvae from treatments DA (disinfected-axenic), DX (disinfected-xenic) and NX (non-disinftected-xenic) on DAH (day after hatching) 0, 5, 11 and 15, with standard deviation bars. Within each age class, different letters represent statistically significant differences.

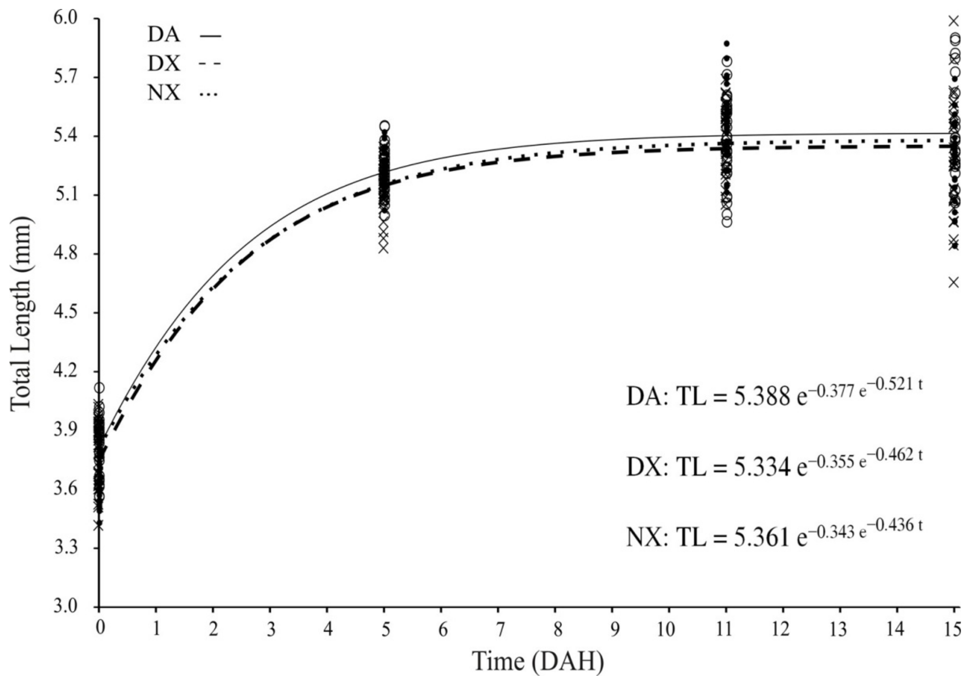

Fig. 4.5: Total length versus time in days after hatching (DAH) for treatments DA (disinfected-axenic), DX (disinfected-xenic) and NX (non-disinftected-xenic), with the Gompertz formulae for each curve. The specimens of treatment DA are being represented with dot markers, DX with circle markers, and NX with x markers.

4.3.3.2. Yolk sac

On DAH 0, there was a significant effect of the treatments ($p<0.001$, ANCOVA), with the yolk sac areas of treatment DX specimens being significantly larger than DA and NX (in both cases: $p<0.01$), with means and standard deviations of 0.38±0.04 mm^2, 0.35±0.05 mm^2 and 0.33±0.03 mm^2, respectively. However, there was no significant effect on DAH 5, 11 and 15 (DAH 5: p=0.34; DAH 11: p=0.379; DAH 15: p=0.701, ANCOVA; Fig. 4.6).

The parameters of the three linear regression models with standard deviation and R^2 values for the three treatments were the following: for DA, slope a=-0.094±0.032, intercept b=0.129±0.044, R^2=89.7%; for DX, a=-0.108±0.039, b=0.131±0.05, R^2=88.7%; for NX, a=-0.092±0.043, b=0.127±0.053, R^2=82.3%. They are being graphically represented in Fig. 4.6. The slopes were shown to be different (extra-sum-of-squares F test: $p<0.01$), with $|a_{DX}|$ larger than both $|a_{DA}|$ and $|a_{NX}|$ ($p<0.05$ between a_{DX} and a_{DA}; $p<0.01$ between a_{DX} and a_{NX}, Tukey's post-hoc test). Therefore, the yolk sac depletion rate was significantly larger in the specimens of treatment DX than both DA and NX.

4.3.3.3. Pectoral angle

On DAH 11, the pectoral angle of treatment DA was significantly larger than that for treatment DX and NX (respectively $p<0.01$ and $p<0.001$, Bonferroni-corrected Watson-Williams test). The means with standard deviations were the following: PA_{DA}=133.8±8.1°, PA_{DX}=124.7±14.6°, PA_{NX}=120.0±11.6°. On DAH 15, there were no significant differences between the means of the treatments (p=0.966 between DA and DX; p=0.99 between DX and NX; p=0.366 between DA and NX; Bonferroni-corrected Watson-Williams test).

4.3.3.4. PCA on lengths and depths

According to the PCA of the measurements of all days, PC1 explained 95.94% of the total variation. According to its factor loadings, all variables were positively correlated, with total length and standard length presenting the largest loadings of 0.535 and 0.499 respectively, except yolk sac length and yolk sac depth which presented negative loadings of -0.196

and -0.095, respectively. Therefore, PC1 was interpreted as a contrast of larvae length to yolk sac size. PC2 explained 1.6% of the total variation, and its major factor loadings were head length and pre-anal finfold length, with values of -0.453 and 0.4, respectively. It was thus interpreted to represent head length in contrast to pre-anal finfold length (Fig. 4.7).

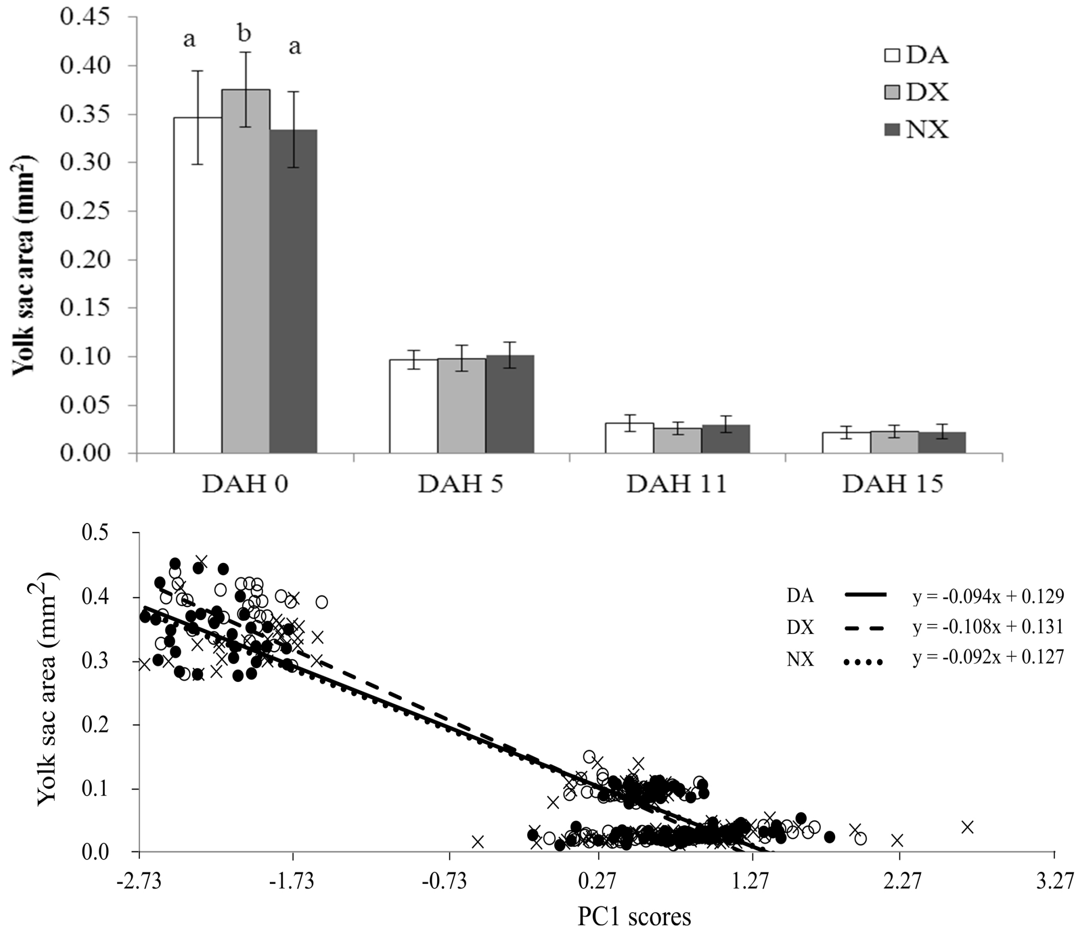

Fig. 4.6: Upper graph: Yolk sac area versus age of fish in DAH, with standard deviation error bars. The only statistically significant difference exists on DAH 0, and is being prepresented with different letters. Lower graph: PC1 scores (size component) from the PCA analysis on all distance measurements of all days excluding pectoral angle, yolk sac length and yolk sac depth, versus the yolk sac area for all treatments, with the linear model formulae for each line. The graph represents the depletion of the yolk sac as the larvae size is increasing, which is being represented on the horizontal axis. Specimens of treatment DA are being represented with dot markers, DX with circle markers, and NX with x markers.

In Fig. 4.7, the main demonstrated morphological changes as the larvae grew older were the longitudinal growth and the depletion of the yolk sac, which were represented across PC1: there was a distinction of clusters between DAH 0 and the rest of the age classes, which indicated that the larval length and yolk sac differences were small after DAH 5. This was particularly evidenced from the overlap of clusters from DAH 11 and DAH 15. Furthermore, no particular overall pattern of treatment effect on size was shown throughout the duration of the experiment.

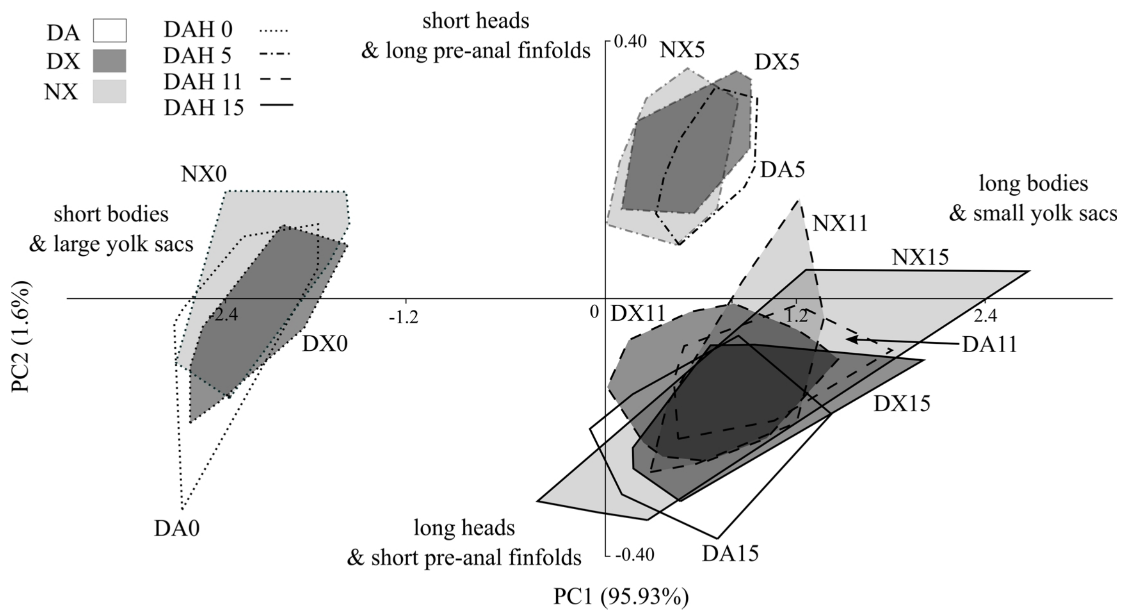

Fig. 4.7: Scatterplot of PC1 vs PC2 scores of the disregard-group PCA of all measured lengths and depths on the combined dataset of all age classes. The outermost specimen points of each group are connected to form its cluster, with specimen markers omitted to improve the clarity of the graph. The names of the treatment groups are placed beside their corresponding clusters. The effects of each individual principal component are noted at the edges of the axes, and the percentage of total size variation explained by each PC is included beside the name of each PC axis.

Regarding the effects in each age class, on DAH 0, PC1 explained 82.67% of the total variation. According to the factor loadings, dorsal finfold length presented the highest loading of 0.585, followed by gut length with a loading of 0.456. Most of the other larvae lengths were also positively correlated, all to a larger degree than the depths with loadings that remained very low, and thus PC1 was interpreted to represent longitudinal growth. There

was a treatment effect on PC1 ($p<0.001$, ANOVA), with "NX" larvae exhibiting longer bodies than "DA" ($p<0.001$, Tukey's post-hoc test), as also indicated by the total lengths analysis which indicated that the total lengths of "NX" were larger than "DA". PC2 accounted for 17.33% of the total variation, with the major loading coming from head length at 0.635, followed by yolk sac length at 0.551 and anal finfold length at -0.321. It was interpreted to represent head lengths and yolk sac lengths in contrast to anal finfold lengths. There was again a treatment effect on PC2 ($p<0.001$, ANOVA), with "DX" larvae having longer heads and yolk sacs but shorter anal finfolds than both "DA" and "NX" (in both cases $p<0.001$, Tukey's post-hoc test).

On DAH 5, PC1 explained 93.88% of the total variation. Standard length presented the largest loading of 0.578. All of the other variables were also positively correlated, except pectoral body depth (PBD) with a loading of -0.309, and therefore PC1 was interpreted to represent size in contrast to pectoral body depth. The treatments induced an effect on PC1 ($p<0.001$, ANOVA), with "DA" larvae having significantly larger bodies with smaller pectoral depths than both "DX" and "NX" (in both cases $p<0.001$, Tukey's post-hoc test). PC2 accounted for 6.12% of the total variation, with the major loadings coming from head depth and head length at 0.53 and 0.505 respectively. It was interpreted to represent head size. The treatments again induced an effect on PC2 ($p<0.01$, ANOVA), with "DX" specimens possessing larger heads than "NX".

On DAH 11, PC1 explained 90.42% of the total variation. Standard length again presented the highest loading equal to 0.539, with all of the other variables positively correlated to a smaller degree. It was interpreted to represent larval size. There was a treatment effect on PC1 ($p<0.001$, ANOVA), with "DA" larvae being significantly larger than both "DX" and "NX". PC2 explained 9.58% of the total variation, with the major loadings originating from anal finfold length and pre-anal finfold length, equal to 0.488 and 0.474 respectively. These two represent the length of the ventral finfold, and are also approximately equal to the length of the notochord. However, there was also a contrast of these two lengths with the larval depths, and therefore PC2 was interpreted to represent the ventral finfold length in contrast to body depths. The treatments induced an effect on PC2 ($p<0.001$, Kruskal-Wallis), with "NX" larvae being longer in their ventral finfolds and notochords but thinner than both "DA" and "DX".

On DAH 15, almost all of the variation was explained by PC1, with a percentage of 96.95%. Pre-anal finfold length exhibited the highest loading of 0.508. All of the other variables were also positively correlated, apart from dorsal finfold depth, predorsal finfold length and anal body depth, but their loadings were very small (equal to -0.106, -0.028 and -0.024 respectively) compared to the loadings of all the other variables, and were thus not taken into account. Consequently, PC1 was interpreted to represent size. As far as PC2 is concerned, it explained 3.05% of the total variation, and was an indication of dorsal finfold depth (DFFD), as it was dominated by it with a loading of 0.615. The treatments did not induce an effect on PC1 or PC2 (p=0.335 and p=0.626, Kruskal-Wallis).

4.3.4. Outline-based shape analysis

Similarly to the distance and angle analysis, the PCA on all treatment groups from all DAH indicated the ontogenetic change of the depletion of the yolk sac as the most distinct shape effect, represented by PC1 which explained 67.39% of the total variation (Fig. 4.8). Throughout the progress of the experiment the clusters of each age class were getting placed further towards the negative end of the PC1 axis which indicated a yolk sac depletion, with DAH 0 being the rightmost one, followed by DAH 5, 11 and 15. This orientation is explained by the yolk sac and oil globule becoming smaller as the larvae grew older, and situated further into the larval body. After DAH 5 and especially after DAH 11, the yolk sacs became almost indistinguishable in the shape outlines. Similarly to the distance and angle-based analysis, an overall treatment shape effect could not be discerned, and it was thus necessary to explore the differences separately on each DAH (Fig. 4.9).

On DAH 0, there was a significant shape difference from the treatments (p<0.01, PERMANOVA), with a difference between groups “DA” and “NX” (p<0.01, Bonferroni-corrected post-hoc test). The PCA indicated there were 8 effective principal components, with PC1 and PC2 explaining 35.87% and 32.03% of the total shape variation. Unlike for the distance analysis, PC1 was not predominantly responsible for explaining the variation, as PC2 played an almost equally important role. PC1 also reflected a treatment effect (p<0.05, ANOVA), with “NX” having significantly lower scores than “DA” (p<0.05, Tukey’s test).

On DAH 0, PC1 reflects subtle levels of body curvature, and PC2 body thickness, with the "NX" specimens appearing to be slightly more curved and thinner than "DA" (Fig. 4.9).

On DAH 5, the treatments induced again a significant effect (p<0.001, PERMANOVA), with shape differences between all groups (p<0.001 between DA and NX; p<0.05 between DA and DX, Bonferroni-corrected post-hoc tests). There were 10 principal components, with PC1 and PC2 explained 34.25% and 24.12% of the total variation. The graph of DAH 5 in Fig. 4.9 indicated that specimens of treatment DA were placed slightly further towards the negative edges of the PC1 and PC2 axes than DX and NX. PC2 indicated a small effect on body thickness in the trunk area, but both PC1 and PC2 seemed to explain very subtle shape effects, which did not suggest a distinct shape difference. Therefore, a difference between the groups was present, but not clearly manifested.

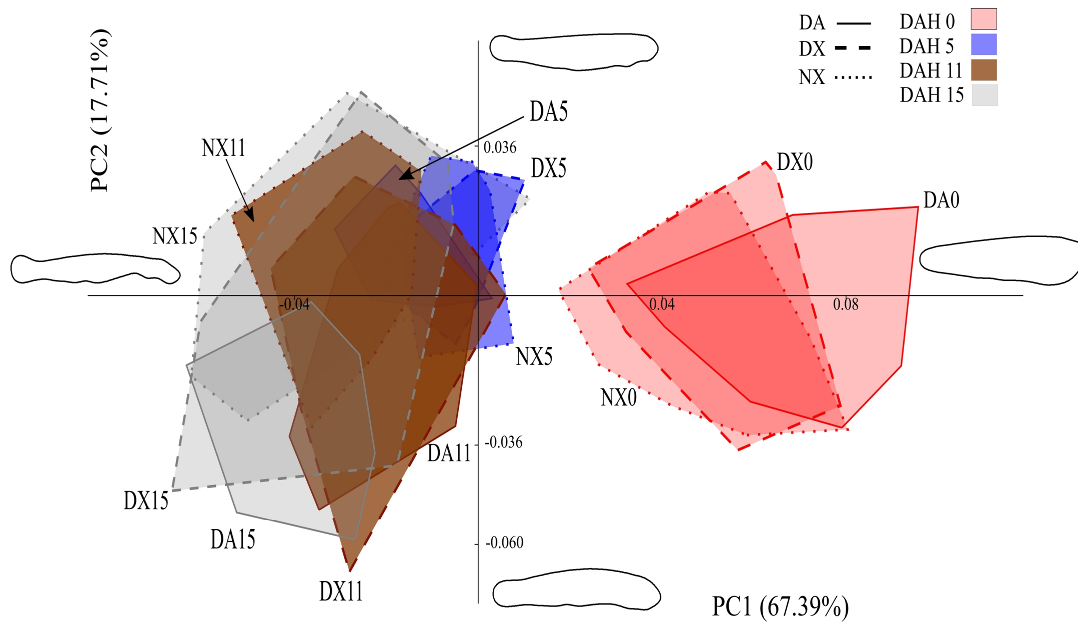

Fig. 4.8: Scatterplot of PC1 vs PC2 scores of the disregard-group PCA on the Elliptic Fourier shape coefficients of the pooled dataset of the treatment groups from all days after hatching (DAH). Similarly to the PC1 vs PC2 graph of distance measurements of Fig. 4.7, the outermost specimen points of each group are connected to form its cluster, but the specimen markers have been omitted to improve the clarity of the graph. The names of the treatment groups are again placed beside their corresponding clusters. At the edges of the axes the ±2 standard deviation (SD) shape outlines of each PC are placed, and the percentage of total variation explained by each PC is included beside the axis name.

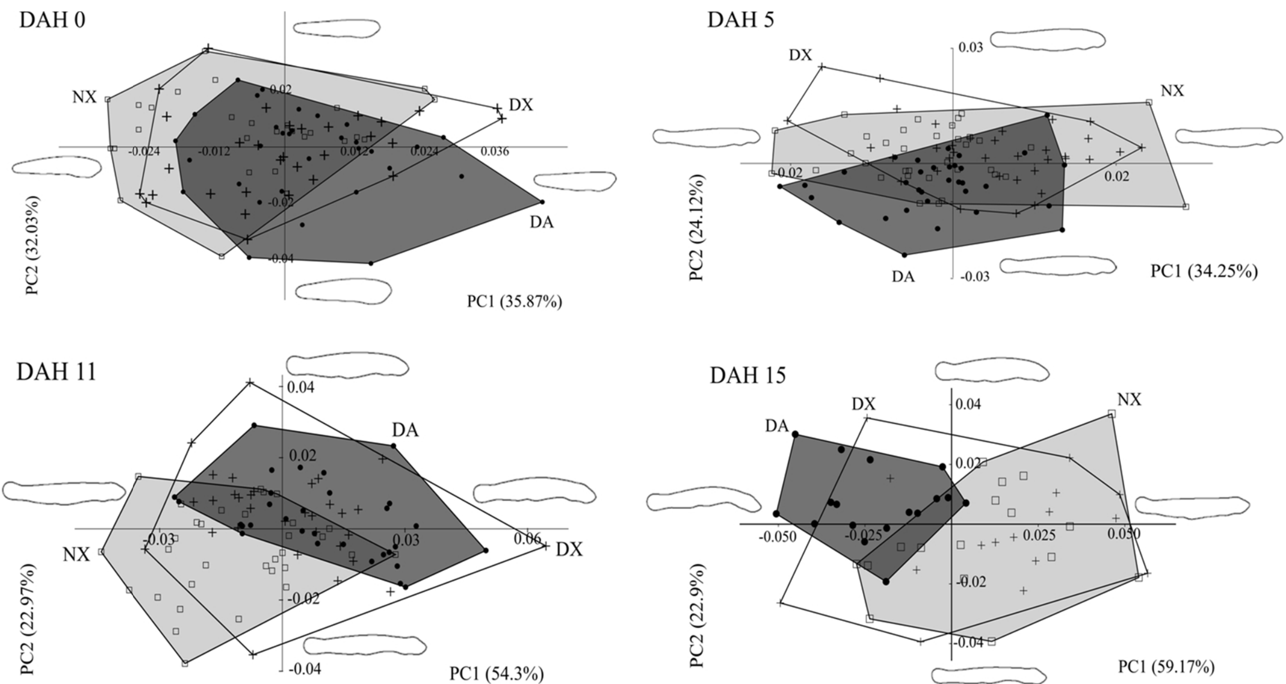

Fig. 4.9: Scatterplot graphs of PC1 vs PC2 scores from disregard-group PCAs on the Elliptic Fourier shape coefficients of all treatment groups from each day after hatching (DAH). The names of the treatment groups are placed beside their corresponding clusters. Treatment DA specimens are being represented with dotted markers and their clusters with dark grey fill colour, DX with cross markers and transparent clusters, and NX with square markers and light grey clusters. At the edges of the axes the ±2 standard deviation (SD) shape outlines of each PC are placed, and the percentage of total variation explained by each PC is included beside the axis name.

On DAH 11, there was a significant effect of the treatments on shape ($p<0.001$, PERMANOVA), with differences between group NX and both DX and DA (in both cases: $p<0.001$, Bonferroni-corrected post-hoc tests). The principal components were 6, with PC1 and PC2 explaining 54.3% and 22.97% of the total variation. PC1 reflected a small degree of body curvature (graph of DAH 11, Fig. 4.9), while PC2 was an indication of body thickness, particularly in the trunk area. There was a treatment effect on both PC1 and PC2 ($p<0.01$, Kruskal Wallis and ANOVA, respectively), with the “NX” group presenting significantly lower scores than “DX” and “DA” (for PC1: $p<0.001$ between “NX and “DX”, $p<0.05$ between “NX” and “DA”, Bonferroni-corrected Mann Whitney U; for PC2: $p<0.01$ between “NX” and both “DX” and “DA”, Tukey’s test). This implied that “NX” specimens were less curved and thinner than both those of “DX” and “DA”.

On DAH 15, similarly to the other three age classes, there was again an effect of the treatments on the shape of the specimens ($p<0.001$, MANOVA), with differences existing between groups DA and both DX and NX (in both cases $p<0.001$, Bonferroni-corrected post-hoc tests). There were 6 principal components, with PC1 and PC2 accounting for 59.17% and 22.9% of the total shape variation. PC1 was shown to be an indicator of body curvature, while PC2, like the PC2 in the other three age classes, showed the overall body depth (Fig. 4.9). There was a treatment effect on PC1 ($p<0.001$, ANOVA), with “DA” presenting significantly lower scores than both “DX” and “NX” (in both cases $p<0.01$, Tukey’s test). Consequently, the “DA” specimens exhibited a tendency for slightly more curved shapes than “DX” and “NX”.

The question arises whether these observed between-group differences in PC1 and PC2 could be the result of differences in the total length of the individuals. The shape analysis through the use of Elliptic Fourier descriptors involves the removal of all size-related variation through the performed standardisation for position, size, orientation of the specimens and starting point of outline coding (Iwata and Ukai, 2002; Nikolakakis et al., 2014). The possibility of the existence of an allometric effect was examined with an ANCOVA of PC1 and PC2 per DAH with TL as a covariate, with Bonferroni-corrected post-hoc tests.

On DAH 0, the witnessed differences in total length were $TL_{NX>}TL_{DA}$. The ANCOVA test indicated there was a shape effect on PC1 ($p<0.05$), with $PC1_{DA}>PC1_{NX}$ ($p<0.01$), Therefore, the aforementioned shape difference in PC1 between groups DA and DX is indeed due to a

treatment effect. As for PC2, the ANCOVA test indicated there was no shape effect ($p=0.543$) when standardising for TL. Therefore, the shape differences of PC2 (reflecting a slight body thickness, mainly in the trunk region) can partly be a reflection of their difference in total length, with the "NX" larvae not only being slightly thicker than "DA", but longer as well. If we take into consideration that total length can be a reflection of their ontogenetic state, this can possibly indicate that the "NX" presented shapes that were ontogenetically more advanced than "DA".

On DAH 5, there was a treatment effect on PC1 ($p<0.05$) with $PC1_{DX}>PC1_{NX}$ ($p<0.05$), but without it corresponding to an effect on total length, as there was no significant difference between TL_{DX} and TL_{NX}. As for PC2, ANCOVA indicated there was a shape effect, with $PC2_{DX}>PC2_{DA}$ and $PC2_{NX}>PC2_{DA}$ ($p<0.01$). However, even though the differences in total length were $TL_{DA}>TL_{DX}$ and $TL_{DA}>TL_{NX}$, due to the ANCOVA's standardisation for TL the witnessed shape effect of PC2 (very subtle thickness in the trunk area) is not thought to have been due to ontogenetic allometry, but due to treatment.

On DAH 11, the length differences were $TL_{DA}>TL_{DX}$, and there was a treatment effect on PC1 ($p<0.01$), but with no difference between $PC1_{DA}$ and $PC1_{DX}$ ($p=0.053$). There was also a treatment effect on PC2 ($p<0.01$), but again with no difference between $PC1_{DA}$ and $PC1_{DX}$ ($p=0.835$). On DAH 15, there were no differences in total length, so the aforementioned differences in PC1 or PC2 are not due to a difference in total length.

Regarding the pairwise shape differences (Fig. 4.10), the axenity factor showed effects that were moderate (DAH 5) or not significant (DAH 0 and DAH 11), but with a peak towards the end of the experiment on DAH 15. The disinfection factor induced shape differences only on DAH 5 and 11, and on these two age classes it exhibited the smallest effect. Finally, the factor of their combination was the only one which maintained a significant shape effect in all age classes, also specifically at the moment of hatching, and showing a trend of increase after DAH 5.

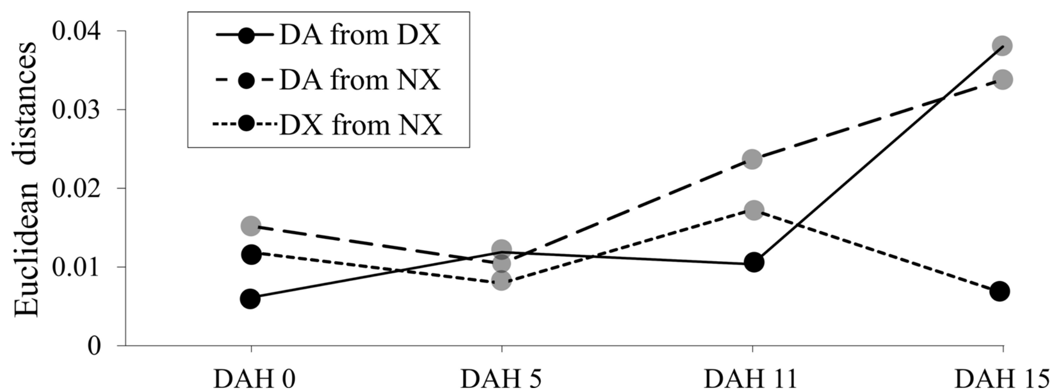

Fig. 4.10: Euclidean distances between the shape centroids of treatments DA, DX and NX on each day after hatching (DAH). Each line corresponds to a pairwise comparison, representing the effect of axenity (between DA and DX), disinfection (between DX and NX) and their combination (between DA and NX). In the cases where the shape differences were significant, the marker is coloured grey.

4.4. Discussion

4.4.1. Hatching and mortality percentages

In the present study, the data did not indicate a significant effect of the disinfection and axenity on the hatching and mortality percentages. In axenic or gnotobiotic studies, glutaraldehyde disinfection has been shown to present better hatchability versus non-disinfected eggs of seabass *Dicentrarchus labrax* (Dierckens et al., 2009) and Atlantic cod *Gadus morhua* (Forberg et al., 2011), but in Nile tilapia *Oreochromis niloticus* (Situmorang et al., 2014) no effect was present.

Numerous reasons might explain not reporting the same beneficial result on hatchability in the current study. The initial bacterial load could have been higher in the present experiment, with the disinfection not reducing it to a point where a difference in hatching percentages would be demonstrated. In literature, the bactericidal effect has been shown to vary with the initial bacterial density: in Salvesen and Vadstein (1995), the fraction of surface sterile eggs of plaice *Pleuronectes platessa* after a standard disinfection treatment was higher in groups with a low initial bacterial load than in groups with a high load.

Similarly to hatchability, the influence of disinfection and axenic conditions has also presented inconsistent effects on survival: glutaraldehyde has been generally known to improve it for many different marine fish species (Skjermo and Vadstein, 1999), but not without exceptions, as for example in the case of larval halibut *Hippoglossus hippoglossus* where no significant differences were induced during the yolk sac stage, although it presented better survival after first feeding (Harboe et al., 1994). In Forberg et al. (2011), egg disinfection by glutaraldehyde and axenity by rifampicin and ampicillin (administered until hatching) improved the survival of Atlantic cod larvae. However, similarly to the present study, in the study of Rekecki et al. (2009) no significant effect due to disinfection by glutaraldehyde and axenity by rifampicin and ampicillin was observed on the survival of axenic seabass larvae, treated following the same axenity protocol of Dierckens et al. (2009), as used here.

In general, the use of disinfectants such as glutaraldehyde has been shown to have varying results on hatching percentages, growth rates and the survival of fish larvae, depending on their type, concentration, duration of exposure, stage of egg development on which they are being applied, and the species (Douillet and Holt, 1994; Escaffre et al., 2001; Ben-Atia et al., 2007; Schaeck et al., 2016). Variation in hatching percentages and survival has been observed between groups of the same glutaraldehyde egg disinfection treatment for seabass (Da Silva, 2007) and gilthead seabream *Sparus aurata* (Escaffre et al., 2001), with the authors suggesting that the toxicity of glutaraldehyde might be lower in eggs with a better initial quality than in others. This quality affects larval performance in the early stages, including but not limited to the hatchability and survival, and has been known to be unpredictable. Many factors are playing a role (Kamler, 1992; Brooks et al., 1997; Rani, 2005), with effects that are non-standardised and confounding. An example of such a factor is parental genetics, or the sensitivity of the eggs to disinfection according to changes in chorion thickness (Escaffre et al., 2001), induced for example by differences in the time of sampling in each spawning season (Hirazawa et al., 1999).

4.4.2. Effects on distances, angles and yolk sac areas

Regarding the overall treatment effect on the lengths and depths of the larvae as represented in Fig. 4.7, in the first days the ontogenetic changes were rapid and drastic, but differences became too small for the clusters of later age classes to remain discrete. There were three overlapping groups: (a) DAH 0, (b) DAH 5, and (c) a separate one with groups of DAH 11 and 15. The evident ontogenetic effects were the increase in total length and head length, and the depletion of yolk sac. In Fig. 4.6 where the depletion of the yolk sac is represented graphically, three cluster groups also appear. This might be happening because the yolk sac depletion is rapid, and what is seen here is likely a cluster for the starting point of DAH 1, another one for DAH 5, and then a final one for both DAH 11 and DAH 15 where there is only an extremely small amount of yolk sac still left. Due to the fact that in the current experiment the larvae opened their mouhths around DAH 4-5 and almost completely depleted their yolk sacs by DAH 15, the overlap for the two aforementioned latter cluster groups might correspond to the vulnerable ontogenetic phase when endogenous and exogenous feeding occur simultaneously.

These ontogenetic effects were stronger than the treatment effects in determining the overall morphological changes, and therefore the differences in each individual DAH needed to be explored in detail. On DAH 0, the axenity and disinfection factors did not induce a significant effect on total length, but their synergistic effect was significant, as the larvae of treatment NX had significantly larger total lengths than the ones from treatment DA. At such an early stage, the larvae have not grown in the axenic medium yet, but have only been exposed to it for the three days of their egg incubation period. Therefore, it might be too soon for any differences to manifest themselves as a result of the axenic incubation or the disinfection alone. However, their combination might be strong enough to inhibit total length growth while the embryos are developing inside the eggs. Chemical uptake is known to occur in the fish egg oocyte (Brooks et al., 1997), and rifampicin and glutaraldehyde have been known to penetrate it prior to hatching (Brown et al., 1990; Escaffre et al., 2001).

Although the lack of disinfection and axenic incubation resulted in longer larvae at the moment of hatching, this was soon reversed for the axenic specimens: the total length growth rate immediately after hatching was higher in treatment DA than NX, with “DA” specimens

exhibiting significantly larger bodies than both "DX" and "NX" on DAH 5 and 11. This could mean that the possible growth-inhibition result of the combined effects of glutaraldehyde disinfection and antibiotics-induced axenity was no longer present after the embryos came into direct contact with their rearing environment outside the egg shell.

Rekecki et al. (2009) also observed xenic seabass larvae having a similar total length with axenic ones at the moment of hatching, but having smaller total lengths on DAH 6 and DAH 14. The reason might be the competition of the xenic larvae with the greater microbiota community for energy and nutrients (Anderson, 2002; Collier et al., 2003), or the increased accumulation of bacterial toxic metabolic byproducts in the gut of the xenic larvae, which have been known to be growth depressing (Muramatsu et al., 1987; Anderson, 2002; Collier et al., 2003; Willing & Van Kessel, 2007). The microflora could even have played a negative role on the normal physiological development. Furthermore, poorer total length growth of the xenic larvae might originate from them having to deal with energy demands that are common in all treatments, but likely elevated in the xenic ones due to the higher bacterial presence. Examples include energy expenditures in activities such as epithelial differentiation and maturation in the gut, which are aided by the gut microbiota (Nayak, 2010). A counter argument would be that the absence of gut microbiota in the axenic specimens may have caused a lack of nutrient absorption to provide energy for growth, resulting in a more inefficient food digestion. However, in the present study and in Rekecki et al. (2009), the overall growth of the axenic larvae was better. It is suggested that activities such as the colonisation of the digestive tract, the triggering of immunology mechanisms and other similar energy costs to combat or assimilate bacteria are indeed increased in xenic larvae, according to the morphological differences in the gut between xenic and germ-free/gnotobiotic larvae of zebrafish *Danio rerio* (Rawls et al., 2004; Bates et al., 2006) and European seabass (Rekecki et al., 2009).

The axenic larvae had a smaller yolk sac than the xenic at the moment of hatching. Since the initial yolk energy is related to the amount of energy available to be consumed daily from yolk by an embryo or larva (Kamler, 1992 and references therein), this means that the xenic were better equipped to handle the aforementioned increased energy demands. Moreover, they consumed their yolk sacs more rapidly than the axenic throughout the experiment. This suggests that these energy demands of the xenic were met with an increased yolk sac

depletion rate. There were indications that this might not have been enough, and that these demands were difficult for the xenic larvae to handle, with them showing signs of an artificial starvation state compared to the axenic, which is induced if an unsatisfied need for energy beyond the internal yolk sac reserves of the larvae exists (Johns and Howell, 1980; Kamler, 1992).

A possible indication of this pseudo-starvation state of the xenic was their significantly higher yolk sac depletion rate, similarly to starved larvae of brown trout *Salmo trutta*, which have been shown to consume yolk more rapidly than fed larvae during the final period of yolk resorption (Raciborski, 1987). Another indication is the pectoral angle of specimens from both xenic treatments of DX and NX, which was significantly smaller than DA on DAH 11, as this smaller angle has been shown to be an indication of starvation in larvae, with the examples of herring *Clupea harengus* and plaice *Pleuronectes platessa* (Ehrlich et al., 1976), and of rock bream *Oplegnathus fasciatus* (Park et al., 2013). However, on DAH 15 there were no significant differences between these angles, which might indicate this starvation state was no longer present, or detectable due to the large mortalities on DAH 15 and 16.

Regarding the effects of the disinfection factor, the disinfected eggs of treatment DX yielded larvae with larger yolk sac areas at the moment of hatching than the non-disinfected of treatment NX, which, as described above, means that they were better equipped to handle the energy demands at the very early stages until they could rely solely on external feeding. This happens after the complete depletion of the yolk sac and oil globule, which in the present experiment occurred towards the end of the experiment around DAH 15. Additionally, the yolk sac depletion rate was higher in the “DX” specimens than “NX”, and therefore the egg disinfection was associated with a more rapid consumption of the larval internal energy reserves. It could be argued that the disinfection allowed a more efficient yolk utilisation for their bacterial energy expenditures, as well as their ontogenetic differentiation (Klaoudatos et al., 1990), maintenance, building up of body tissues and accumulation of reserves in various organs (Pilar Olivar et al., 2000). There is a considerable lack of information in literature regarding the association of disinfection regimes with the yolk sac consumption rate. In Fry et al. (2015), four treatments (ozone at low, standard and high concentrations, and Perosan) exhibited no effect on the yolk sac conversion efficiency of rainbow trout *Oncorhynchus*

mykiss larvae of DAH 0 to 30. As discussed above, these effects are usually species, disinfection agent and concentration-specific.

In contrast to these beneficial effects of the disinfection, there was a possible negative: “DX” larvae had smaller anal finfolds than “NX” on DAH 0 and 11, and smaller dorsal finfolds on DAH 11. The dorsal and ventral portions of the embryonic finfold are respiratory surfaces in pelagic fish larvae (Klaoudatos et al., 1990 and references therein). Reduced finfold surface area has been linked with detrimental physical and physiological effects such as decreased blood flow to tissues, interference with nervous system function and increased energy expenditures (Von Westernhagen, 1988 in Boglione et al., 2013b), which agrees with the aforementioned increased yolk sac consumption rate of “DX” larvae versus “NX”. It must be noted that the above morphological observation was not witnessed consistently in all age classes. Nevertheless, it was present at the moment of hatching, and when this is evaluated together with the above conclusion of the lack of disinfection and axenic incubation allowing the untreated larvae to display larger total lengths on DAH 0, it suggests a possible adverse effect of this secondary disinfection process during hatching.

4.4.3. Outline-based shape effects

Disinfection and axenity were achieved through the use of glutaraldehyde as a disinfectant, and rifampicin and ampicillin as antibiotics. To the best of our knowledge, no severe abnormalities on fish larvae from the use of these agents in the concentrations and exposure times used in the present study have been documented. In Escaffre et al. (2001), there was no significant difference in deformities upon hatching between gilthead seabream *Sparus aurata* larvae originating from eggs non-disinfected and disinfected with glutaraldehyde, in the concentration and exposure time used here.

As far as an overall outline-based shape effect is concerned, again in a similar fashion to the previous analysis, the ontogenetic effects of yolk sac depletion were distinct while there was no clear overall treatment effect on all sampling points, and therefore it was necessary to examine the individual effects on each DAH. Significant shape differences due to the antibiotics-induced axenity were observed between “DA” and “DX” specimens on DAH 5 and 15, but the shape effects were not clearly evident. According to the Euclidean distances

between the shape centroids of the two groups, shape differences manifested slowly but peaked towards the end of the experiment, with "DA" specimens exhibiting a slight tendency for thicker but more curved shapes than "DX" and "NX", with a curvature that may be considered abnormal. Prolonged exposure to antibiotics has been known to have the potential to induce malformations in the progeny of spring chinook salmon *Oncorhynchus tshawytscha* (De Cew, 1972). Furthermore, they can be considered an extra stress factor detrimental to eggs that come from a suboptimal batch quality (Schaeck et al., 2016), which is a problem frequently encountered with European seabass, also according to our observations.

It is important to note that the curved shapes of "DA" larvae might not be a deformity effect, but an ontogenetic one: similarly curved specimens have been known to represent a natural state of shape, after the stage of point-of-no-return (Yin & Blaxter, 1986) and around the time they are ready to transition from the pre-flexion to the post-flexion stage (Barnabe, 1976; Zavala-Munoz et al., 2016), which in seabass is usually happening around DAH 20 (Koumoundouros et al., 2002a). Therefore, the curved shapes of the "DA" larvae might be a sign of a slightly more advanced ontogenetic stage compared to "DX", which might be induced by the presence of axenic conditions. However, even though there were signs that this started to occur on DAH 5, this might have happened only towards the end of the experiment, since the "DA" seemed less ontogenetically advanced than "NX" on DAH 0. The presence of fixation artefacts was minimised to the best possible degree, with no size or shape differences between the live and fixed specimens until five months post fixation (Nikolakakis et al., 2014), with the analyses completed before that time.

The witnessed increased yolk sac consumption rate of the xenic larvae might have left the axenic free to invest the saved energy into rapid growth, and consequently into the manifestation of such a slightly advanced ontogenetic stage. Such a pattern was observed by Pilar Olivar et al. (2000): after DAH 13, there was a sudden increase in the growth rate following a growth plateau (as described by the Gompertz model) of fed seabass larvae versus starved, with the fed having the energy reserves to support it. The same 2-cycle Gompertz function is also described for Pacific herring larvae by McGurk (1985), with the first cycle occurring from hatching until DAH 10, and the second until approximately DAH 50. Pilar Olivar et al. (2000) suggest that the sudden increase in growth rate after the end of the first cycle means that the animals, after using the yolk sac energy for maintenance, are

free to invest their energy reserves in building up body tissues or accumulating reserves in organs. This is probably happening after the establishment of successful external feeding and living past their point of no return, which Barnabe (1976) places around DAH 15 for 15°C.

A shape effect due to disinfection was not present at hatching and at the end of the experiment, and it was significant but very moderate on DAH 5 and DAH 11. On both these days, based on the Euclidean distance values, the disinfection factor induced the smallest shape differences from the other two factors of axenity and the synergistic effect of axenity and disinfection. The most distinct shape effect was on DAH 11, where “NX” specimens appeared slightly less curved than those of “DX”, but it was still very moderate. Major disinfection effects were not observed in the current study, which might be due to the gentle action of glutaraldehyde, which is known to have acceptable values for seabass notably lower than those recommended for other fish species (Da Silva, 2007), where more pronounced effects have been observed. An example is from ozone on larval gilthead seabream *Sparus aurata* (Ben-Atia et al., 2007), where enlarged heads and curved spines at the mid-body, tail and at the base of the head were encountered, which caused it to be angled dorsally.

The synergistic effect of the axenity and egg disinfection factors was significant in all age classes, with a clear trend of increase after DAH 5. On DAH 0 it was the only factor inducing an effect, with the NX specimens appearing thinner and slightly more curved than DA. Similar to the treatment NX with significantly larger total lengths than the ones from treatment DA at the moment of hatching, it is noted again that at this stage it might be too soon for any shape differences to manifest themselves from axenity or disinfection alone, but their combined effect was significant. On DAH 11 and 15, the shapes of NX specimens were again thinner than DA, but less curved. This seemed to indicate that the specimens untreated with the disinfection and antibiotic agents exhibited smaller dorsoventral growth during the progress of the experiment, but also less abnormal shapes, possibly indicating a beneficial shape effect from the lack of secondary disinfection and antibiotic-induced axenic conditions.

It must be noted that the validation of the axenic conditions, according to the protocol by Dierckens et al. (2009), was performed with culture-based media (marine broth and marine agar), and therefore the presence of viable but non-culturable bacteria might have not been detected. However, in the original protocol, bacterial contamination was also checked

through DNA analysis. Together with these methods, flow cytometry can provide an extra verification as it can identify and quantify organisms formerly undetectable by culture techniques, with the added benefits of speed, reproducibility and the ability to handle large sample sizes, as illustrated in the axenic seabass larvae rearing protocol of Schaeck et al. (2016).

Water quality could have played a part in the results of this study. Unfortunately, data were not available for it, due to the following reason: the present study was performed using the rearing protocol followed by Nikolakakis et al. (2014), based on Dierckens et al. (2009). These protocols require the absolute minimum interaction with the rearing vials in order to avoid larvae stress and bacterial contamination, and for this reason there were no measurements of O_2 and ammonium nitrogen taken. It is noted however that tests in preliminary experiments had shown low levels of the latter. Additionally, due to the fact that the larvae were individually chosen one by one with a pipette and stocked in vials filled with filtered autoclaved seawater with rifampicin (in the case of treatment DA), and with xenic seawater (in treatments DX and NX), the water quality was likely different only between treatment DA and the two other treatments of DX and NX, as the rearing medium is thought to have been the major determining factor. Therefore, together with the differences in bacterial load, the water quality could have played a part in the witnessed size and shape differences between treatments DA-DX and DA-NX. However, this effect is unclear, since to the best of our knowledge, there is a lack of substantial literature data for any phenotypic effects from water quality in such early larval stages. There is an exception for the effect of heavy metals on embryonic and larval development, but it was judged to be largely irrelevant, as there were no potential sources of contamination with heavy metals in the present study. In conditions of low water exchange, such as the ones in the vials, abnormal swimming behaviour and deformities have been known to appear in rainbow trout *Oncorhynchus mykiss* (Davidson et al., 2011). However, this was witnessed on specimens (a) larger (of at least 18g) than the ones of the present study, (b) with advanced skeletal development while there was none in the seabass specimens of DAH 15 (Rahman, 2008; Darias et al., 2010), (c) under chronic exposure to deteriorated water quality of elevated concentrations of dissolved potassium and nitrate nitrogen (at least 9 weeks after the mentioned starting size of 18g), and (d) with increased mortalities, which was not the case in the present study, since as mentioned above there was no clear pattern of any treatment

consistently presenting an effect on mortalities or hatching percentages. Therefore, it is argued that while bacterial conditions can have a size and shape effect in such a short study time at this stage, water quality conditions such as the aforementioned may require a prolonged exposure for the manifestation of morphological abnormalities, and are usually met with increased mortalities, which were not witnessed here.

To summarise, regarding the effect of antibiotics-induced axenity, axenic specimens exhibited larger bodies than both xenic and untreated (NX) on DAH 5 and 11. They also had a smaller yolk sac than the xenic at the moment of hatching, but consumed it more slowly, probably due to the reduced energy demands from lack of bacterial colonisation of the digestive tract, and other similar reduced energy expenditures to combat or assimilate bacteria. This could allow them to invest that energy better towards growth, ontogenesis, and accumulation of reserves in various organs. Towards the end of the experiment, the axenic larvae were thicker, but to a small degree more curved than the xenic and untreated, which may be considered an abnormal shape, or a slightly more advanced ontogenetic stage.

As far as the egg disinfection is concerned, it had significant but very moderate shape effects on DAH 5 and 11. It was also associated with a more rapid consumption of the larval internal energy reserves. An adverse effect was that it induced smaller anal finfolds on DAH 0 and 11, which might be associated with a reduced respiratory ability, with consequent increased energy expenditures that could explain the increased yolk sac depletion rate.

Regarding the synergy of the axenity and disinfection factors, on the day of hatching they did not induce a significant effect on total length individually, but their combined effect allowed the untreated larvae to exhibit significantly larger total lengths than the axenic from disinfected eggs at the end of incubation. As for the shape effect, the lack of secondary disinfection and axenity induced thinner, but less curved, shapes.

As a conclusion, the secondary egg disinfection in the laboratory, after the first in the hatchery, and the administration of antibiotics in the incubation and rearing media in order to establish an axenic environment for the rearing of seabass larvae, were not seen to induce adverse effects on the hatching percentages, survival and larval morphology. Additionally, the axenity factor induced significant shape effects, which suggest the existence of bacterial

mechanisms that control them. These might be associated with the known metabolic processes that affect growth, ontogenesis and the allocation of energy towards them, but they need to be investigated further, especially in the crucial early larval stages with the aid of geometric morphometrics.

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Chapter 5

Effects of antibiotic-induced differences in bacterial load on growth and shape of early larval European seabass (*Dicentrarchus labrax* L.)

This chapter is based on:

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Abstract

The aim of the present study was to quantify the effects of antibiotic-induced differences in bacterial load on the size and shape of fish larvae, using *Dicentrarchus labrax* of day after hatching (DAH) 3 as a case study. They were split in two treatment groups and reared in 50 ml vials until DAH 14, with the control treatment ("NA") including larvae reared in filtered autoclaved seawater without antibiotics, while the second ("A") included larvae reared in filtered autoclaved seawater with rifampicin, ampicillin, kanamycin, trimethoprim and gentamicin, with a concentration of 10 mg l^{-1} each. They were sampled for bacterial presence on DAH 4, 7 and 14, and had their mortalities recorded, their total lengths, gut lengths, anal body depths, eye diameters, head depths, yolk sac lengths and yolk sac depths measured, and their outlines analysed on DAH 7 and 14. Treatment NA exhibited the highest mortalities on DAH 14. The antibiotics had a significant size effect, yielding larvae with larger total length on DAH 7 and 14, larger bodies on DAH 7, and on DAH 14 larger anal body depth and greater variance in body size. Their effect on outline shape was also significant in both age classes, with increasing differences from DAH 7 to DAH 14. On DAH 7, "A" specimens were more uniform in their dorso-ventral development, and on DAH 14 "NA" had more slender shapes. The beneficial total length and size effects and the witnessed shape effects might be associated with the low bacterial presence.

5.1. Introduction

Specific attention has recently been given to the study of fish larvae reared in gnotobiotic environments, in which a known microbial community is inserted as a means to study host-microbial interactions. Examples of such gnotobiotic rearing systems include the use of zebrafish *Danio rerio* (Hamilton) (Rawls et al., 2004), European seabass *Dicentrarchus labrax* L. (Dierckens et al., 2009) and cod *Gadus morhua* L. (Forberg et al., 2011). The protocol by Dierckens *et al.* (2009) involves the use of antibiotics (rifampicin and ampicillin) for the egg incubation, subsequent to an egg disinfection with glutaraldehyde. Hatched larvae are then stocked in filtered autoclaved seawater with rifampicin, and monitored until DAH 15 when increased mortalities usually occur.

So far, the study of morphological differences between germ free or gnotobiotic and xenic larvae has focused mainly on the development and maturation of the gastrointestinal tract, such as in zebrafish (Rawls *et al.* 2004; Bates et al., 2006) and European seabass (Rekecki et al., 2009). As far as size is concerned, Forberg et al. (2011) did not witness a difference in the total length between gnotobiotic and xenic cod larvae, while Rekecki et al. (2009) found a reduced total length growth of xenic European seabass larvae versus germ-free on days after hatching (DAH) 6, 9, 12 and 15. However, beyond these differences in total length, knowledge of the effects of bacterial load on the size and shape of larvae in detail is absent. Consequently, the aim of this study was the quantification of the effects of antibiotic-induced differences in bacterial load on the size and shape of the larvae, as a proxy of their effects on fish performance during their rearing.

The aforementioned studies start from the egg stage, and after hatching an axenic rearing process is applied. However, the production of larvae is subject to frequent fluctuations in egg batch quality, and the use of antibiotics or other disinfectants may cause adverse effects on the eggs, as unwanted and unknown interactions with the target organism may occur (Marques et al., 2006). Furthermore, apart from the beneficial use of disinfectants such as glutaraldehyde on hatching percentages (Dierckens et al., 2009; Forberg et al., 2011), they can be considered an extra stress factor detrimental to eggs that come from a suboptimal batch quality (Schaeck et al., 2016). Therefore, the exclusion of any possible adverse effects of the axenic process on the eggs advocated the omission of the egg stage and start with xenic larvae of DAH 3, with the essential use of antibiotics to keep the bacterial presence and proliferation as low as possible.

European seabass was chosen as a case study due to being a very popular and successful species in Mediterranean aquaculture, and also due to the considerable study focus on its morphological abnormalities that many have been known to appear or strongly suspected to have their origins in the first life stages (Koumoundouros, 2010; Boglione et al., 2013b). An additional reason for the selection of the species was the existence of the necessary protocols for larval axenic rearing (Dierckens et al., 2009) and size and shape quantification (Nikolakakis et al., 2014). The latter is a procedure which takes into account and minimises the impact of handling, anesthetisation and fixation, and has shown not to induce any

significant size or shape effect between the fixed and live specimens up to five months after fixation.

In order to quantify the effect of the treatments on growth and larval performance, we took distance and outline shape measurements that represent body parts with important functional roles during ontogeny. Total and gut lengths were chosen in order to detect any effects on the longitudinal growth of the body and of the gut. A greater length of the latter can be an indication of a more efficient digestion, as increased gut length represents extended duration of food exposure to digestive enzymes such as proteases (Kamler, 1992). The yolk sac area was chosen as an indication of how quickly their primary endogenous feeding energy reserves were depleted. Anal body depth was used as a proxy of the perpendicular body growth. Eye diameter can be an indicator of visual performance (Rodriguez and Gisbert, 2002; Gisbert and Doroshov, 2006). It is also linked to brain growth during the early life history of fish (Packard and Wainwright, 1974). Finally, head depth can provide insight on the development of the sensory and ingestion capacity (Khemis et al., 2013). As for the shape analysis, it is becoming a very useful tool for the description of subtle variation that traditional morphometric analysis is not well suited to capture (Adams et al., 2004). Ideally, it complements metric data and is particularly applicable in the study of morphological differences in the earliest life stages of fish (Nikolakakis et al., 2014).

5.2. Materials and methods

5.2.1. Rearing and sampling

European seabass larvae of DAH 3 were obtained from the Ecloserie Marine de Gravelines hatchery in France. Upon arrival at the lab and after their acclimatisation, they were split in two treatment groups, and stocked in 50 ml vials (12 per vial) until DAH 14. The stocked larvae were vigorously swimming individuals chosen one by one with the aid of a 400μl micropipette, using pipette tips with a cut edge to avoid distorting the larvae (Nikolakakis et al., 2014). The first treatment included larvae reared in filtered autoclaved seawater with a mix of rifampicin, ampicillin, kanamycin, trimethoprim and gentamicin with a concentration of 10 mg l^{-1} each (treatment "A"), added on the stocking day (DAH 3) and every three days after that. The second treatment included larvae reared in filtered autoclaved seawater

without antibiotics (treatment “NA”). The larvae were split in four groups, A7 and A14 from treatment A, and NA7 and NA14 from treatment NA. Groups A7 and NA7 were monitored until DAH 7 (15 vials per group), and A14 and NA14 until DAH 14 (51 vials per group). The larvae were kept in a temperature controlled room at 16.0±0.5^{o}C, under constant dim light of 10 cd sr m^{-2} in a salinity of 37 g l^{-1}, and were fed with 30 axenic *Artemia franciscana* nauplii (Kellogg) per vial on DAH 8, 10 and 12, prepared according to the protocol of Marques et al. (2004).

Dead larvae in the vials of all four groups were counted and removed on DAH 7, and on DAH 14 for groups A14 and NA14. The protocol of Nikolakakis et al. (2014) was used for the handling of the larvae, their anesthetisation with MS222 and fixation in 3% phosphate buffered glutaraldehyde. The present study was conducted by personnel certified in animal experiments by the Belgian State, in compliance with the EU Directive 2010/63/EU.

For the bacteriological sampling, 6 vials were randomly chosen from each of the four groups. Two water samples of 50 µl per vial were taken on DAH 4, 7 and 14, plated with a Spiral Systems DU Spiral Plater on 10% marine broth (BD Difco, New Jersey, USA) + 15% agar (Bacteriological Grade, MP Biomedicals, Santa Ana, USA), incubated in 28°C for 96 h and counted to determine the number of CFU ml^{-1}.

5.2.2. Analysis of distance morphometry

The distance morphometry analysis of the specimens was conducted according to the protocol of Nikolakakis et al. (2014). In summary, the measurements were performed on pictures of the larvae photographed with an Olympus Altra 20 digital camera mounted on an Olympus SZX9 microscope via AnalySIS GetIT software (www.olympus.com). The distances were measured using ImageJ v1.51j (Abramoff et al., 2004), with an accuracy of 0.001 mm. On all four groups (A7 and NA7 sampled on DAH 7, and A14 and NA14 sampled on DAH 14), measurements of total length were performed on 120 specimens per group, and of gut length, anal body depth, eye diameter, head depth, yolk sac length and yolk sac depth (Fig. 5.1) on 30 specimens per group, selected randomly from all available vials in equal numbers (15 for each of the two treatments on DAH 7, and 51 on DAH 14). These measurements were performed and defined in chapter 4 as well, but for clarity their

definitions are repeated here: total length (TL) from the tip of the upper jaw to the distal margin of the caudal fin fold; gut length (GL) along the TL from the tip of the upper jaw up to the level of the anus; anal body depth (ABD) perpendicular to the TL at the level of the anus between the uppermost and lowermost points of the body, following the "posterior dorsal depth" of Holden and Raitt (1974) and the "anal body depth" of Theilacker (1978) and McGurk (1985); eye diameter (ED) as the largest vertical distance of the eye; head depth (HD) taken as an extension of ED between the lower and the upper part of the cranium, as used by Yin and Blaxter (1986); yolk sac length (YSL) as the longest horizontal distance between yolk sac walls; and yolk sac depth (YSD) as the longest perpendicular distance between yolk sac walls. Blaxter and Hempel (1963) calculated yolk sac volume (YSV) from YSV= $\pi/6$ YSL*YSD^2. However, in order to increase accuracy, since the photos only give a two dimensional representation of the yolk sac without ensuring it is a perfectly homogeneous ellipsoid, a calculation was made for the yolk sac area (YSA) that is clearly visible by using the general formula for an ellipse: YSA= $\pi/4$ YSL*YSD.

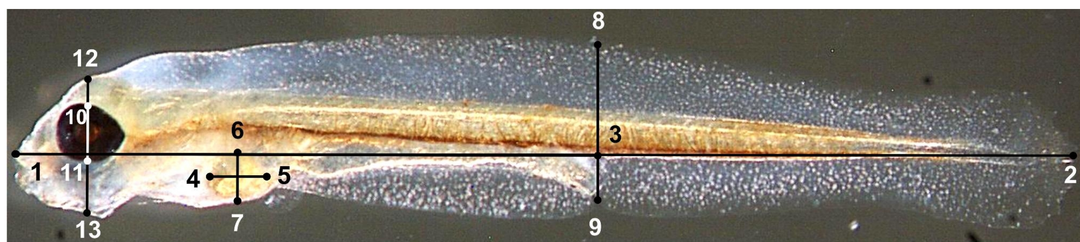

Fig. 5.1: Representation of the distance measurements taken on a body of a larva between individual numbered points, with the corresponding measurements being: total length (TL) between points 1-2, gut length (GL) 1-3, anal body depth (ABD) 8-9, head depth (HD) 12-13, eye depth (ED) 10-11, yolk sac length (YSL) 4-5 and yolk sac depth (YSD) 6-7.

The significance level of all statistical tests was set at the nominal signifance level of alpha=0.05. The total lengths on DAH 7 and 14 on both age classes were tested for the assumptions of normality of distribution with Shapiro Wilk's test and homogeneity of group variances with Levene's test. In case these were met, they were compared with a t-test for equal means, and when they were not, with a Mann-Whitney U test for equal medians. In order to explore the statistical differences between the means of the other distance variables within each age class, analysis of covariance (ANCOVA) tests of each variable with TL as a covariate were used on the individual datasets of DAH 7 and 14 to determine whether

treatments induced significantly different means for each age group. For ANCOVA, the assumptions of equality of linear regression slopes for all groups was tested with an F test, normality of distributions with Shapiro Wilk's test, and homogeneity of variance with Levene's test. In the cases where Levene's test rejected the null hypothesis of equal variances, it was noted in the ANCOVA results, but univariate group analyses are considered to be robust to violations of homogeneity of variance as long as the group sizes are equal (Nimon, 2012; Field, 2013).

A disregard-groups principal components analysis (PCA) was also carried out to find the most important variables explaining the largest part of the variation among the measured morphometric characters, and to explore and visualise any patterns of multivariate allometry. The variance-covariance matrix of all distance measurements was used on the datasets of each age class separately, and on a dataset with the measurements of both classes pooled. On this pooled dataset, the variation of the PC scores of the two major principal components PC1 and PC2 was compared between the two treatment groups of A and NA on each age class with an F test, with equal variance as the null hypothesis.

5.2.3. Analysis of outline morphometry

Regarding the analysis of outlines, it was conducted as described in detail in Nikolakakis et al. (2014) on 100 to 120 specimens from all groups (A7 and NA7 on DAH 7, and A14 and NA14 on DAH 14), randomly sampled from all available vials of each treatment, as mentioned above. Briefly, the outlines of the larvae were traced manually on their images by using Corel Draw 12 (Corel Corp.; www.corel.com), and analysed using Shape 1.3 (Iwata & Ukai 2002). To reduce the number of variables, a disregard-groups principal component (PC) analysis (PCA) was performed on the elliptic Fourier coefficients using Shape. All further statistical analyses were performed using PAST 3.16 (Hammer et al., 2001). The analysis of between-group variation was performed through a one-way multivariate analysis of variance (MANOVA) on the scores of all effective PCs (Iwata and Ukai, 2002; Nikolakakis et al., 2014). Regarding its assumptions, multivariate normality was checked with Mardia's multivariate skewness and kurtosis test, and the equivalence of covariance matrices with Box's M test. In the cases these were not met, a one-way non-parametric multivariate analysis of variance (PERMANOVA) was performed with 10000 permutations, with

Euclidean distances as the distance measure. The variation of the PC scores of the two major principal components PC1 and PC2 was compared between the two treatment groups of A and NA on each age class with an F test, with equal variance as the null hypothesis. Finally, the Euclidean distance between the shape centroids of the two treatment groups in each age class was calculated in order to determine in which age class the shapes were more similar, with a lower distance measure indicating a higher similarity. For that purpose, the PC scores of each age class were copied in Microsoft Excel 2010, and the Pop Tools 3.2 add-in (www.poptools.org) was used.

5.3. Results

5.3.1. Bacterial presence

In the present study, the antibiotics mix did not create axenity, but was effective in keeping the bacterial presence very low from the start. In both groups of treatment A colonies were visible on the culture plates. According to the measurement protocol of the spiral plater, however, they were below detection limit (less than 30 CFU ml^{-1}) on DAH 4 and 7, and $0.10*10^5$ CFU ml^{-1} (standard deviation of the observations=$0.09*10^5$ CFU ml^{-1}, standard error=$0.04*10^5$ CFU ml^{-1}) on DAH 14 (Fig. 5.2). To test for the differences between groups, non-parametric Kruskal-Wallis tests were performed, with Bonferroni-corrected p levels for post hoc Mann-Whitney pairwise comparisons. There was a highly significant effect of the antibiotics mix on the number of counted bacterial colonies ($p<0.001$ for DAH 4 and 7; $p<0.01$ for DAH 14). Post-hoc comparisons indicated that in treatment NA bacterial presence was always significantly higher at all sampling points ($p<0.05$ for DAH 4 and 7; $p<0.01$ for DAH 14), while it did not differ between groups of the same treatment.

5.3.2. Mortalities

Regarding mortalities (Fig. 5.3), there was a highly significant effect of the antibiotics mix (Kruskal Wallis: $P<0.001$), however that was not consistent on all age classes. Bonferroni-corrected Mann Whitney post-hoc comparisons indicated that on DAH 7, group NA7 had significantly more mortalities than the two groups of treatment A, but also more than group NA14 ($p<0.001$ in all cases). Therefore, the pattern of the NA treatment exhibiting higher

mortalities on DAH 7 was not clear. However, during the course of the experiment mortalities of the NA treatment increased, and became the largest on DAH 14 ($p<0.001$ versus all other groups).

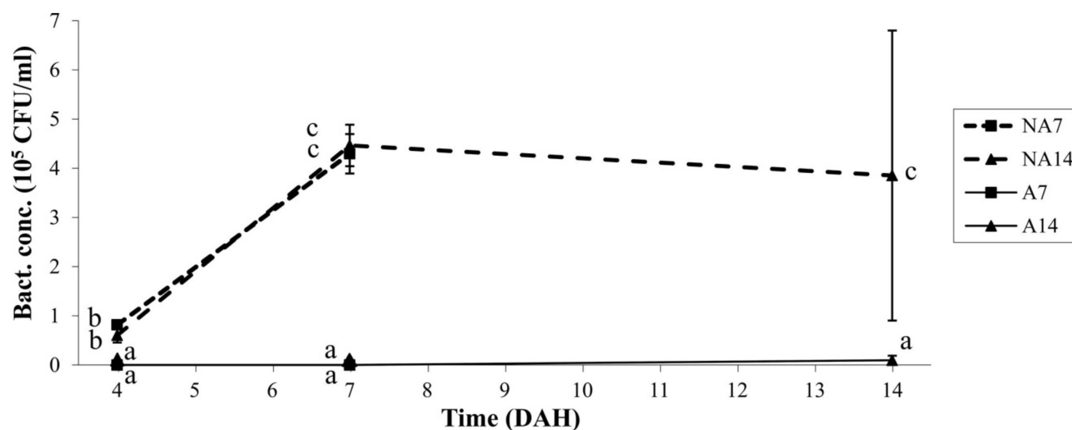

Fig. 5.2: Bacterial concentration in the rearing water of groups NA7, NA14, A7 and A14, with error bars representing the standard deviation of the observations. Groups NA7 and A7 were sampled on DAH 4 and 7, and groups NA14 and A14 on DAH 4, 7 and 14. The concentrations of treatment A are being connected with a solid line, which is barely visible as it lies very close to the horizontal axis, due to being close to 0. Different letters indicate statistically significant differences.

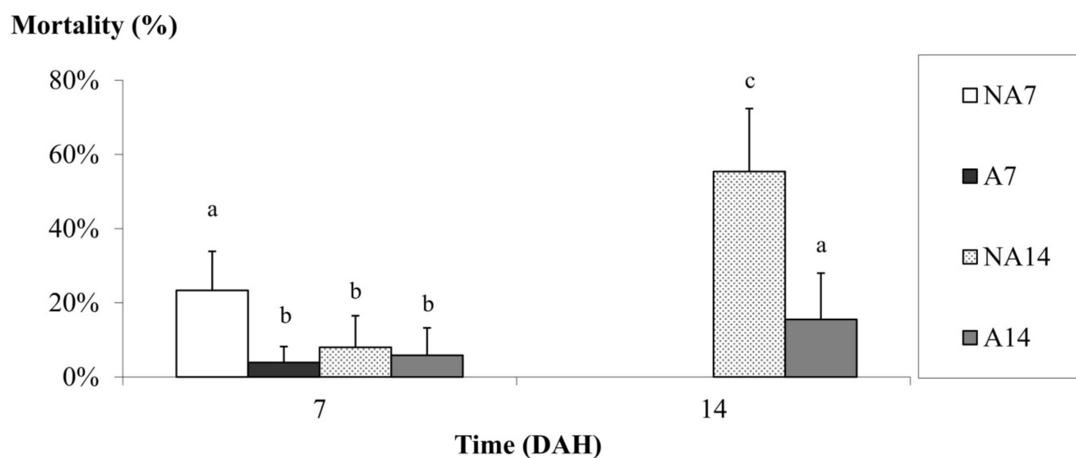

Fig. 5.3: Graph of the mortalities of each of the four groups on DAH 7 and 14, with error bars representing the standard deviation of the observations. Groups NA7 and A7 were sampled on DAH 7, and groups NA14 and A14 on DAH 7 and 14. Different letters indicate a statistically significant difference.

5.3.3. Analysis of distance morphometry

There was a significant effect of the antibiotics mix on the total length of the larvae on DAH 7 (t-test: $p<0.001$), on DAH 14 (Mann-Whitney U: $p<0.05$), and on their anal body depth on DAH 14 (ANCOVA: $p<0.001$, condition of homogeneity of variances not met). The larvae of treatment A had significantly larger average TL on both DAH 7 and 14, and significantly larger ABD on DAH 14 (Fig. 5.4).

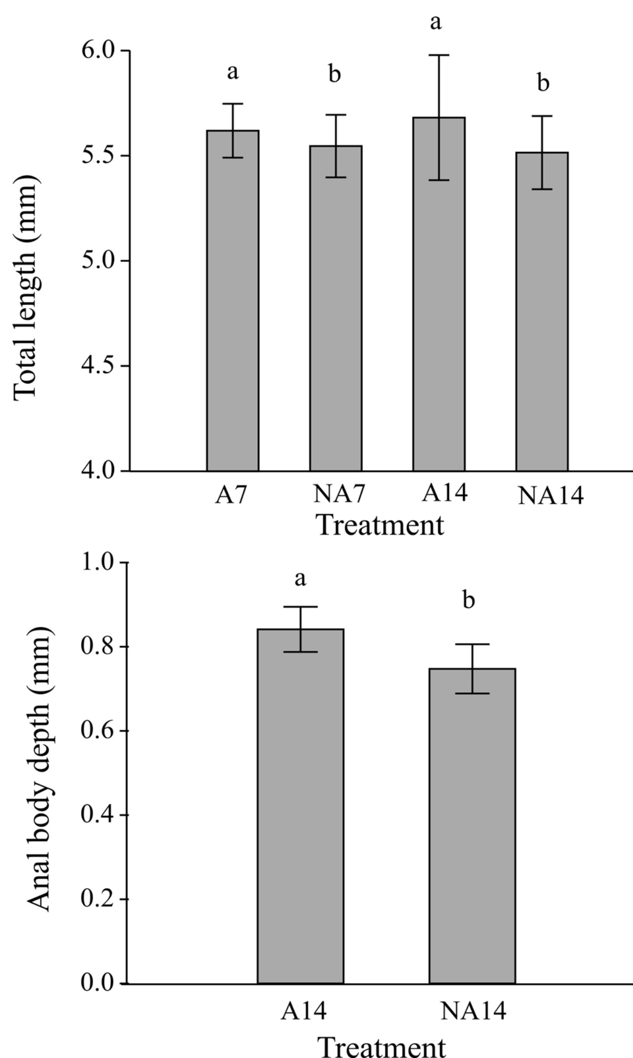

Fig. 5.4: Graph of the total lengths of the larvae on both DAH 7 and DAH 14 (upper graph), and of their anal body depth (lower graph) on DAH 14, with with error bars representing the standard deviation of the observations. In each graph, different letters indicate statistically significant differences.

Regarding the principal component analysis on the distance measurements of DAH 7, PC1 accounted for 80.32% of the total variation. It was dominated by total length and gut length, with loadings of 0.83 and 0.51, respectively. All other variables were also positively correlated, except anal body depth and head depth, but all of their loadings were very small compared to total length and gut length. PC1 was therefore interpreted to represent mainly longitudinal growth. PC2 accounted for 5.99% of the total variation. It was dominated by anal body depth with a loading of 0.84, while also presenting positive loadings of head depth and eye depth, and thus represented body depth. PC3 explained 4.98% of the total variation, and was a contrast between yolk sac depth and length on one hand and head depth on the other, with loadings of 0.66, 0.48 and -0.36, respectively. It represented yolk sac size in contrast to head depth. The graph of PC1 vs PC2 (Fig. 5.5, upper graph) indicates that the specimens of treatment A are positioned more towards the positive values of PC1, are significantly larger in their PC1 scores than NA (t-test: $p<0.01$), and are therefore showing more longitudinal growth. It is also interesting to see in the graph of PC2 vs PC3 (Fig. 5.5, lower graph) that the cluster of the specimens of treatment A lies within that of NA, meaning that the body depth, yolk sac and head depth of larvae reared in the antibiotics mix are less variable. However, the difference may be marginal since the NA cluster is not larger across the bottom right side, which according to the biplot represents the difference in head depth.

As far as the PC analysis of DAH 14 is concerned, PC1 represents 92.67% of the total variation. All distance variables were positively correlated, but since the major loadings came from total length and gut length with values of 0.82 and 0.53 respectively, it was interpreted as longitudinal growth. PC2 explained 3.54% of the total variation, being dominated by anal body depth (loading of 0.95), and with positive loadings by head depth and eye depth. It was therefore interpreted to represent body depth. In the graph of PC1 vs PC2 (Fig. 5.6, upper graph), the PC1 scores mainly show that the A specimens have a significantly larger length range than the NA specimens (F test: $p<0.01$), with both treatments producing short larvae but with treatment A exhibiting significantly longer bodies (unequal variance t-test: $p<0.05$), and also with significantly larger depths (t-test: $p<0.01$) as seen from their positioning across PC2.

Finally, regarding the PC analysis of the combined dataset of distance measurements for both age classes, PC1 explained 75.3% of the total variation. All variables were again positively correlated similar to DAH 14, with total length and gut length presenting the largest loadings of 0.83 and 0.54 respectively, but with the rest of the variables having higher correlations to PC1 than in the dataset of DAH 14. Therefore, the positive correlations of all variables with PC1 suggest a simultaneous increase in all dimensions, and thus PC1 was interpreted as a size component. PC2 explained 17.19% of the total variation and was dominated by yolk sac length and yolk sac depth, with loadings of 0.73 and 0.66. This suggested that it represented yolk sac size. In the graph of PC1 vs PC2 (Fig. 5.6, lower graph), as expected the orientation of both DAH 14 groups towards the negative values of PC2, in contrast to the two DAH 7 groups, represents the depletion of the yolk sac. Furthermore, apart from "A" specimens showing longer bodies on both classes, as shown from the above PCAs, on DAH 7 the "A" also had larger bodies (Kruskal Wallis on PC1 scores: 0.004, Bonferroni-corrected Mann Whitney test between A7 and NA7: $p<0.05$). Additionally, the graph shows that there is an age effect in treatment A, with the variation of PC1 scores reflecting body size being significantly higher than for NA on DAH 14 (F test: $p<0.01$), while on DAH 7 the variations of the two treatments in body size were not shown to be different (F test: $p=0.82$). Therefore, according to the graph of PC1 vs total length of DAH 14 (Fig. 5.7), this seemed to suggest that at the end of the experiment, treatment A yielded longer specimens, and with greater variance in their body sizes.

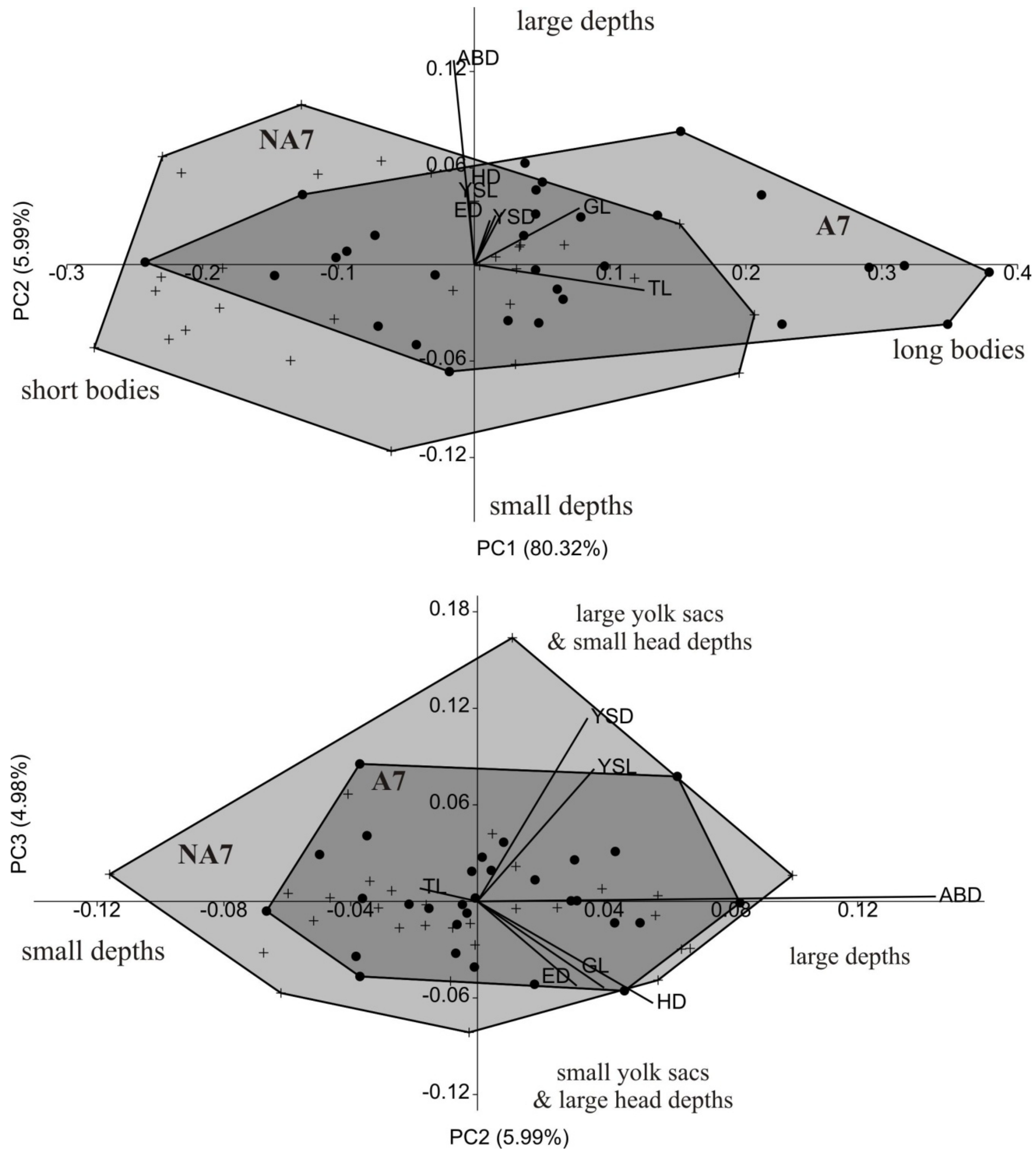

Fig. 5.5: Graph of DAH 7 for PC1 vs PC2 and PC2 vs PC3 of the principal component analysis of all distance variables, with the names of the treatment groups inside the corresponding clusters. Treatment A specimens are being represented with dotted markers, and NA with cross markers. The effects of each individual principal component are noted at the edges of the axes. Biplots of the loading of each individual variable towards each PC are also included, with the variables represented as lines starting from (0,0). Their length represents the amount of correlation to each PC, and their orientation towards each PC axis their positive or negative correlation to each one.

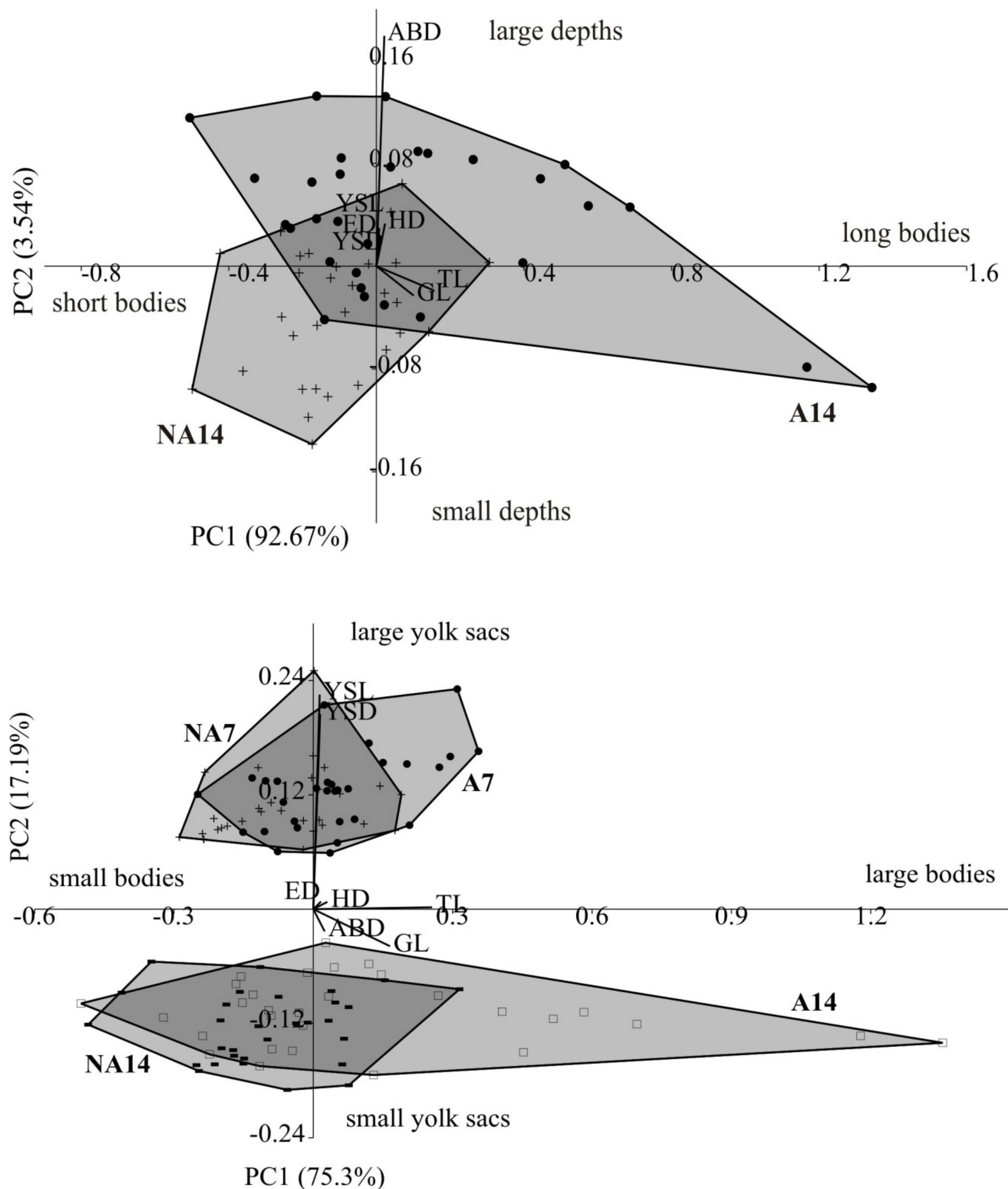

Fig. 5.6: Graphs of PC1 vs PC2 for DAH 14 (upper graph), and for the combined dataset of both age classes (lower), of the principal component analysis of all distance variables. The names of the treatment groups are placed beside the corresponding clusters. In the upper graph, treatment A specimens are being represented with dotted markers, and NA with cross markers. In the lower graph, group A7 is being represented with dotted markers, NA7 with cross markers, A14 with square markers, and NA14 with dash markers. Similarly to Fig. 5, the effects of each individual principal component are noted at the edges of the axes, with included biplots of each individual variable.

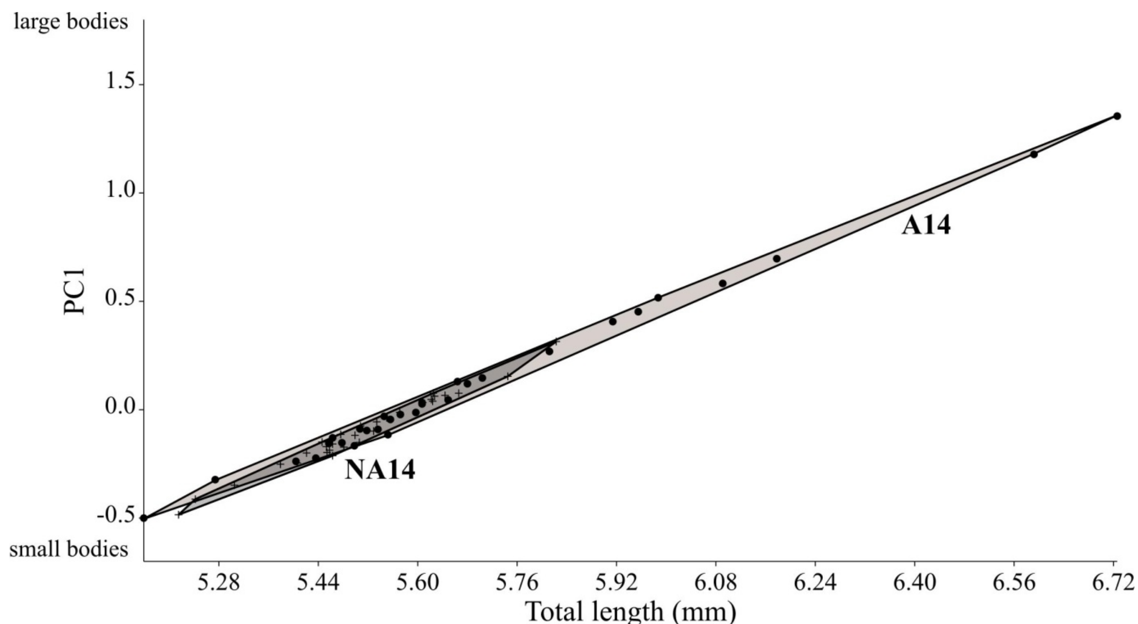

Fig. 5.7: Graph of PC1 scores of the principal component analysis of all distance measurements of the combined dataset for both age classes vs total length for treatment groups A14 and NA14. Their names are placed beside their corresponding clusters. The effects of PC1 are noted at the edges of the axis. Treatment A specimens are being represented with dotted markers, and NA with cross markers.

5.3.4. Analysis of outline morphometry

On both DAH 7 and DAH 14 there was a highly significant effect of the antibiotics mix on outline shape, similarly to total length. Larvae of treatments A and NA had significantly different shape (for DAH 7: PERMANOVA: $p<0.05$; for DAH 14: PERMANOVA: $p<0.001$). On DAH 7, the effective principal components explained 92.95% of the total shape variation. PC1 explained 47.99%, and mostly reflected the variation of the dorsal area (Fig. 5.8). Furthermore, it was shown that the variation of PC1 scores on DAH 7 was significantly smaller in specimens of treatment A (F test: $p<0.001$), indicating a more uniform development across the middle part of the body. PC2 explained 19.56%, and reflected the variation in the ventral area. As far as PC2 scores are concerned, unlike PC1 their variation in

treatment A was not shown to be significantly smaller than NA's (F test: p=0.94), but the major effect comes from PC1 since the total shape variation is mostly being explained by it. As for the rest of the principal components, PC3 explained 8.3%, PC4 7.37%, PC5 3.6%, PC6 2.33%, PC7 2.03% and PC8 1.77%.

On DAH 14, the effective principal components explained 93.07% of the total shape variation. PC1 explained 51.5%, and similar to PC1 of DAH 7, it reflected the variation of the dorsal area, but with a small degree of curvature. PC2 explained 24.15% of variation and reflected the variation of the pelvic, anal & pectoral area (Fig. 5.9). As noted, in both age classes the two treatments were shown to produce larvae with different shapes, but in the case of DAH 14 the overlap of the two clusters in the graph seemed smaller than DAH 7, which suggested that their shape differences were increasing. This was true, since the Euclidean distance between the shape centroids of the two groups on DAH 7 was 0.005 and on DAH 14 0.023. Regarding the rest of the principal components, PC3 explained 7.7%, PC4 3.89%, PC5 2.83%, PC6 1.66% and PC7 1.35%.

Similarly to DAH 7, the shape outlines of PC1 on DAH 14 lined up in the ventral area while variation was manifested across the dorsal area, which suggests that PC1 explains a difference in the dorsal area and body depth. The opposite was shown with the outlines of PC2: they lined up in the dorsal area and variation was shown in the ventral, so this might be an indication of PC2 explaining the variation in the ventral area. The NA specimens were positioned towards the shape outlines of -2SD of PC1, suggesting a more slender shape. They also presented variable development across PC2, but without the variation of PC2 scores being statistically different than treatment A's (F test: p=0.15). Larvae of treatment A were positioned more towards +2SD of PC1, suggesting a slightly better development in the broader trunk region, a trend that was supported by the distance analysis that indicated "A" specimens presented larger body depths on DAH 14.

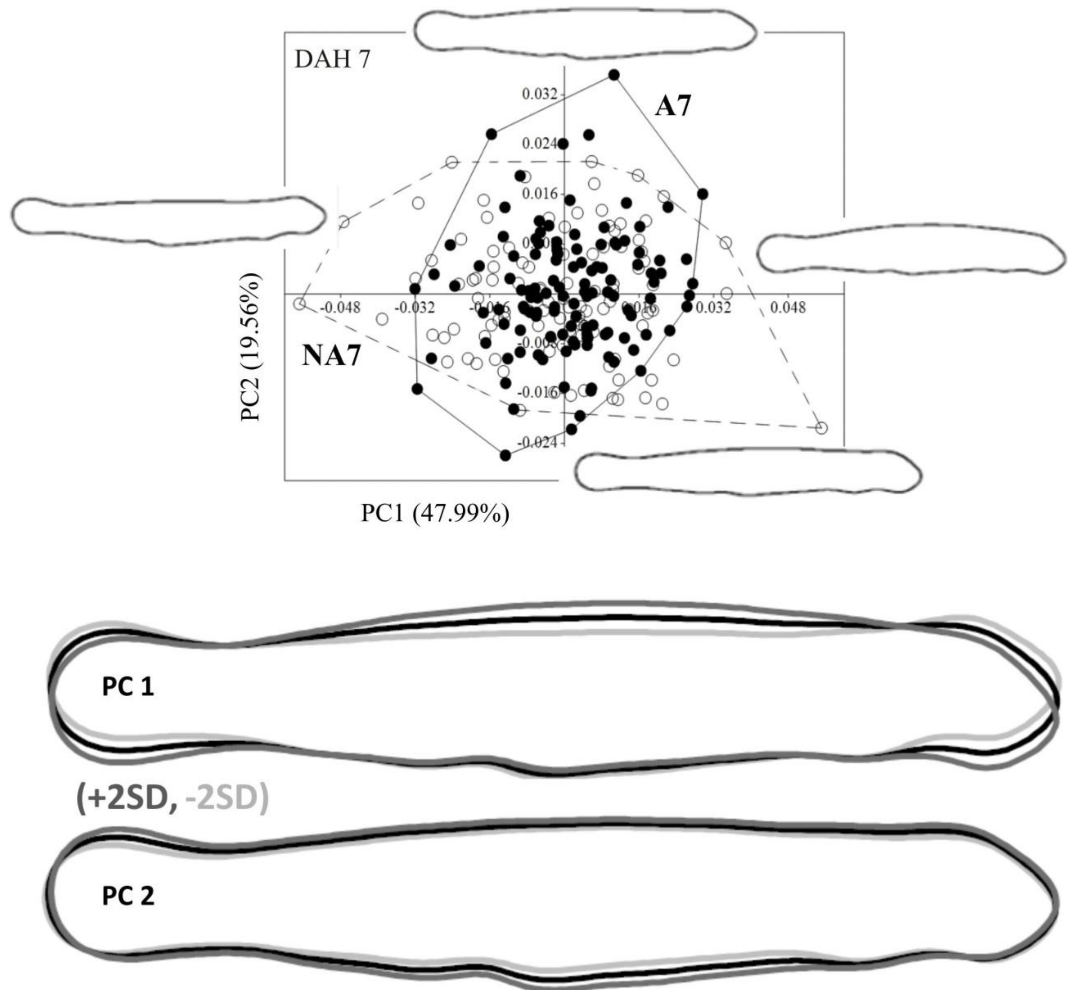

Fig. 5.8: Scatterplot graph of principal component PC1 vs PC2 scores of DAH 7, with the outermost specimen points of each treatment connected to form the clusters. Treatment A is being represented with a solid line and black dots for specimens, and NA with a dashed line and white dots. At the edges of the axes the ±2 standard deviation (SD) shape outlines of each PC are placed, and also placed at the bottom of the graph as an illustration of the specific shape variation explained by each PC. They are being represented with a dark grey line for +2SD and a light grey for -2SD, and superimposed together with the mean shape outline in black.

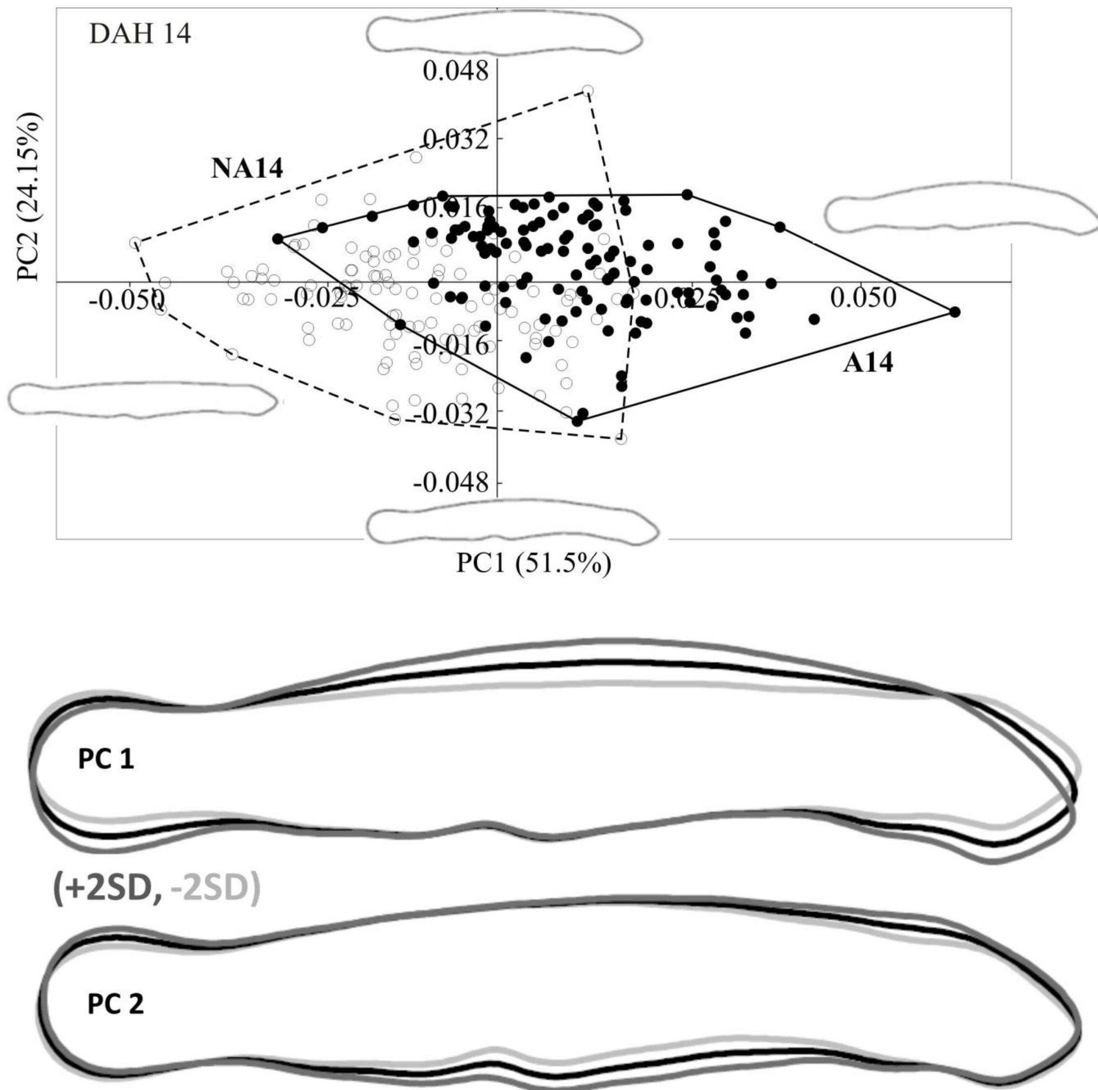

Fig. 5.9: Scatterplot graph of principal component PC1 vs PC2 scores of DAH 14, with the outermost specimen points of each treatment connected to form the clusters. Similarly to Fig. 5.8, treatment A is being represented with a solid line and black dots for specimens, and NA with a dashed line and white dots. At the edges of the axes the ±2 standard deviation (SD) shape outlines of each PC are placed, and also placed at the bottom of the graph, with a dark grey line for +2SD and a light grey for -2SD, and superimposed together with the mean shape outline in black.

5.4. Discussion

Rifampicin, ampicillin, kanamycin, trimethoprim and gentamicin are antibiotics commonly used in aquaculture, and in the concentrations used have proven successful in maintaining axenic conditions in tilapia *Oreochromis niloticus* L. larvae that were axenically hatched (Situmorang et al., 2014). In the present study, the antibiotics mix did not maintain axenity but severely restricted the bacterial proliferation.

Rifampicin and ampicillin have proven to be effective in the concentrations used in this study in maintaining axenity in larvae until DAH 15 (Dierckens et al., 2009). In other similar protocols (Munro et al., 1995; Rawls et al., 2004; Forberg et al., 2011), antibiotics have been used to maintain axenic conditions. In the studies of Forberg et al. (2011) and Schaeck et al. (2016), antibiotics were also used until the hatching of seabass larvae, but not after it. The use of antibiotics in the rearing medium in the present study was judged necessary in order to combat the bacterial load that the larvae maintained from the hatchery, while maintaining the aforementioned benefits of not using them during the egg stage.

To the best of our knowledge, there have been no reports of teratogenic effects of these antibiotics in fish larvae, in concentrations similar to the ones used in the present study. Kanamycin has been shown to be teratogenic to zebrafish larvae of DAH 2 in a concentration of 0.72-1.44 mM upon exposure at the embryonic stage (Song et al., 2010), which is higher than the 10 mg l^{-1} (0.02 mM) used here after DAH 3. Teratogenesis is considered an egg-larvae phenomenon largely due to the exposure of eggs to teratogenic agents during the egg stage, and with its time of induction at the yolk sac stage when the larvae are feeding on contaminated yolk sac reserves. Once external feeding begins, the potential for teratogenic effects declines and soon disappears (Von Westernhagen, 1988; Lemly, 2002). Since the antibiotics were added after hatching, the potential for the manifestation of such an effect was minimised. Furthermore, one of the typical effects occurring after sublethal exposure to teratogenic compounds is gross eye and cephalic deformations (Von Westernhagen, 1988). Such drastic shape changes were not witnessed on any of the specimens.

Normally, the eggs should not be considered more sensitive to antibiotics or to other antimicrobial agents than the larvae, since the chorion is playing a protective functional role in the egg. However, as also discussed in section 4.4, these agents can be considered an extra stress factor detrimental to eggs that come from a suboptimal batch quality (Schaeck et al., 2016), which is a problem frequently encountered with European seabass, also according to our observations. Parental genetics have also been known to play a role, as well as the sensitivity of the eggs to disinfection according to changes in chorion thickness (Escaffre et al., 2001). There are also two more cases where the eggs can at least be as sensitive to these agents as the larvae: (a) if the parent fish pass on contaminated yolk sac reserves to the eggs (Lemly, 2002), (b) there are antibiotics that have been known to either penetrate the chorion considerably, such as chloramphenicol, or to a small degree such as kanamycin, while associating and binding their molecules with the outer membrane surface and altering the membrane structure, which may affect the flow/rotation of phospholipids, transport of membrane proteins, import of necessary substances and export of metabolic wastes. In other words, a high concentration of kanamycin (as commented, in concentrations much higher than the one used in the present study) may form an adhesion shell enclosing the membrane, leading to physical membrane damage, e.g. obstruction of extracellular signal transmission, impairment of membrane transport and asphyxiation of the cells (Song et al., 2010).

The lack of axenity in the present study might be due to an inadequate bacteriostatic and bactericidal action of the antibiotics in the concentrations used. In aquaculture, they have been known to occasionally present a reduced potential of affecting common bacteria strains. Rifampicin is one of the most potent and broad spectrum antibiotics against bacterial pathogens, however bacteria can still develop resistance to it with high frequency (Campbell et al., 2001). Examples of pathogenic bacteria and their resistance to the used antibiotics in aquaculture include the following: *V. alginolyticus* can be resistant to ampicillin and kanamycin (Korun et al., 2013), *V. anguillarum* to ampicillin and rifampicin (Pedersen, et al., 1995), and *V. anguillarum*, *V. ordalii* and *V. harveyi* to kanamycin and trimethoprim, with *V. ordalii* also resistant to ampicillin (Korun and Timur, 2008). Gram-negative bacteria commonly found in European seabass have also been found to be resistant to trimethoprim-sulphamethoxazole (Matyar, 2007), and many other *Vibrio spp.* found in gilthead seabream *Sparus aurata* L. to ampicillin, gentamicin, kanamycin and trimethoprim (Scarano et al., 2014). The bodies of larvae are environments that support bacterial attachment and growth

(Goulden et al., 2013), and there is a strong possibility the gut of the larvae was also colonised with bacteria from the hatchery. European seabass larvae open their mouths around DAH 3, and after exposure to *Vibrio anguillarum* on DAH 4 their swim bladder and gut are colonised 2h and 48h post exposure, respectively (Rekecki et al., 2012). It is therefore possible the antibacterial action of the antibiotics was particularly inadequate against the microflora present in these internal larval areas, where the contact between the antibiotics and the bacteria is smaller than the external.

In order to explain the differences in growth and mortalities, it is necessary to focus on the development of the immune system of the larvae. It has been shown that it is immature, relying primarily on the innate immune response (Bricknell and Dalmo, 2005). At mouth opening, the commensal microbiota colonises the gastrointestinal tract and stimulates immune activities, at which time it becomes one of the most important and intimate sites of interaction with the external world, and one of the major portals for pathogenic invasion in fish (Olafsen, 2001; Dimitroglou et al., 2011).

This bacterial invasion and colonisation might be involved in the mortalities that start to occur after DAH 7. The time of the transition from endogenous to external feeding is recognized as the critical period, and is characterised by high vulnerability to external factors and increased mortality (Kamler, 1992). It must be noted that the pattern of treatment-dependent mortality effects was not clear, as there was a significant difference between the mortalities of groups NA7 and NA14 on DAH 7. The mortalities of group NA14 on DAH 14 were the highest among all other groups, including group A14's, but the data cannot support the hypothesis that there was a mortality effect related to the treatment. These high "NA" larvae mortalities on DAH 14 might be a consequence of the encounter of more opportunistic pathogens, since in their rearing water the bacterial numbers remained significantly higher due to the bacteriostatic and bactericidal effect of the antibiotics in treatment A. In axenic conditions, larval European seabass (Rekecki et al., 2009) and turbot *Scophthalmus maximus* L. (Munro et al., 1995) have been reported to present lower mortalities than xenic.

Water quality could have played a part in the results of this study. Unfortunately, data were not available for it, but it is thought not to have been a compromising issue, due to the following reasons: The present study was performed with the rearing protocol followed by

Nikolakakis et al. (2014), based on Dierckens et al. (2009). These protocols require the absolute minimum interaction with the rearing vials in order to avoid larvae stress and bacterial contamination, and for this reason there were no measurements of O_2 and ammonia nitrogen taken. However, the potential concentration of un-ionised ammonia nitrogen, based on the following calculations and references, is thought to have been around 0.15 mg/l, which is much lower than the toxic levels described for seabass, being 1.7 mg/l (LC50-96h, Cerda-Reverter 2015).

Larvae from both treatments were stocked in filtered autoclaved seawater, so the initial concentration of ammonia nitrogen should have been close to zero. Data for the nitrogen excretion rates in the larval stages of various seawater species are scarce, and to the best of our knowledge, are lacking for *Dicentrarchus labrax*. From the species that these literature data exist, gilthead seabream *Sparus aurata* is the one with the most similar biology to *Dicentrarchus labrax*, and it has a nitrogen excretion rate of 0.15 $nmol \cdot ind^{-1} \cdot h^{-1}$ after the start of external feeding around DAH 4 (Ronnestad et al., 1994). Therefore, as an overestimation with 100% survival (which was not witnessed, with mortalities removed on DAH 7 and 14), 12 larvae in each vial for the 10 days of the experiment would produce 0.15 nmol x 12 x 10 x 24h = 4.3 10^{-7} mol in each 50 ml vial, which equals 8.6 x 10^{-6} mol/l = 0.15 mg/l. Additionally, as mentioned above, there was no clear pattern of any treatment consistently presenting higher mortalities throughout the experiment, so it is unlikely that they induced a significant treatment difference on the water quality.

On both DAH 7 and DAH 14, the seabass larvae reared in the antibiotics mix had larger average total lengths, and on DAH 14 greater dorso-ventral development translated mainly into larger anal body depth, with the difference starting to manifest itself just four days after stocking all vials with the larvae from the hatchery. This might be explained from the relationship between growth and metabolism during the larval stages. A fish egg contains a predetermined amount of yolk energy reserves. During the endogenous feeding period this yolk energy is allocated between body growth, metabolism and excretion, and there is competition between the conflicting demands of growth and metabolism. Increased metabolism inevitably results in a relative decrease in growth (Kamler, 1992). The growth in treatment NA was decreased compared to treatment A, and this might mean the NA larvae are focusing on their metabolism instead. Energy measurements were not performed in the

present study, but if "NA" larvae are indeed focusing on their metabolism instead of their growth, it can originate from them having to deal with energy demands that are common in both treatments, but likely elevated in NA due to the significantly higher bacterial presence. Examples include energy expenditures in activities such as epithelial differentiation and maturation in the gut, which are aided by the gut microbiota (Nayak, 2010). In literature, there have been documented morphological differences in the gut between germ-free / gnotobiotic and xenic larvae of zebrafish (Rawls et al., 2004; Bates et al., 2006) and European seabass (Rekecki et al. 2009) that suggest these activities are increased in xenic larvae. Additionally, Rekecki et al. (2009) also found a reduced total length growth of xenic European seabass larvae versus germ-free on DAH 6, 9, 12 and 15, which can be analogous to the reduced total length of "NA" larvae on DAH 7 and 14. It must be noted however that the above studies were not between bacteria-poor and xenic specimens, such as the ones examined in the present study. Due to the presence of bacteria, "A" and "NA" larvae had their gastrointestinal tracts colonised and their immune activities stimulated, whereas this happens to a much smaller degree in gnotobiotic larvae (Nayak, 2010).

Another possible increased energy demand in "NA" larvae comes from dealing with putative negative effects of the microbial colonisation of the gut, such as the existence of bacterial toxic metabolic byproducts that have been known to be growth depressing, or the competition of the organism with the microbiota community for energy and nutrients (Anderson, 2002; Collier et al., 2003). This competition is thought to be low in axenic conditions (Rekecki et al., 2009), and assumed to be low in treatment A as well.

Studies have shown that ontogenetic state can be tightly correlated with size, as expressed by measures such as larval standard or total length (for example in Adriaens and Verraes, 2002). Therefore, it might be argued that the witnessed differences in size and shape of DAH 14 between the two treatments are due to a difference in ontogenetic stages. For example, in the upper graph of Fig. 5.6, "A14" specimens have total lengths and PC1 scores (representing overall longitudinal development) that are significantly larger than "NA14". However, for the same PC1 range of -0.5 to 0.3, the two groups also significantly differ in their scores of PC2, which is a major part of the observed variation that is uncorrelated to total length (for the dataset of DAH 14: Pearson correlation statistic=-0.02, p_{uncorr}=0.86, r^2=0.0006; for the combined dataset of DAH 7 and DAH 14: Pearson correlation statistic=-0.03, p_{uncorr}=0.81,

r^2=0.001). Therefore, if we assume that ontogenetic state is tightly correlated with the larval total length, they differ in their PC2 scores that represent larval depths, but this difference is not related to the difference in total length, and likely unrelated to their ontogenetic state.

Towards the end of the experiment on DAH 14, treatment NA specimens had smaller anal body depth, and according to the PC analysis of the distance measurements, a smaller dorso-ventral development in general. A hypothesis to explain this is that it might be a size-related ontogenetic effect of growth allometry: since the "A" are longer than "NA", if we assume that size is a good estimate of developmental stage, this means they are ontogenetically more advanced. As larvae undergo growth, the dorsal and anal fins start to form, with a resorption of the finfold at the level of anal body depth (Larouche et al., 2017). Therefore, the "A", as more developed, would be expected to present a smaller anal body depth. However, the opposite was witnessed, so the argument that it was an effect due to growth allometry does not seem plausible.

A different hypothesis that can explain the observation is based on the possible association of the witnessed smaller depths with a pseudo-starvation state in treatment NA. A decrease in thickness of the larval fish body, including but not limited to anal body depth, has been correlated with starvation (Theilacker, 1978 and references therein; Yin and Blaxter, 1986; Shan et al., 2009), which is induced when an unsatisfied need for energy beyond the internal yolk sac reserves of the larvae exists (Johns and Howell, 1980; Kamler, 1992). This can be due to the aforementioned elevated energy demands of the NA larvae compared to A, such as the colonisation of the digestive tract by an increased bacterial load, and other energy expenditures to combat or assimilate bacteria. In the present study, larvae from both treatments were fed equally, but treatment A larvae exhibited lower mortalities on DAH 14 and longer bodies on both DAH 7 and 14, so it is likely that this hypothetical starvation state did not affect them as much as larvae from treatment NA. When these differences are considered together with the larger variance in body sizes of "A" larvae on DAH 14, this might indicate that the larvae reared without antibiotics started to present high mortalities without being able to reach large sizes, while the larvae in antibiotics grew bigger, and perhaps started to develop their natural size variation.

The argument of the longer treatment A specimens is supported by their significant differences in total length conducted on 120 specimens per treatment per DAH, and also from the principal component analysis on all body distance measurements on at least 30 specimens per treatment per DAH. However, large differences in standard deviation of total length combined with higher mortalities can represent a highly variable sample, and thus should also be examined. There was no significant difference between the standard deviations of the total lengths on DAH 7 (F test, $p=0.108$), and on DAH 14 it was significantly smaller in treatment NA ($p<0.01$). Therefore, on DAH 14 the larvae of treatment NA exhibited higher mortalities, were shorter, and more uniform in total length. That might mean that they were consistently shorter, perhaps due to the aforementioned argument of increased energy requirements. Additionally, what might have happened on treatment NA on DAH 14 is that the higher mortalities meant fewer surviving specimens with a smaller total length variation, due to their selection as the fittest survivors.

Furthermore, in literature it has been shown that after the final yolk absorption, unfed larvae show negative growth due to tissue resorption, which is their only way to meet their metabolic demands if starvation continues, and then die from prolonged starvation while the fed larvae show rapid growth (Kamler, 1992). This seems to agree with the aforementioned mortality and size differences between the two treatments. We must note however that the specimens did not consume all the provided *Artemia*, but that occurred in both treatments, and might have originated from the poor larval visual detection capacity of prey (Villamizar et al., 2011) or from the inherent inability of larvae to always obtain sufficient food, due to the hydrodynamic limitations of their feeding mechanism (China and Holzman, 2014).

As mentioned above, larvae from both treatments were fed equally, and the specimens did not consume all the provided *Artemia*. Consumption differences were difficult to quantify, due to the aforementioned minimum interaction with the rearing vials in order to avoid larvae stress and bacterial contamination. There was a witnessed difference in total length in favour of the antibiotics treatment, and any differences in *Artemia* consumption could have played a part. *Artemia* were provided in order to cover any needs of external feeding, but until DAH 13, European seabass larvae are thought to rely mostly on their internal energy reserves, partly due to the prey capture inefficiency on the first days of feeding (Pilar Olivar et al., 2000). Therefore, the possible existence of any *Artemia* consumption differences is thought

to have played a minor role, and any associated total length differences should also not have played a role in the outline differences, as there was a size standardisation process involved in the outline analysis.

Similarly to the distance morphometry analysis, outline shape differences were present in both age classes. Problematic swimbladder inflation has been associated with deformities (Koumoundouros, 2010), but it did not seem to play a role, since the percentages did not differ significantly between the two treatments: on DAH 14 it was completed in a percentage of 74.07±18.72% (SD) for treatment A and 75.6±26.23% (SD) for treatment NA (t-test for significant differences between treatments: p=0.86). Interestingly, the outline differences were already present on DAH 7, just four days after the starting point of the stocking of larvae. The major difference lies across PC1, which largely reflects the variation across the dorsal area. The head and tail shifting upwards for the negative PC scores and downwards for the positive PC scores indicates there might be a possibility that the shape effect explained by PC1 is due to a fixation artefact. However, such a possibility is small or non-existent, as it was shown in Nikolakakis et al. (2014) that there is no shape change occurring between the live and fixed specimens when the protocol is used, up to five months post-fixation.

The smaller variation of PC1 scores of treatment A on DAH 7 suggested a more uniform development across the middle part of the body. This might be an indication that the overall dorso-ventral development across the broader middle area was different, with higher uniformity in the bodies of the larvae reared in the low in numbers, and likely more standardised, bacterial environment of the antibiotics mix. This is also supported by the distance principal component analysis of PC2 vs PC3 on DAH 7, which seemed to suggest that the body depths, yolk sacs and head depths of "A" specimens were indeed more uniform in development, although as noted in the Results it might be a minor effect. On DAH 14 a similar smaller variation in either PC1 or PC2 shape scores was not witnessed, but "NA" specimens presented a more slender shape, while "A" specimens presented a slightly better development in the trunk region. This agrees with the results from the distance analysis that indicate significantly larger depths in larvae of treatment A on DAH 14.

On DAH 14, larvae reared in antibiotics had shapes that were similar to witnessed abnormalities of the anterior dorsal part of the primordial finfold in common dentex *Dentex*

dentex L. (Koumoundouros et al., 2001) and European seabass (Koumoundouros, 2010; Fragkoulis et al., 2017b). When they are paired with posterior notochord deformities, they can be early signs of conditions such as saddleback syndrome and caudal fin anomalies that start to appear at the flexion stage. Such notochord deformities were not observed in the pre-flexion stage larvae of the present study, and the short time frame did not allow any verification of whether abnormal conditions appear in later stages under these experimental conditions. However, according to the Euclidean distance analysis, the antibiotics treatment induced increasing shape differences from DAH 7 to DAH 14. The reasons are not known, but they might be an early precursory indication of abnormal development. Early fin anomalies have been described in European seabass as early as 9.4 mm of standard length (Marino et al., 1993) which is reached around DAH 23, in sole *Solea senegalensis* K. on DAH 24 (Gavaia et al., 2002), and notochord anomalies in gilthead seabream of 3.4 – 3.9 mm total length on DAH 2 (Koumoundouros et al., 1997b).

In conclusion, it is possible that the low bacterial presence and/or the presence of antibiotics have the potential to influence size and shape, at least partly by altering the response of the organism rapidly through different colonisation patterns and intensity. Under such a controlled environment in bacterial composition, total length and size were adversely affected when bacterial presence was higher, and the use of antibiotics in a research context induced significant and increasing shape effects that need to be investigated further. However, these effects could not be specifically attributed to the presence of antibiotics, to low bacterial levels due to the antibiotics, or to a possible interaction effect. To determine this, more detailed studies are needed over a longer period, also in order to provide deeper insight on any possible bacterial mechanisms affecting shape in commercial hatchery conditions.

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Chapter 6

General discussion, conclusions and future perspectives

6.1. Introduction

The larva is the first and most critical stage in the life of fish. Its development marks the transition from the egg into an organism that initially has to rely on its yolk and oil globule reserves as its only energy supply, until it is able to catch and digest prey organisms. In nature, its survival is mainly dependent on food supply and the avoidance of predators, with a small percentage of survival as it undergoes metamorphosis to become a juvenile fish that can be recruited into the fish stock (Helik et al., 2009). Under aquaculture hatchery conditions, this survival would be expected to be higher, due to the regulated food supply and absence of predators. However, even under such controlled conditions, the mortalities are still high and variable between batches, as the egg and larval quality remains inconsistent, due to the susceptibility of the larvae to every biotic or abiotic factor that can affect them at these sensitive very early stages of their lives.

In contrast to mammalian farm animals, whose corresponding stages occur inside the mother in a constant, protected environment with a steady nutritional supply, fish have to deal with a very fluctuating and harsh environment. In many ways, the success of dealing with it will determine their later life. Their sensitivity during this period is the reason that some of the most important and critical challenges in aquaculture today, such as the improvement of survival rates and the production of robust, high quality juveniles, are rooted in the larval stage (Helik et al., 2009). Its importance is underlined from the fact that even small improvements in the larval growth and quality can have a large beneficial impact in the adult stages.

This sensitivity during the larval time period, especially at the critical transition from endogenous to external feeding, is characterised by high vulnerability to various factors with an environmental, nutritional, genetic or biotic origin. The morphology of the larva is also rapidly changing, and is very sensitive to the individual or synergistic effect of these factors. Fish larvae have a greater susceptibility to them than adults, due to (a) their smaller tolerance and increased sensitivity to abnormal levels of various stressors, such as for example elevated temperatures, during the period of the occurrence of rapid ontogenetic changes, due to their

very early and immature developmental state (Komoroske et al., 2014), and (b) the fragility of their bodies, which suffer the mechanical stress of shear, compression and torsion (twisting) forces in the water currents of the tanks, providing drag on the delicate larval body in different directions (Helvik et al., 2009).

When considerable and abnormal deviations from the standard developmental phenotypic variation are occurring, the term “morpho-anatomical abnormality” is used to describe any deviation of external morphology, which is usually - but not always - associated with defects of internal anatomy (Koumoundouros et al., 2009). In spite of impressive progress that has been made over the recent years in the larval rearing methods, these abnormalities still pose a very significant problem in aquaculture, compromising the welfare of the fish, and eventually a substantial loss of effort, energy, time and funds in hatcheries. They represent a significant financial loss for the farmers, not only due to the deformed fish being discarded, but also due to the reduced predictability and profitability associated with the variable quality of larvae and juveniles (Helvik et al., 2009). In the last decade, the mean reported frequency of abnormalities in Mediterranean hatcheries has been 7-20%, but with occasional increased incidences of 45-100% (Koumoundouros et al., 2010).

In the present study, a protocol was presented that deals with the quantification of size and shape variation on newly hatched European seabass larvae. It was then applied on the study of bacterial effects, originating from axenity, egg disinfection and from differences in bacterial load, on the phenotype of the larvae. The following discussion will focus on two main areas: the knowledge gained from (a) the use of this protocol, and (b) its application on the study of bacterial effects on growth and deformities. The shortcomings of both in providing information on specific areas of high interest will be addressed, as well as how these drawbacks can be improved as a focus of further studies.

6.2. Suggested protocol for the quantification of abnormalities

Application of the protocol for size & shape variation quantification of Chapter 3, and issues related to its application for the quantification of deformities

Many of the aforementioned abnormality conditions have been known or strongly suspected to have their origins or first manifestations at the embryonic, yolk sac and first feeding stages. Over the past years, there has been a considerable focus of ongoing research on the determination of the causative factors, but particular advances have not been made on the establishment of protocols of early assessment, quality verification and improvement, as well as on the development of a scale of quality for the precise distinction of commercially severe deformities from those of scientific interest only (Koumoundouros et al. 2010). The protocol described in Chapter 3 can be a step towards this direction of the early identification of abnormalities. It focuses on the yolk sac stages until DAH 15, and is applicable on later time points as well, occurring before the usual moment of first big scale quality control monitoring in hatcheries, which in the case of European seabass happens around DAH 60. Instead of relying on empirical visual estimations by the researchers, it can allow an accurate mathematical quantification of shape variation, while taking into account and minimising the detrimental size and shape effects of anesthetisation, handling and fixation, which are issues frequently overlooked in morphometric studies. This is accomplished by a procedure that does not induce any size or shape effects between live and fixed specimens until five months post fixation in 3% phosphate-buffered glutaraldehyde. It provides an objective quantification of size and shape variation in these stages, originating from any biotic or abiotic factor that has the potential to induce it, and a visualisation of this variation. The procedure that follows it is an attempt to translate any detected deviation from the standard ontogenetic variation into a biological meaning that can be used to draw conclusions in a research context, and in a commercial hatchery environment. One drawback, however, is that the direct connection of localised phenotypic differences on the larval bodies to a specific condition and a causative factor can be very difficult, since there is a lack of concrete evidence from literature.

In the application of this protocol, there are some additional issues that will be addressed in the following sections: the establishment of a reference point for a "normal" aquaculture phenotype for the species of interest, the establishment of a clear set of decision rules to

classify specimens as “normal” or “deformed”, and their application in a real-world scenario. Furthermore, the suggested protocol is providing information on specimens of the larval stages until DAH 15, but in order to suggest a classification of deformities at this very early time point, it must be proven that specific phenotypes are indeed the first signs of abnormalities that will be fully manifested later. Therefore, it is very important to investigate whether any detected deviations from the normal phenotypic variation at this stage, induced by a particular factor or a combination of abnormal conditions, are also manifested in the later stages of the species, at the end of the hatchery phase and beyond. This is a key issue that will also be discussed in the sections that follow.

6.2.1. Definition of a “normal” phenotype

In spite of the benefit of the objectivity from the mathematical quantification of shape variation with the aid of geometric morphometrics, there is still subjectivity involved on what a “normal” specimen is. The method discussed in Chapter 3 can provide information on size and shape differences between groups. However, it does not specify whether the specimens can be classified as "deformed" or not. That firstly depends on our own definition of what a normal, ideal, non-deformed specimen is and its associated shape variation, which should be established in an objective way without any bias from the researcher. A way to solve this problem is discussed by Fragkoulis et al. (2017a) for gilthead seabream: a reference shape can be determined by taking into account wild phenotypes and consumer preferences (the study included the evaluation of the body shape of 100 fish by 65 participants, according to a five-point Likert scale ranging from 1 for “fair” up to 5 for “excellent”). However, this was accomplished for specimens of market size, and not in the early larval stage such as the one studied in the present thesis. Another method for the determination of an ideal species-specific phenotype for different age classes until the end of the metamorphosis could be the calculation of an average shape from a large number of samplings from the wild, and in the cases where that is difficult or impossible, from egg batches originating from different hatcheries and across a wide time range that would include several spawning seasons. The hatcheries that could be used as a reference should belong to a type that has been shown to produce a very small or no occurrence of deformities, which is the case with the “mesocosm” technique, achieving this by largely resembling the natural environment, and by keeping the densities low (Gisbert et al., 2015).

6.2.2. Classification of deformed specimens

Are all shape variations abnormalities?

Similarly to the approach of the present thesis, the first step and function of the protocols that use methods based on geometric morphometrics is the quantification of size and shape variation. The next step is an effort to provide answers to the questions “Does the observed shape correspond to normal or abnormal variation?”, “Is this variation biologically relevant?” “Does it correspond to a known deformity type?”, and possibly “What can be characterised as a cause?”. An additional question that arises is whether the witnessed shape effect from a specific factor under observation, such as axenity, egg disinfection or bacterial load, is a natural morphological adaptation in the context of phenotypic/developmental plasticity, or if it can be indeed characterised as a deformity. It can be very difficult, or even impossible, to determine an objective criterion that allows to safely distinguish between these two interpretations. What makes this even more difficult is the presence of confounding experimental factors, in spite of the best efforts that can be made to exclude them under controlled conditions. These can be biological and also genetic, since in most similar experiments the eggs originate from multiple parents, as well as individuals coming from the same parents show genetic variation. However, under the necessary acknowledgment of the limitations of similar research efforts, some conclusions might still be drawn regarding the attempt at the characterisation of a witnessed shape variation as an abnormal development. This might be achieved through the discussion of the potential association of this variation (a) to deformities by using a reference point discussed on page 150, and (b) to erratic, undesirable skeletal or fin development through radiography or staining, present on that time point or as precursory signs that can be fully manifested later by establishing a link (discussed on page 160). The arguments to support this discussion can originate from studies on similar species, in non in-vitro conditions, and on a larger time scale, as discussed on page 166.

Identification of outlier shapes

A question that arises beyond the definition of a normal phenotype of a certain age class is how an objective classification of deformed specimens can be made. A basic way that this could be done is through the identification of shapes that correspond to outliers. Once the

shape analysis of a specific batch or treatment is complete, a selection process can be applied for specimens with "outlier" shapes. An outlier point has a value outside the upper or lower quartile of a boxplot plus 1.5 times the inter-quartile range (IQR) of the middle 50% of the observations. Furthermore, any case greater than the upper or lower quartile plus 3 times the IQR is labelled as "extreme" (Field, 2013). The values that can be used for this purpose are the PC1 and PC2 shape scores of a certain specimen group, and this distinction could allow the characterisation of specimens as "mildly deformed" or "severely deformed". However, a drawback could be that only one very simple set of rules would be involved in the decision-making process.

Fuzzy logic and its application in aquaculture

A sophisticated way to deal with this problem would be the setting up of an algorithm, where multiple parameters are taken into consideration in rule-based systems that do not simply rely on object membership of "yes" or "no", "deformed/not deformed", or 0/1. An example is fuzzy logic, which describes the membership of an object by a number in the unit interval [0, 1] as opposed to either 0 or 1 (member or non-member), as in classical set theory. A very good illustration of it is provided by Lee et al. (2000): "One fuzzy set might be «young». One might define young as follows: 10 years old is young with membership 1, 30 years old is young with membership 0.45, and 50 years old is young with membership 0.1. That is, everybody is young to a degree." Fuzzy logic is particularly suited to complex systems, because it is emulating human expert knowledge and experience, incorporating ambiguity and contradiction (Lee et al., 2000).

In practice, it employs an automated process that takes a number of input variables, programmed to give certain qualitative responses based on an if-then set of rules according to whether a measured value falls below, within or above a range of preset optimal reference values of the input parameters, and finally applies the control process. An example of these input variables from the study of Soto-Zarazua et al. (2011), with their preset optimal values and the resulting decisions, is given in Fig. 6.1. The described decision of "opening of proportional valve" corresponds to the water treated by the recirculation system. Other control responses were also programmed, such as the parameters of pump and blower motors, various electrovalves, and the ultraviolet module. This is an illustration of an aquaculture system and its control processes based on modelling, with preset ranges of parameters that

Fuzzy sets configuration

Turbidity (cm of visibility)	Temperature (°C)	Dissolved oxygen (% of saturation)
[100:100:80]	[20:20:24]	[20:20:40]
[100:80:60]	[20:24:26]	[20:40:60]
[80:60:40]	[24:26:28]	[40:60:80]
[60:40:20]	[26:28:30]	[60:80:100]
[40:20:20]	[28:30:30]	[80:100:100]
Feeding (% of biomass)	**TAN (mg/l)**	**Opening proportional valve (%)**
[1.5:1.5:3]	[0:0:2.5]	[0:0:25]
[1.5:3:5]	[0:2.5:5]	[0:25:50]
[3:5:7.5]	[2.5:5:7.5]	[25:50:75]
[5:7.5:10]	[5:7.5:10]	[50:75:100]
[7.5:10:10]	[7.5:10:10]	[75:100:100]

Accept Cancel Save

Rule-based configuration

if TUVL TUL TUME TUH TUVH & TEVL TEL TEME TEH TEVH & OVL OL OME OH OVH & FVL FL FME FH FVH & TANVL TANL TANME TANH TANVH

then VVC VC VMEO VO VVO

1 IF TUVL & TEVL & OVL & FVL & TANVL THEN VVC

2 IF TUVL & TEL & OL & FL & TANL THEN VC

3 IF TUME & TEME & OVME & FVMME & TANME THEN VMEO

4 IF TUH & TEH & OH & FH & TANH THEN VO

5

Accept Cancel Save

Real input and output values changed to linguistic values to construct the fuzzy sets

Turbidity (cm of visibility)	Temperature (°C)
100 (TUVL, very low turbidity	20 (TEVL, very low temperature)
80 (TUL, low turbidity)	24 (TEL, low temperature)
60 (TUME, mean turbidity)	26 (TEME, mean temperature)
40 (TUH, high turbidity)	28 (TEH, high temperature)
20 (TUVH, very high turbidity)	30 (TEVH, very high temperature)
Dissolved oxygen (saturation %)	**Feeding (%)**
20 (OVL, very low oxygen)	1.5 (FVL, very low feeding)
40 (OL, low oxygen)	3 (FL, low feeding)
60 (OME, mean oxygen)	5 (FME, mean feeding)
80 (OH, high oxygen)	7.5 (FH, high feeding)
100 (OVH, very high oxygen)	10 (FVH, very high feeding)
TAN (mg/L)	**Proportional valve opening (%)**
0 (TANVL, very low TAN)	0 (VVC, very closed valve)
2.5 (TANL, low TAN)	25 (VC, closed valve)
5 (TANME, mean TAN)	50 (VMEO, mean open valve)
7.5 (TANH, high TAN)	75 (VO, open valve)
10 (TANVH, very high TAN)	100 (VVO, very open valve)

Optimal ranges of water quality for fish production

Parameter	Optimal range	Reference
Turbidity (cm visibility)	>30 cm visibility	Soto-Zarazúa et al. (2010a)
Temperature (°C)	26–28	Azaza et al. (2008)
Dissolved oxygen (saturation %)	>70%	Buentello et al. (2000)
pH	7–9	El-Sherif and El-Feky (2009a, b)
TAN (mg/L)	<4	Shnel et al. (2002)

Fig. 6.1: A fuzzy set and rule-based configuration, with its corresponding codes and optimal parameter values (Soto-Zarazua et al., 2011).

can be deformity-related, as will be discussed in the following part of this section. In aquaculture, fuzzy logic has been a modelling technique widely used in various studies. These have focused on the feeding requirements of gilthead seabream larvae under intensive rearing conditions (Papandroulakis et al., 2000), on automation in a recirculation aquaculture

system of tilapia *Oreochromis niloticus* based on parameters such as temperature, dissolved oxygen, feeding, total ammonia nitrogen etc (Soto-Zarazua et al., 2011), in various other control systems for freshwater aquaculture (Rana and Rani, 2015) with an emphasis on their denitrification process (Lee et al., 2000), and also in machine vision systems for the purpose of real-time identification of individual animals and estimation of their activity (Whitsell et al., 1997).

Models used for the classification of deformities in humans

Regarding the initial question of the classification of deformities, models such as artificial neural networks, genetic algorithms, fuzzy logic, correlation, regression etc. have been used in studies focusing on the computer-aided classification of digitised medical images of the spine for various human deformities, such as scoliosis (Duong et al., 2006; Birtane and Korkmaz, 2014 etc.) These images can be 2D such as X-rays, or even 3D spine reconstructions (Duong et al., 2006) by using wavelet transformation to reduce the huge amount of shape information to just twenty wavelet approximation coefficients as 3D descriptors, in a similar fashion to the elliptic Fourier transformation that produces a small number of 2D descriptors in the outline shape analysis used in the present study. Most of the shape information of the fish larvae at these early stages is two-dimensional, and can therefore be analysed through the simpler, cheaper and quicker processes of outline or landmark analysis. However, 3D reconstruction is just an example of a wider set of rules that can be entered in a model-based decision system, which will result in a greater knowledge we can have of the original system we are trying to describe.

Models for the occurrence and characteristics of malformations in aquaculture

Regarding the study of skeletal anomalies in aquaculture, self-organising maps is an unsupervised learning algorithm that has been used for that purpose (Russo et al., 2010 and 2011). The input variables used in these two studies were standard length, meristic counts such as the numbers of vertebrae, number of rays of the left pectoral fin, of the anal fin, of upper and lower caudal lepidotrichia etc., and a wide range of skeletal deformities such as scoliosis, lordosis, kyphosis, swim bladder anomalies etc., together with the body region where they occurred. The models were used to determine the occurrence and characteristics of specific malformations associated with different rearing methods such as mesocosm or intensive culture, in order to evaluate the effect of different rearing procedures on fish

quality, and to assess the quality of aquaculture products in terms of distance from the wild-like phenotype used as a reference.

Basic example of a fuzzy logic model for classification of deformities with parameters from protocol of Chapter 3

The study presented in Chapter 3 is using a protocol that provides information on size and shape of larvae in their earliest life stages, in terms of various parameters such as total length, anal body depth, gut length, yolk sac area etc., and PC scores that correspond to a specific outline shape effect from any factor that has the potential to induce it, according to the visualisation provided by the method. These can be the input parameters in models similar to the ones used in the aforementioned studies of Russo et al. (2010, 2011). As a very basic fuzzy logic example, according to the example of Fig. 6.1, the algorithm could be programmed into: IF «pectoral body depth» < 1.2 mm AND IF «anal body depth» < 4 mm AND IF «score of shape PC associated with curved specimens» < -0.3 AND IF «temperature» > 20°C AND IF «tank water speed» > 30 cm sec^{-1} THEN «deformed» (example values). This formulation would give equal weight to each of these parameters for the determination of the outcome, but that could be changed according to our desired optimisation.

The most significant of these parameters will need to be identified, along with defined ranges that correspond to specimens that we will have to characterise as “mildly” or “severely” deformed, with a range of varying degrees. In order for these goals to be accomplished, separate studies on the effect of specific factors that can be controlled in a hatchery on the selected phenotypic characters will have to be conducted. The hatchery parameters can be water temperature, speed and orientation of tank currents, oxygen saturation levels etc., and the phenotypic characters can be for example anal body depth, pectoral body depth, shape PC scores associated with curved shapes (if we decide based on evidence that curved specimens are a precursory sign of deformities), number of rays in various fins, lordotic angles (Russo et al., 2010 and 2011; Costa et al., 2013; Boglione et al., 2013b) etc. The classification will be associated with the reference phenotype, and an acceptable range of shape variation will have to be determined around it. This phenotype, as mentioned above, can be one from the “mesocosm” culture technique, according to the species and ontogenetic stage (or age in DAH) of interest. This approach would be a good step towards the desired aforementioned

wide set of rules, which would deal in an integrated manner with as much data associated with deformities as possible, especially since the manifestation of deformities is a multi-factorial problem where many variables are playing a role (Boglione et al., 2013b).

Our own characterisation of the value ranges of these parameters will, admittedly, still be subjective. Such a degree of subjectivity, regardless of the expert knowledge associated with it, will inevitably have to be incorporated in such a decision making process. However, that is actually an advantage of modelling techniques such as fuzzy logic due to the discussed benefits of the emulation of human expert knowledge and experience, and the incorporation of ambiguity and contradiction (one input variable might suggest a mildly deformed specimen, while another with added weight will not). Furthermore, such a model will eventually be an unsupervised one based on the precise quantification of its input parameters, and therefore it can be far better than the simple empirical visual estimation of a specimen according to each individual researcher that is making the assessment, as is the case in most hatcheries today.

6.2.3. Usefulness of suggested protocol in a commercial setting & application of other classification methods

Feedback on the effect of biotic or abiotic factors in the hatchery

As mentioned above, the protocol of the quantification and visualisation of size and shape variation presented in Chapter 3 can provide valuable information at a very early larval stage, before the time that the main evaluation of deformities is taking place in hatcheries. This information can be useful in a research context: for example, a producer or a company might be interested in performing trials on new feeds that are aiming in improving growth, feed conversion ratios, survival, or on the application of pre/probiotic feed additives. In that case, feedback can be provided on the size and shape differences between the tested treatments and the controls. Feedback can also be provided on the effect of one biotic or abiotic factor in the hatchery, such as bacterial load or temperature, pH, oxygen levels, tank water currents etc., or a combination of them, on the size and shape of the larvae, in order to test the detailed effects of improvements in the rearing protocols.

Application in cases of quick or real-time decisions

Apart from providing the aforementioned feedback in the academic or commercial sector, the question arises on whether it can still be useful when very quick, or even real-time, decisions are required in hatcheries. Unfortunately, due to the involved level of detail and the labour-intensive and time-consuming procedures of fixation, handling, stereoscope picture-taking, outline tracing and data analysis, it is not judged to be practical in this context. In these very early larval stages, the suggested method deals with the quantification of very subtle differences, and probably the only safe way to do it is manually, in the suggested detailed and labour-intensive manner, and not in an automated, unsupervised way. The reason is the fragility of the larvae, their consequent sensitivity to handling that might introduce deformity artefacts, and the fact that outline tracing must be supervised to avoid detection mistakes that can play a significant role in creating shape variation noise. Regarding this, an additional difficulty is that the larvae are transparent in the beginning of this stage, and therefore do not have adequate contrast with the background in the stereoscope in order for the outline detection process to be fast and properly automated. Stainings can be used, as suggested in Chapter 3, but that would be impossible to apply on live specimens.

Application on older fish (fingerlings or adults) with the use of machine vision

For older fish with an advanced skeletal development that are towards the end of their metamorphosis or even sometimes after it, which for the two main Mediterranean aquaculture species of European seabass and gilthead seabream happens, according to temperature regimes and the individual rearing protocols, approximately around DAH 90 (Moretti et al., 1999), numerous automation procedures through the use of machine vision have been suggested in literature. The main elements of a machine vision system are displayed in Fig. 6.2. It is based on the use of various optical sensors, and is being used primarily for fish classification, monitoring of fish behaviour such as the presence or absence of schooling in sea cages, monitoring of the individual food intake or swimming speed in response to environmental changes such as stocking, feeding, net cleaning and grading, and also counting, grading, de-heading, the quantification of various morphometric traits, and biomass estimation. Even more novel uses have been accomplished, such as the estimation of the state of freshness, due to changes in fish flesh texture as a result of its freezing and thawing, through the use of magnetic resonance imaging, and for the real-time detection of

microbial spoilage and prediction of the presence of lactic acid bacteria through the use of near-infrared hyperspectral imaging (Mathiassen et al., 2011; Navarro et al., 2016; Saberioon et al., 2016 and references therein). Nowadays, machine vision is being used extensively for object sorting and generally for decision making in other industrial sectors such as in mining, for sorting vegetables etc., but the adoption of its aforementioned benefits in aquaculture has been slow. A detailed review for its current use in products of the aquatic animal sector can be found in Saberioon et al. (2016).

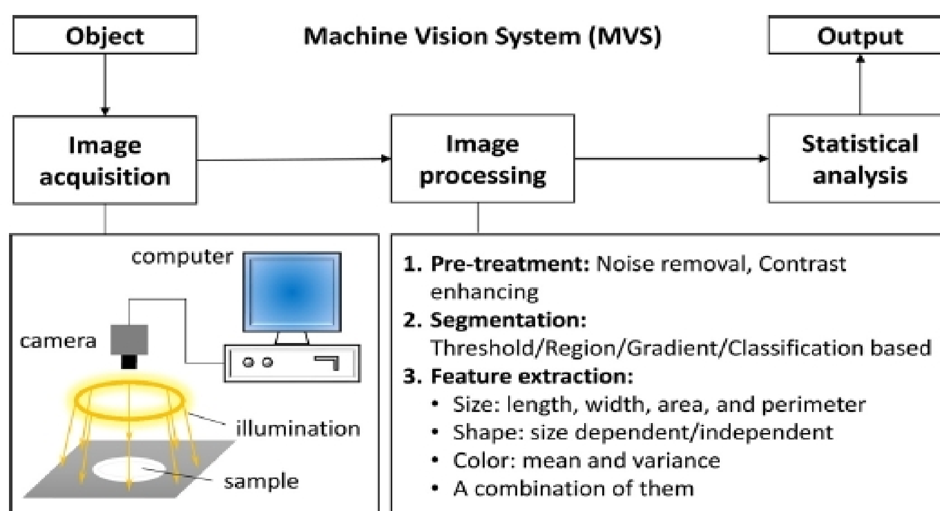

Fig. 6.2: Essential elements of a machine vision system (Hong et al., 2014).

Their secondary use is for the detection and sorting of deformed fish, but to the best of our knowledge, there is a considerable lack of evidence in literature. In a similar fashion to the suggested larval protocol of Chapter 3, image analysis through outline morphometry (Elliptic Fourier analysis on outline coordinates) has been used for the detection of spinal deformities of European seabass (Costa et al., 2013). In that study, spinal deformities were assessed by visual inspection of the vertebral column after removal of the left fillet, and by manually measuring the angle between the antero-posterior axis (the axis passing through the mouth and the middle fish height) and the axis of the caudal peduncle. Afterwards, a classification was accomplished through the use of partial least squares modelling, and the two approaches were compared. An important difference of this study with the one presented in Chapter 3 is that it was conducted on adult specimens with a mean weight of 250g. Another difference is that the model was built on training based on the estimation of vertebral deformities, but

these were usually obvious and easy to quantify through simple visual inspection, and not very subtle like the ones examined in the protocol presented here. Nevertheless, it is a study that portrays a promising theoretical system, based on the suggested combination of outline detection and multivariate modelling, which can be implemented in machines performing a real-time detection of gross skeletal deformities.

In the past, there have been a few attempts to program machine vision into sorting out deformities, by companies such as Marexi, Palinox and Vaki. An example from Marexi (product "AQUADEF") can be seen in Fig. 6.3. The live fingerlings are automatically brought and placed, one by one through water pipes and conveyor belts, on a camera-equipped flat surface, and after the photo of each one is taken, they are removed again through the use of water tubes, and sorted according to their deformity classification. This is accomplished through the use of modelling techniques, such as artificial neural networks and support vector machines. Another very recent example of a product is by the company Cermaq for sea cages (product iFarm, https://www.cermaq.com/wps/wcm/connect/cermaq/news/mynewsdesk-press-release-2226712/ [Accessed 23/11/2017]). In an underwater sensor chamber in the cage, each individual fish is being recognised and logged based on the dot pattern of its cranium. The number of fish, their size, weight, length and K factor (condition factor from the individual's weight and length) are also logged. After this, an automatic evaluation and sorting of fish occurs without stress. Attributes for sorting include fish that are deformed or with a low condition factor and possible signs of disease, such as problematic eyes/gill cover/dorsal fin, snout damage, bleeding, discoloration and presence and number of lice, with a removal by laser. The sorting of healthy fish can then be performed on a basis of weight, (a) in individual size ranges and transferred automatically via underwater ducts to new cages, or (b) above a certain threshold for harvesting.

Until now, machines like these have not been widely accepted from the producers, likely due to their cost, or to not meeting the expected good performance (Costa et al., 2013). Possible reasons might be the complexity of the image recognition and deformity classification procedures. The producers are also likely reluctant to accept major and very costly changes in rearing protocols that have been established from years of experience and technical know-how without concrete promising results. These would be necessary in order to convince them that there is a specific financial benefit from their use that would justify the associated

expenses, personnel, training hours and the process of incorporating them into their long-standing protocols. However, reaching the full potential of the discussed processes of machine vision, extraction of size and shape information, and deformity classification through modelling might be just a question of optimising these procedures that already exist, and of a better marketing campaign that can present tangible benefits to the producers, associated with a proven financial profit in a large scale-hatchery.

Fig. 6.3: Automatic system for detection and separation of fry with morphological defects (Marexi Aquadef) (picture from http://www.marexi.com/aquadef.html [retrieved on 17th August 2017]).

6.2.4. Link between early precursory conditions and manifestation of late deformities

Investigation of whether early phenotypic traits are precursory signs of later deformities, and benefits of establishing that link

In the introduction of this chapter, the issue of investigating the link between any witnessed deviations from the normal phenotypic variation in larval fish, induced by a particular factor or a combination of abnormal conditions, and late ones in the fingerling stage (or beyond) was raised. In order for this to be accomplished, long-term studies will have to focus on establishing this link between early conditions, defined by phenotypic traits such as particular distance, angle, area or shape variables corresponding to extreme ranges, and the manifestation of similar or more pronounced conditions in later stages. The final goal would be to determine, with an acceptable minimum degree of certainty, whether these phenotypic traits are indeed precursory signs of later deformities. If this was achieved, then it could

provide researchers with a new set of rules to be incorporated in a model system for discarding larvae with non-reversible phenotypic defects that would have to be rejected later. Furthermore, an additional benefit of a model with such a set of rules that takes into account the manifestation and development of deformities across time, is that it can have the potential of being incorporated in recognised existing food quality protocols, such as the ISO 9000 or 22000 family of management systems, especially with the adoption of model decisions as critical control points in the rearing procedure.

It must also be noted that this research of establishing a link between early phenotypic traits and later deformity conditions could indicate the opposite: that the specimens have a potential for recovery, if their abnormal rearing parameters are corrected back into their optimal levels. This would be an equally interesting conclusion. Such ontogenetic convergences of shape as fish continue to develop into later juvenile stages have been shown for European seabass, after the elimination of temperature differences occurring in their egg and larval stages (Georgakopoulou et al., 2007). Lordotic seabass fingerlings of DAH 54 have also been known to develop a normal skeletal system by DAH 110, if they are reared in rectangular tanks of 400m^3 instead of cylindrical tanks of 12m^3 (Kayim et al., 2010). Also, gilthead seabream specimens of DAH 140 with monolateral opercular defects can recover after 16 months, without a change in their rearing environment, in 61% of the cases (Beraldo and Canavese, 2011).

Limitations in the investigation of the discussed link

In order to investigate the existence of this link, one important difficulty in the experimental design will have to be addressed: ideally, the same individual specimens would have to be monitored and sampled repeatedly in their tanks, with their abnormalities recorded in a non-lethal way, and with the minimum amount of stress. Various acoustic, radio and passive integrated tags have been used on fish in several studies, but they are all applicable on fish of at least 68 mm of total length, and with small individuals having lower survival. Apart from negative effects on the survival and the retaining of the tag, the swimming performance is also reduced (Jorgensen et al., 2017; Smith et al., 2017 and references therein) which could cause additional deformities. The aforementioned machine vision techniques could be used to follow specific individuals, but they would be costly, difficult to implement, and the

problems of non-lethal sampling and stress may still be present. However, in a similar way to the above recovery studies, this research could be performed on a population level instead.

6.3. Bacterial effects on growth and deformities

6.3.1. Phenotypic action of specific strains and bacterial load, and suggestions for further research

Knowledge gap on bacterial phenotypic effects on larvae and juveniles

Additionally to the small volume of research dedicated to the monitoring and quantification of deformities in the very early larval stages, and to their integration with machine learning processes for the classification of deformities, there is also a knowledge gap on how the bacterial characteristics of the live prey and larval cultures are involved in the creation of morphologically robust larvae and juveniles. In the present thesis, the possibility of a bacterial mechanism affecting the phenotype of the larvae is illustrated. Additionally, a hypothesis was tested that the smaller average growth of the xenic larvae compared to the axenic or the ones reared in a very small bacterial load might be associated with increased energy expenditures and metabolic activity in xenic larvae. It is necessary for further studies to look deeper and more closely at how these mechanisms work, and any association this might have with the manifestation of size and shape effects in the larval organism.

Possible bacterial mechanisms affecting the phenotype

Regarding these mechanisms and their phenotypic effect, as mentioned in Chapter 2, there is, to the best of our knowledge, lack of evidence in literature on the effect of bacterial load on deformities. One example is the witnessed bacterial invasion of the larval head tissues, associated with the anterior parts of the jaw cartilages being pushed apart, and a disintegration of the thin oral membrane leading to a development of a gaping jaw condition (Morrison & MacDonald, 1995). However, there is evidence connecting the effect of specific bacteria to the manifestation or reduction of deformities. Pathogenic bacteria can cause malformations in adult fish, such as *Mycobacteria neoaurum* and *Aeromonas salmonicida* in Atlantic salmon (the former upon spread of the infection to the skeleton, and the latter upon

contraction of the collagen of the scar tissue), and *Arcobacter cryaerophilus* inducing upper jaw deformations (Austin & Austin, 2007; Plumb & Hanson, 2011). One other example is related to osteogenesis: vitamin K_2 is one of the three forms of vitamin K, and is produced by bacteria. Its deficiency affects the synthesis of plasma and bone proteins, and causes cartilage calcification, low bone mineral densities and bone deformations in developing bones of various animals. In spite of the fact that little is known about the requirements, deficiency signs and metabolism of vitamin K in fish, its deficiency has been shown to affect the quality of bone matrix in haddock *Melanogrammus aeglefinus* L. (Lall and Lewis-McCrea, 2007 and references therein).

On the other hand, there are also examples of the beneficial effect of some bacteria, such as several probiotic lactic acid bacteria, on the larval deformities in several species. *Pediococcus acidilactici* has been associated with the reduction of the manifestation of deformities such as vertebral compression syndrome in rainbow trout, and spinal deformities of sea bass fry, specifically lordosis. However, not all lactic acid bacteria have shown these beneficial effects: for example, *Lactobacillus casei* has been associated with a high incidence of spinal deformities in seabass larvae. It seems that these two lactic acid bacteria influence bone mineralization in different ways, possibly involving the mechanisms of Ca^{2+} absorption at the time of vertebral column ossification, which in seabass occurs around DAH 22 (Lamari et al., 2013 and references therein). Several hypotheses to explain the beneficial effect of some probiotic lactic acid bacteria have been formulated: their prevention of infection by pathogenic bacteria, the improvement of gut integrity and acidification, the subsequent improvement in the uptake of minerals and vitamins, and the resulting lower oxidative stress and occurrence of inflammation, which could affect the integrity of the spine (Autin et al., 2012; Lamari et al., 2013 and references therein).

More detailed studies are needed over a longer period than the first 15 days after hatching of the present thesis, also in order to provide deeper insight on any possible bacterial mechanisms affecting shape in commercial hatchery conditions. Such studies should focus on both the bacteria-centric and host-centric mechanisms that have been known or suspected to be involved in the action of antimicrobial growth promoters, which have been historically used in agriculture, and are still being used today. Bacteria-centric hypotheses propose that changes to bacterial communities induced by antimicrobial growth promoters lead to

enhanced growth by modulating the microbiota to create a more efficient system. This may include altering competition for nutrients, preventing pathogen colonisation, and/or selecting for bacteria that are able to extract more energy from the diet. Host-centric hypotheses focus on the immunomodulation potential of antimicrobial growth promoters, stating that they dampen physiological inflammation at the intestinal mucosa. This decreases the catabolic costs of maintaining an immune response, thereby allowing more resources to be dedicated to anabolic processes (i.e. muscle development) (Brown et al., 2017).

Consequently, these studies should focus on the metabolic energy requirements of the animals, and fish larvae in particular. Regarding seabass, in literature they have been illustrated to a considerable extent (for example in Peres et al., 2005). A possible insight into the way that these metabolic processes affect developmental plasticity can perhaps be provided by very recent data that have been presented as a link between the energy requirements and the witnessed metabolic depression and consequent reduced growth rates of juvenile seabass, induced by conditions such as hypoxia occurring on seabass larvae between DAH 28 and 43 (Cadiz et al, 2018). In that study, it is suggested that metabolic pathways in the liver are playing a role through the expression of hepatic genes such as *phd3*, which is thought to be involved in the regulation of fish fitness. Additionally, a significantly lower production of trypsin was witnessed in the hypoxia treatment, with that enzyme known as an indicator of food consumption and digestion.

Complexity of deformity-inducing mechanisms

All of these examples are providing some partial insight on the mechanisms that bacteria are influencing the phenotype of animals, and of fish in particular. However, they also illustrate their complexity and their contradictory effects, since some are beneficial, while others can be harmful. The complexity of these processes, their identification, and the problematic nature of a standard knowledge and control of their effect, are highlighted from the fact that various deformity factors are expressing their effects in a non-standardised and non-universal way: the same factor can induce different anomalies in different fish species, the sensitivity to a factor may change dramatically during ontogeny, and the same factor may provoke a high incidence of anomalies in some skeletal elements, but not in others with the same bone type and ossification, in the same individual (Boglione et al., 2013a and references therein).

Therefore, the study of these effects should be species-specific, for the specific ontogenetic stage of interest, with an investigation of if and how these effects are also manifested later, and with the necessary acknowledgment of the uncertainty involved in the manifestation of that effect across different skeletal elements in the same organism.

Effects of egg disinfection, axenity and difference in bacterial load witnessed in the present study

Regarding the size and shape effects of egg disinfection, axenity and the difference in bacterial load presented in this study, these firstly include the tendency of the larvae that were axenic or reared in a very low bacterial load to be longer, slightly thicker, and with a more uniform dorso-ventral development. The axenic larvae also consumed their yolk sac more slowly, probably due to (a) reduced energy demands from the lack of bacterial colonisation of their digestive tract and from other similar reduced energy expenditures to combat or assimilate bacteria, and (b) not having to deal with putative negative effects of the microbial colonisation of the gut, such as the existence of bacterial toxic metabolic byproducts that have been known to be growth depressing. The axenic larvae were also to a small degree more curved than the xenic. This may be considered a slightly more advanced ontogenetic stage, or an abnormal shape due to the prolonged exposure to the antibiotics, as they can be considered an extra stress factor detrimental to eggs that come from a suboptimal batch quality, which is a problem frequently encountered with European seabass. This detrimental effect should be taken into consideration in the cases where antibiotics are administered at this stage in axenic/gnotobiotic models, and can explain the varying hatching percentages encountered on a per-batch basis, which frequently determine whether an experiment can be performed or not.

As far as the secondary egg disinfection with glutaraldehyde after the primary one with iodine is concerned, on DAH 5 and 11 it presented significant but very moderate shape effects. It was also associated with a more rapid consumption of the larval internal energy reserves of the disinfected specimens, and it is argued that this allowed a more efficient yolk utilisation for their bacterial energy expenditures, as well as their ontogenetic differentiation, maintenance, building up of body tissues and accumulation of reserves in various organs. There was also a disinfection effect that can be interpreted as negative: “DX” larvae had

smaller anal finfolds than “NX” on DAH 0 and 11, and smaller dorsal finfolds on DAH 11. This might be significant, since the dorsal and ventral portions of the embryonic finfold are respiratory surfaces in pelagic fish larvae, and reduced finfold surface area has been linked with detrimental physical and physiological effects such as decreased blood flow to tissues, interference with nervous system function and increased energy expenditures, which agrees with the aforementioned increased yolk sac consumption rate of “DX” larvae versus “NX”. Egg disinfection has the acknowledged beneficial effects of controlling the intense bacterial load, and of being the first effective barrier against the transmission of fish diseases. However, based on the above ubiquity of whether a secondary application has beneficial effects or not in the case of European seabass, it cannot be safely suggested in a commercial setting.

Time limitation of the studies of Chapters 4 and 5

This study is focusing on the morphological effects of bacterial load in the larval rearing medium, but not on the action of specific bacteria and their phenotypic effects on size and shape. It did not include the identification of various strains on the fish, especially in their gastrointestinal tract. Other studies have focused on the investigation of morphological effects of axenity on a histologic and cellular level (for example Rekecki et al., 2009 for European seabass), and they have been commented on in the presented arguments, but to the best of our knowledge, beyond this thesis there has been no focus on the detailed investigation of any associated phenotypic effects. Furthermore, there is a need to determine whether that associated size and/or shape effect has a specific biological impact, and whether that can be interpreted as (a) positive, (b) simply an ontogenetic effect, or (c) negative. In case it is negative, it can be questioned whether it can be a precursory causative condition leading to the manifestation of deformities at a later stage. The present study, due to the limited time that it allows the fish larvae to be monitored in this experimental setup before the onset of mortalities, cannot safely determine, with a minimum amount of certainty, that the witnessed bacterial effects point to abnormal morphological conditions. This would only be feasible if the rearing protocol could be improved for that time to be prolonged, in order to allow these abnormalities to develop fully. A step towards this could be increasing the volume of the rearing medium, in order to deal with concerns over the water quality deterioration by the absence of its renewal (it is not renewed in order to minimise the risk of bacterial contamination). This can be achieved by using aerated glass bottles instead of vials.

In that case, if securing axenic conditions and achieving the prolongation of the study time prove to be difficult, larvae of DAH 3 obtained by the hatchery can be reared in a medium with an antibiotics mix, similarly to the protocol of chapter 5, to examine them under conditions of very low bacterial load. This might actually not be a necessary compromise instead of using axenic conditions, but a method that can present advantages, which are discussed in section 6.3.2.

Investigation of size and shape effects of specific bacterial strains or methods of bacterial control

Therefore, another suggestion for future research is to examine the size and shape effects of specific bacterial strains, in the species and ontogenetic stages of interest. A way to monitor these bacterial effects on specific individuals across time would be rearing the larvae one by one. This has been done axenically for seabass larvae until DAH 16 in 24-well tissue culture plates in a in barrier isolator with a glove system, each well with 2 ml of filtered autoclaved seawater without antibiotics, with 1 ml changed every second day (Schaeck et al., 2016). However, this method is costly, provides a limited water volume to each larva, and is again time-limited until DAH 16. An additional problem is the lack of non-destructive methods for the identification of individual temporal microbial community structures. However, in a commercial hatchery environment, the protocol of the quantification of size and shape variation could prove useful to assess the impact of the application of different methods for bacterial control such as ozonation, UV-irradiation and biofiltration, and with various predetermined degrees of efficiency as an additional factor, on the phenotype of various aquaculture species in their early stages, until the end of metamorphosis.

For example, as discussed in section 2.5.2, the use of ozonation and UV-irradiation can promote the selection of bacteria that are r strategists (De Schryver et al., 2012). In the experiments of Chapters 4 and 5, due to the fact there was no identification of specific strains, there were no indications whether there could have been a selection of bacteria of the former or the latter category. K strategists are slow growers that need time to establish a community whereas these experiments were limited in time. Also, there was no use of matured water in order to promote that selection. Additionally, r strategists need a high nutrient-to-microbial community ratio and frequent microbial perturbations, which were

conditions not present, or at least not verifiable, in our experiments. However, the methods presented in this thesis could be used to study a potential size and shape effect of r or K strategists, for example in experiments in recirculation-system environments with conditions specifically designed to select for either category, such as in the study of Attramadal et al. (2014) with Atlantic cod larvae.

Use of antibiotics and prophylactic treatments

It must be noted that the beneficial effects witnessed in the larvae reared in an environment that is either axenic or very low in bacterial load, achieved through the use of antibiotics, do not mean that their administration is recommended in a commercial setting, due to strict regulations on their use, and due to many adverse effects associated with it such as potential toxicity, residues and development of resistance (Shaeck et al., 2016). It is also not suggested that a bacterial load as low as possible will be beneficial, since there are already many steps taken to control it in aquaculture systems, with the presence of many stochastic factors making this control difficult. In hatcheries, the protection of the larval animals at this delicate time is usually a function of good husbandry, such that the management systems prevent contact with pathogens rather than reacting to their presence (Bowden and Bricknell, 2013). There is also a growing research interest in the application of prophylactic disease treatments such as probiotics, prebiotics and other immuno-stimulants or antimicrobial peptides (Merrifield and Carnevali, 2014).

6.3.2. Protocol for antibiotics-reared larvae of DAH 3 as an alternative to axenic/gnotobiotic models

In the context of larval research on host-microbe interactions in fish, axenic or gnotobiotic models are being used, where eggs are obtained from the hatchery, disinfected, and then incubated and reared under axenic conditions, as was the case in Chapter 4. For Chapter 5, an alternative model was used, which involved the application of an antibiotics mix in the rearing medium, where larvae of DAH 3 from the hatchery were reared until DAH 14. There was no axenity, but a very small bacterial load of less than 30 CFU ml^{-1} on DAH 4 and 7, and 0.10 ± 0.09 (SD) x 10^5 CFU ml^{-1} on DAH 14. Apart from the use of this protocol in the study of the existence of a bacterial mechanism affecting the size and shape of larvae, it can also be

useful in providing information on the aforementioned host-microbe interactions, and the specific contribution of microbial communities to the biology and pathobiology of the host, as a method alternative to the use of gnotobiotic models. Gnotobiotic animals such as mice have been known to present numerous deficiencies in their immune response to certain bacterial, viral and parasitic pathogens, due to the lack of gut microbiota (Round and Mazmanian 2009). Such a larval rearing protocol allows the presence of a small bacterial community that will most likely enable at least some basic immunological triggering during host colonisation, thus mitigating the effects of the above immune deficiency drawbacks, while minimising the presence of high bacterial numbers, and consequently their increased interference.

It can thus be used to explore the effect of a high concentration of a single or mixed bacterial strain on the size and shape development of the larvae, through the establishment of an antibiotics-induced very low bacterial load. The antibiotics, due to their bacteriostatic or bactericidal action, will likely limit or eliminate the strain under testing, however, its microbe-associated molecular patterns (MAMPs) of known origin will still be present, with their ability to act as agents of endogenous danger signal recognition and innate immunity activation (Galindo-Villegas and Mulero, 2015). In this context, European seabass is useful as a case study, due to the availability of multiple immunological tools for it (Galindo-Villegas and Mulero, 2015). Furthermore, this larval rearing protocol also has the additional advantage of its simplicity, and due to its application on larvae of DAH 3, the circumvention of any unknown adverse effects of the use of antibiotics on the embryos during the egg stage, which is common in many gnotobiotic models.

6.4. Conclusions

In the present thesis, a protocol for the quantification of size and shape variation in the early larval stages of seabass larvae until DAH 15 was presented, with no size or shape effects between the live and fixed specimens until 5 months post fixation. It can provide very useful information on the effect of any biotic or abiotic factor that has the potential to induce size and shape differences. It is suggested that its usefulness can be greatly improved if it is paired with a model-based deformity classification method that takes into account not only

parameters of size and shape, but also any parameter known or suspected to play a role in the manifestation of deformities. This can be a good step towards a need which has been acknowledged in literature: addressing deformities in a manner which integrates all available data, such as the frequencies, timing and typologies of abnormalities, and the effects of environmental, physiological, biomolecular, histological and genetic factors, in the context of establishing objective quality standards that can be incorporated in existing quality certification schemes.

Larvae that were axenic or reared in a very low bacterial load tended to be longer, slightly thicker, and with a more uniform dorso-ventral development. The axenic larvae consumed their yolk sac more slowly, which might be due to reduced energy expenditures to combat or assimilate bacteria, and possibly due to avoiding the bacterial toxic metabolic byproducts found in xenic larvae that have been known to be growth depressing. The axenic larvae were also to a small degree more curved than the xenic. This may be considered a slightly more advanced ontogenetic stage, or an abnormal shape due to the prolonged exposure to the antibiotics, as they can be considered an extra stress factor detrimental to eggs that come from a suboptimal batch quality. Furthermore, the secondary egg disinfection with glutaraldehyde after the primary one with iodine presented significant but very moderate size and shape effects, which might be associated with a more efficient yolk utilisation, or detrimental physical and physiological effects such as decreased blood flow to tissues, interference with nervous system function and increased energy expenditures.

These results, together with a few studies that provide sporadic information on the beneficial or adverse effects of specific bacteria on the manifestation of abnormalities, further suggest the existence of bacterial mechanisms that influence the size and shape of the larvae. The witnessed phenotypic effects might be precursory signs of abnormalities that will be manifested later, and may also be associated with metabolic processes that affect growth, ontogenesis and the allocation of energy towards them. They need to be investigated more extensively, on different species, on a greater time span, and ideally in a commercial environment, especially in the crucial early larval stages. In this investigation, the use of geometric morphometrics has been shown to be an ideal tool that achieves the objective and precise mathematical quantification of these effects.

Appendix

Throughout the present thesis, according to the requirements of the journals that chapters 3 and 5 have been published in and chapter 4 submitted to, the p probability values of the statistical hypotheses are reported at the nominal significance level of alpha=0.05 with the following rule: if a p value is significant it is mentioned as $p<0.05$, and if it is highly significant as $p<0.01$ or $p<0.001$. Furthermore, if it is insignificant or borderline significant (for example $p=0.049$), it is reported numerically. However, it is more advisable to report it numerically in the cases of significance as well, according to the requirement of good statistical practice.

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Summary

Dicentrarchus labrax (European seabass) is one of the most commercially important marine species in aquaculture, and of special and high significance to Europe and the Mediterranean. In spite of the impressive progress that has been made over the past years in its rearing methods, its production, along with all other major fish aquaculture species, is severely affected by deformities. These have been known or strongly suspected to have their cause and first manifestation in the first larval stages.

In Chapter 1, an introduction is provided on the importance of aquaculture production today, especially as a means of alleviating the pressure on natural resources. Focus is provided on the existence of abnormalities and deformities in aquaculture, which in spite of the impressive progress that has been made in the rearing methods, they are still a significant problem producing considerable financial losses. Additionally, the need for objective deformity quantification methods is highlighted, as well as the choice of European seabass as a case study.

Chapter 2 (Literature review) provides an overview of the biology and the aquaculture techniques of European seabass, the importance of its early larval stages, the specific deformity conditions encountered today, and the means for their identification and detection. Their drawbacks are examined. One of the most significant is that the existing methods for the identification of deformities in a commercial hatchery environment are currently mostly taking place in late stages of the larval development, and in the case of seabass usually at least 50 or 60 days after hatching, when the morphology has reached a stage that it largely resembles the adult stages. One of the main goals of this PhD study is the identification of a protocol that can focus on the objective estimation and quantification of size and shape variation, and consequently of those abnormalities, at a much earlier stage (from incubation

until 2 weeks post hatching), where it can produce a timely and very interesting insight on the impact of factors that induce abnormal development. Additionally, an analysis is provided on how such a size and shape quantification protocol in these stages can also provide feedback on the morphological effects of axenity, bacterial load and the application of egg disinfection agents. Furthermore, the current status of knowledge on the phenotypic effects of these factors is illustrated. An analysis is then provided on the focus of the thesis on the investigation of growth and shape effects of European seabass *Dicentrarchus labrax* larvae in incubation and rearing environments that are normal (xenic) versus axenic or very low in bacterial presence, during the first 15 days after hatching.

In Chapter 3, this size and shape quantification protocol is investigated on newly hatched European seabass larvae from DAH (day after hatching) 2 until DAH 14. It is based on the use of traditional morphometrics and outline analysis to determine the shape variation that can be induced by a range of parameters such as fixation, mounting, and the possible detrimental influence of antibiotics or egg disinfectants. It includes the examination of the effect of fixatives and mounting on their total length and body shape (comparison between live and fixed specimens five months post fixation). The four fixation treatments chosen for comparison are: (1) 8% formalin, (2) 70% ethanol, (3) 8% formalin for 48 h and then to 70% ethanol, and (4) 3% phosphate-buffered glutaraldehyde. Furthermore, an effort is made to identify the error introduced by anesthetisation and handling which are issues frequently overlooked in morphometric studies. This error is minimised to the best possible degree, ensuring that no size or shape changes occur on the specimens even five months after fixation. The protocol was then used to quantify size and shape effects, as reported in chapters 4 and 5.

In Chapter 4, the hypothesis that the difference in axenic conditions in the incubation and rearing environment of European seabass larvae induces size and shape effects on the specimens is tested. This difference is studied between xenic and axenic seabass larvae of DAH 0, 5, 11 and 15, the latter incubated and reared with the use of the protocol by Dierckens et al. (2009). The aforementioned axenic rearing protocol of Dierckens et al. (2009) also involves the use of glutaraldehyde disinfection on the eggs in the lab, additionally to the primary iodine disinfection at the hatchery, and the hypothesis that this secondary disinfection induces size and shape effects is also tested. In order to accomplish this, three

egg and larvae treatments are included: “DA” (Disinfected Axenic), “DX” (Disinfected Xenic”) and “NX” (Non-disinfected Xenic).

Regarding the effect of antibiotics-induced axenity, DA specimens exhibited larger bodies than both DX and NX on DAH 5 and 11. They also had a smaller yolk sac than the DX at the moment of hatching, but consumed it more slowly, probably due to the reduced energy demands from lack of bacterial colonisation of the digestive tract, and other similar reduced energy expenditures to combat or assimilate bacteria. Towards the end of the experiment, the DA larvae were thicker, but to a small degree more curved than the DX and NX, which may be considered an abnormal shape, or a slightly more advanced ontogenetic stage. As far as the egg disinfection is concerned, it had significant but very moderate shape effects on DAH 5 and 11. It was also associated with a more rapid consumption of the larval internal energy reserves. An adverse effect was that it induced smaller anal finfolds on DAH 0 and 11, which might be associated with a reduced respiratory ability, with consequent increased energy expenditures that could explain the increased yolk sac depletion rate.

The study of Chapter 4 was performed on the egg stage as a starting point. However, the application of antibiotics and other disinfection agents has been known to produce adverse effects on the eggs, with the main ones being an impairment of their hatchability and of the larvae survival. Furthermore, it is also suspected to play a role in the occurrence of abnormalities. Hence, in order to avoid these adverse effects of the axenic process on the eggs, a new study was performed in Chapter 5 on specimens obtained as larvae of DAH 3 from the hatchery. In it, the hypothesis under testing is that the difference in bacterial load, as induced by antibiotics, induces size and shape effects on European seabass larvae between DAH 3 and 14. The aim was to quantify the effects of antibiotic-induced differences in bacterial load on the size and shape of fish larvae, using *Dicentrarchus labrax* of day after hatching (DAH) 3 as a case study. They were split in two treatment groups and reared in 50 ml vials until DAH 14, with the control treatment (“NA”) including larvae reared in filtered autoclaved seawater without antibiotics, while the second (“A”) included larvae reared in filtered autoclaved seawater with rifampicin, ampicillin, kanamycin, trimethoprim and gentamicin, with a concentration of 10 mg l^{-1} each. They were sampled for bacterial presence on DAH 4, 7 and 14, and had their mortalities recorded, their total lengths, gut lengths, anal

body depths, eye diameters, head depths, yolk sac lengths and yolk sac depths measured, and their outlines analysed on DAH 7 and 14.

Treatment NA exhibited the highest mortalities on DAH 14. The antibiotics had a significant size effect, yielding larvae with larger total length on DAH 7 and 14, larger bodies on DAH 7, and on DAH 14 larger anal body depth and greater variance in body size. Their effect on outline shape was also significant in both age classes, with increasing differences from DAH 7 to DAH 14. On DAH 7, "A" specimens were more uniform in their dorso-ventral development, and on DAH 14 "NA" had more slender shapes. The beneficial total length and size effects and the witnessed shape effects might be associated with the low bacterial presence.

Finally, in Chapter 6, the advantages and drawbacks of the size and shape quantification protocol are being evaluated. Its practical application in a commercial hatchery setting is discussed, along with the shortcomings presented by its time-consuming and labour-intensive nature, and the possibility of automation through the use of image recognition technology is argued. Furthermore, the significance of the manifested size and shape effects due to the presence of axenic conditions, the antibiotic-induced differences in bacterial load and the application of glutaraldehyde disinfection on the eggs is discussed, in the context of possible suggestions that can be made regarding the critical factors that need to be closely monitored in hatcheries. Additionally, the need to establish a direct link between the manifestation of abnormalities in the earliest larval stages and adult stages at the end of metamorphosis in aquaculture is highlighted. Finally, suggestions for further study are made regarding the need to investigate the existence of deformity-inducing bacterial mechanisms, and their possible causative link with metabolic changes induced by increased bacterial load, in hatchery conditions for a longer period of time than the first 15 days after hatching, as examined in the present thesis for European seabass.

Samenvatting

Dicentrarchus labrax (Europese zeebaars) is één van de meest commercieel belangrijke mariene soorten in aquacultuur en is heel belangrijk in Europa en het Mediterraan gebied. Ondanks de impressionante vooruitgang die werd geboekt in de voorbije jaren in verband met de kweekmethodes en de productie, blijft hij samen met andere belangrijke aquacultuur vissoorten, zwaar getroffen door misvormingen. Van deze is geweten, of heeft men een sterk vermoeden, dat hun oorzaak ligt en eerste manifestatie zich voordoet in de eerste larvale stadia.

In **Hoofdstuk 1**, wordt een inleiding gegeven over het belang van de huidige aquacultuurproductie, voornamelijk als een middel om de druk op de natuurlijke bronnen te verlichten. De focus wordt gelegd op het voorkomen van abnormaliteiten en misvormingen in aquacultuur, welke ondanks de impressionante vooruitgang die werd gemaakt in de kweekmethodes, nog steeds een significant probleem vormen met belangrijke financiële verliezen tot gevolg. Daarenboven wordt de nood voor objectieve methodes voor het kwantificeren van misvormingen in de verf gezet, evenals de keuze voor Europese zeebaars als casus.

Hoofdstuk 2 (Literatuur overzicht) geeft een overzicht van de biologie van en de aquacultuurtechnieken voor Europese zeebaars, het belang van zijn vroege larvale stadia, de specifieke misvormingen die tegenwoordig voorkomen en de beschikbare middelen ter identificatie en detectie. Hun tekortkomingen worden onderzocht. Eén van de meest significante is dat de bestaande methoden voor identificatie van misvormingen in een commerciële kwekerij meestal pas gebeuren in latere stadia van de larvale ontwikkeling, en in het geval van zeebaars, gewoonlijk tenminste 50 of 60 dagen na ontluiking, wanneer de morfologie een stadium heeft bereikt dat grotendeels lijkt op dat van de adulte stadia. Eén van de belangrijkste doelen van deze doctoraatsstudie is de identificatie van een protocol dat kan focussen op de objectieve schatting en quantificatie van grootte en vormvariatie, en bijgevolg, van deze abnormaliteiten, op een veel vroeger stadium (vanaf incubatie tot twee weken na ontluiking), waardoor er een vroegtijdig en zeer interessant inzicht op de impact

van factoren die een abnormale ontwikkeling induceren, kan bekomen worden. Daarbij wordt er een analysis aangeboden over hoe een protocol voor het kwantificeren van grootte en vorm quantificatie in deze stadia inzichten kunnen geven op de morfologische effecten van axeniciteit, bacteriologische belasting en het gebruik van agentia voor de desinfectie van eieren. Het huidige kennisniveau over fenotypische effecten wordt verder ook geïllustreerd. De focus van de thesis op het onderzoek van groei- en vormeffecten bij Europese zeebaars *Dicentrarchus labrax* larven wordt daarna uitgelegd, bij incubatie en kweekomstandigheden die normaal (xenisch) versus axenisch zijn of met heel lage bacteriële densiteiten, en dit gedurende de eerste 15 dagen na ontluiking.

In **Hoofdstuk 3** wordt deze protocol voor quantificatie van grootte en vorm geëvalueerd op pas ontloken Europese zeebaarslarven van twee dagen na ontluiking (DNO) tot DNO14. Het is gebaseerd op het gebruik van traditionele morfometrie en contouranalyse om de vormvariatie te kwantificeren, die kan geïnduceerd worden door een reeks van parameters zoals fixatie, inbedding. Deze methode kan ook aangewend worden om de mogelijk negatieve invloed van antibiotica of disinfectantia voor eieren te bepalen. Het omvat onderzoek naar het effect van fixatieven en inbedding op hun totale lengte en lichaamsvorm (vergelijking tussen levende en gefixeerde specimens vijf maanden na fixatie). De vier gekozen fixatie-behandelingen zijn: (1) 8% formaline, (2) 70% ethanol, (3) 8% formaline voor 48 u en daarna naar 70% ethanol, en (4) 3% fosfaat-gebufferde glutaraldehyde. Verder werd er een poging gedaan om de discrepantie die wordt teweeggebracht door de verdoving en manipulatie, te identificeren, zaken die vaak over het hoofd worden gezien in morfometrische studies. Deze fout wordt zo goed als mogelijk geminimaliseerd, verzekerend dat er geen grootte- of vormveranderingen optreden in de specimens, zelfs tot vijf maanden na fixatie. Het protocol werd daarna gebruikt om de grootte en vormeffecten te quantificeren, zoals gerapporteerd in hoofdstukken 4 en 5.

In **Hoofdstuk 4** werd de hypothese dat de verschillen in axenische condities tijdens de incubatie en kweekomgeving van Europese zeebaarslarven grootte- en vormeffecten induceren bij de specimens, getest. Dit verschil werd bestudeerd bij xenische en axenische zeebaarslarven van DNO 0, 5, 11 en 15, de laatste werden geïncubeerd en opgekweekt conform de protocol volgens Dierckens et al. (2009). Het bovenvermelde axenische groeiprotocol van Dierckens et al. (2009) maakt ook gebruik van glutaraldehyde disinfectie

van de eieren in het lab, bovenop de eerste jodiumdesinfectie in de kwekerij. Ook de hypothese dat deze tweede desinfectie grootte- en vormeffecten induceert, is getest. Om dit te verwezenlijken, werden drie ei- en larvenbehandelingen toegevoegd: “GA” (Gedesinfecteerd Axenisch), “GX” (Gedesinfecteerd Xenisch”) en “NX” (Niet-gedesinfecteerd Xenisch).

Wat het effect van antibiotica-geïnduceerde axeniciteit betreft, axenische specimens hadden een groter lichaam dan zowel xenische (GX) als onbehandelde (NX) op DNO 5 en 11. Ze hadden ook een kleinere dooierzak dan de xenische op het moment van ontluiking, maar verbruikten deze trager, waarschijnlijk omwille van de lagere energievraag door het uitblijven van bacteriële kolonisatie van het spijsverteringsstelsel en andere gelijkaardig lager energieverbruik om bacteriën te weren of te assimileren. Naar het einde van het experiment waren de axenische larven dikker, maar lichtjes meer gebogen dan de xenische en onbehandelde, wat als een abnormale vorm mag gezien worden of een lichtjes verder gevorderd ontogenetisch stadium. Wat betreft de desinfectie van de eieren, deze had significante maar zeer matige vormeffecten op DNO 5 en 11. Dit was ook geassocieerd met een sneller verbruik van de larvale energiereserves. Een ongunstig effect was dat het kleinere vinplooien induceerde op DNO 0 en 11, wat eventueel kan geassocieerd worden met een lagere ademhalingscapaciteit, met verhoogde energieverbruik als gevolg, wat dan de verhoogde depletiesnelheid van de dooierzak kan verklaren.

De studie in **Hoofdstuk 4** werd uitgevoerd op het eistadium als startpunt. Echter, het is gekend dat het gebruik van antibiotica en andere desinfectie agentia kan leiden tot ongunstige effecten op de eieren, vooral dan het bemoeilijken van hun ontluiking en van de overleving van de larven. Verder wordt ook vermoed dat het een rol kan spelen bij het optreden van misvormingen. Om deze ongunstige effecten van het axenisch proces op de eieren te vermijden, werd een nieuw onderzoek gedaan in Hoofdstuk 5 op specimens bekomen als larven van DNO 3 uit de kwekerij. Daarin was de geteste hypothese dat een verschil in bacteriologische belasting, door gebruik van antibiotica, grootte- en vormeffecten induceert in Europese zeebaarslarven tussen DNO 3 en 14. Het doel was om de effecten van antibiotica-geïnduceerde verschillen in bacteriologische belastingop grootte en vorm van de vislarven van *Dicentrarchus labrax* van DNO 3 te quantificeren. Ze werden opgesplitst in twee behandelingsgroepen en gehouden in 50 ml recipiënten tot DNO 14, met als controle behandeling (“NA”) larven gehouden in gefilterd, geautoclaveerd zeewater zonder

antibiotica, terwijl als tweede ("A") larven gehouden in gefilterd, geautoclaveerd zeewater met rifampicine, ampicilline, kanamycine, trimethoprim en gentamicine, met een concentratie van elk 10 mg l^{-1}. Er werd gecontroleerd op aanwezigheid van bacteriën op DNO 4, 7 en 14. Daarnaast werden mortaliteit, totale lengte, lengte vna het darmkanaal, lichaamshoogte ter hoogte van de anale opening, oogdiameter, kophoogte, dooierzaklengte en dooierzakhoogte gemeten en hun contouren geanalyseerd op DNO 7 en 14. Behandeling NA had de hoogste mortaliteit op DNO 14. De antibiotica hadden een significant grootte-effect, wat resulteerde in larven met een grotere totale lengte op DNO 7 en 14, grotere lichamen op DNO 7, en grotere lichaamshoogte ter hoogte van de anaalopening en grotere variantie in lichaamsgrootte op DNO 14. Hun effect op de contour was eveneens significant in beide leeftijdsklasses, met stijgende verschillen van DNO 7 tot DNO 14. Op DNO 7 waren de "A" specimens uniformer wat betreft hun dorso-ventrale ontwikkeling, en op DNO 14 "NA" hadden ze slankere vormen. De positieve effecten op totale lengte en grootte, evenals de waargenomen vormeffecten kunnen mogelijks geassocieerd zijn met lage bacteriële densiteit.

Finaal worden in **Hoofdstuk 6**, de voor- en nadelen van het grootte en vorm quantificatieprotocol geëvalueerd. De practische toepassing ervan in een commerciële kwekerij wordt bediscussieerd, samen met de tekortkomingen omwille van zijn tijdsrovend en arbeidsintensief karakter. Ook wordt de mogelijkheid tot automatisatie door het gebruik van beeldherkenningstechnologie besproken. Verder wordt het belang van de vastgestelde grootte- en vormeffecten omwille van de geldende axenische condities, de antibiotica-geïnduceerde verschillen in bacteriële densiteit en gebruik van glutaraldehyde desinfectie van de eieren bediscussieerd in de context van mogelijke suggesties die kunnen gemaakt worden in verband met de kritische factoren die nauwlettend moeten opgevolgd worden in kwekerijen. Daarnaast wordt de nood benadrukt om een directe link vast te kunnen stellen tussen het voorkomen van misvormingen in de vroegste larvale stadia en in de adulte stadia op het eind van de metamorfose. Op het eind worden suggesties voor verder onderzoek gemaakt met betrekking tot het bestaan van misvorming-inducerende bacteriële mechanismen en hun mogelijks oorzakelijk verband met metabolische veranderingen geïnduceerd door een verhoogde bacteriële densiteit, in kwekerijomstandigheden voor een langere periode dan de eerste 15 dagen na ontluiking, zoals werd onderzocht in deze thesis voor Europese zeebaars.

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Curriculum Vitae

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Born in Athens, Greece on 18/9/1977
Greek Nationality, registered Belgium resident since 2005
Holder of EU driving licence
Greek Military service (compulsory): May 2006 – February 2007

WORK EXPERIENCE

PhD researcher – Laboratory of Aquaculture & Artemia Reference Center in collaboration with the Research Group of Evolutionary Morphology of Vertebrates, Ghent University, Belgium — May 2007 - now

Thesis: "Analysis of size and shape in early larval stages of European seabass (*Dicentrarchus labrax*): effects of egg disinfection, axenity and bacterial load"

- Specialisation in the study & mathematical quantification of morphological effects, including deformities
- Use of statistics & morphometrics software (SPSS, Statistica, PAST, GraphPad Prism, Shape, TPS, Excel)
- Supervision of theses of 3 Master students of the MSc in Aquaculture - Ghent University
- Greek State Scholarships Foundation scholar (single position awarded after written exams)

Aquaculture farm estimator of financial biological assets (freelance) — January - April 2013

Inspection of aquaculture facilities, estimation of total value of inspected livestock according to bibliographic & market references, and production of detailed reports with supporting financial data

Research assistant – Laboratory of Environmental Toxicology & Aquatic Ecology, Ghent University, Belgium — November 2005 - April 2006

Research topic: "In-situ aquaculture for fish community restoration in rivers based on mathematical modelling"

Research assistant – Marine Fish & Algae Culture Lab, January - September 2005
Dept. of Aquaculture and Fisheries,
Technological Educational Institute of Epirus, Greece

- POCEFF (Preservation of Copepod Eggs for Fish Farming) EU project
- Design, construction & maintenance of marine closed water recirculation and copepod egg culture systems

Aquaculture Farm Manager – Llyn Aquaculture Ltd., April - October 2001
Chwilog, North Wales, U.K.

- Monitoring/maintenance of intensive recirculation aquaculture systems of European seabass, emergency situation response, physicochemical - feeding - growth data processing, planning of feeding regime and harvesting schedule, supervision of technical personnel

EDUCATION & TRAINING

MSc in Marine Resource Development & Protection – 2003-2004
School of Life Sciences, Heriot Watt University, Edinburgh, Scotland

- Thesis: «The effects of barite in discharged drill cuttings from offshore oil production on physiological aspects of the biology of Mytilus edulis and Cerastoderma edule»

BSc in Aquaculture and Fisheries –
1997-2003
Dept. of Aquaculture & Fisheries, Technological Educational Institute of Epirus, Greece

- Degree mark : **8.24/10**
- Thesis: «Biology - culture techniques of the microalgae of the Tetraselmis family and the application of Tetraselmis suecica in fish aquaculture – small scale experimental lab culture». Mark awarded: **10/10**

Additional training courses:

qPCR analysis and methods – April 2017
Faculty of Bioscience Engineering, Ghent University, Belgium

Laboratory Animal Science I & II – September - October 2013
Faculty of Medicine and Health Sciences, Ghent University, Belgium
Animal Experiments Director course recognised by
the Belgian Federal Public Service of Health, Food Chain Safety & Environment

Applied Biostatistics – April - May 2010
Marine Biology Dept., Ghent University, Belgium

Data Analysis using Matlab – March 2006
Earth Sciences Dept., Ghent University, Belgium

PC operator & Microsoft Office Certificate – February - May 2002
Organisation for Workforce Employment,
Educational Centre of Pallini, Athens, Greece

Food Hygiene Certificate – August 2001
Chartered Institute of Environmental Health,
Coleg Menai, Bangor, U.K.

ARTICLES & CONGRESS PRESENTATIONS

- **"Effects of antibiotic-induced differences in bacterial load on growth and shape of early larval European seabass (*Dicentrarchus labrax* L.)"** - Spyridon Nikolakakis, Kristof Dierckens, Peter Bossier and Dominique Adriaens (2017). Research article in journal of Aquaculture Research (http://onlinelibrary.wiley.com/doi/10.1111/are.13546/full)
- **"Study of effects of antibiotics and low bacterial presence on growth and shape of early larval European seabass (*Dicentrarchus labrax*)"** - Spyridon Nikolakakis, Kristof Dierckens, Peter Bossier and Dominique Adriaens (**oral presentation** at Aquaculture Europe 2015 congress of the European Aquaculture Society, 20 – 23 October 2015, Rotterdam, Netherlands)
- **"Protocol for quantitative shape analysis of deformities in early larval European seabass *Dicentrarchus labrax*"** - Spyridon Nikolakakis, Peter Bossier, Grigoris Kanlis, Kristof Dierckens & Dominique Adriaens (2014). Research article in Journal of Fish Biology (http://onlinelibrary.wiley.com/doi/10.1111/jfb.12284/full) + 1 new article submitted for publication
- **"Effects of axenity and disinfection on growth and deformities of early larval seabass (*Dicentrarchus labrax*)"** – Spyridon Nikolakakis, Peter Bossier and Dominique Adriaens (**oral presentation** funded by COST 2011 EU in the framework of Larvanet Network at Aquaculture Europe Congress of the European Aquaculture Society, 18 – 21 October 2011, Rhodes, Greece)
- **"Greek Aquaculture: Status, progress and challenges"** – Spyridon Nikolakakis (**oral presentation** in the framework of European Aquaculture Society Student Group at Aquaculture Europe Congress of the European Aquaculture Society, 18 – 21 October 2011, Rhodes, Greece)
- **"Protocol for standardized shape analysis and mounting of early larval seabass (*Dicentrarchus labrax*)"** – Spyridon Nikolakakis, Dominique Adriaens and Peter Bossier (**oral presentation** at World Aquaculture Congress of the World Aquaculture Society, 25 – 29 September 2009, Veracruz, Mexico)
- **"Quantitative shape analysis of fixative induced deformations in early larval seabass (*Dicentrarchus labrax*)"** – Spyridon Nikolakakis, Dominique Adriaens and Peter Bossier (**poster** at Larvi 2009 – 5th Fish & Shellfish Larviculture Symposium, 7 – 10 September 2009, Ghent, Belgium)
- **"Characterization of phenotypic variation in response to xenic vs. axenic rearing conditions in larval seabass (*Dicentrarchus labrax L.*)"** – Spyridon Nikolakakis, Md. Moshiur Rahman, Eyasu Shumbulo Shuba, Kristof Dierckens, Peter Bossier and Dominique Adriaens (**poster** at 15th Benelux Congress of Zoology, 30 & 31 October 2008, Liège, Belgium)

LANGUAGES

- **Greek** as a native language
- **English** (certified tutor of the English language by the Hellenic Ministry of Foreign Affairs, holder of Lower, Advanced, IELTS & Proficiency degrees by the University of Cambridge, UK)
- **Dutch** (level 6 certificate "Nederlands Tweede Taal", Vlaamse Gemeenschap, Dept. Onderwijs en Vorming, België), **French** (elementary)

HOBBIES

Music (theory, solfege & flute studies in Zografou Municipal Music School, Athens, Greece & participation in choir, orchestra and other music bands as guitarist, flutist, drummer etc), music production software & equipment, history of cinema, science fiction literature, table tennis