# CHAPTER I

# INTRODUCTION

## 1. What is egg quality?

Fish populations, both in aquaculture and in the wild, depend of the production of good quality eggs (Brooks *et al*., 1997), because poor egg quality may decrease the survival potential of the hatched larvae (Kjørsvik *et al.,* 1990).

The general accepted definition of egg quality seems to be "the egg's potential to produce viable fry" (Kjørsvik *et al*., 1990; Nissling *et al.,* 1998). According to developmental biology, the quality of an egg is determined by its intrinsic properties, by its genes, by the maternal mRNA transcripts and nutrients contained within the yolk provided by the mother. After fertilization, the quality of the egg (embryo) will also be determined by the contribution of the paternal genes. But not only paternal contributions may affect egg quality. In fact, the environment in which the eggs are incubated, both in the wild and in aquaculture, also affects the success in production of viable offspring (Brooks *et al.,* 1997). These general criteria for egg quality also are applicable to some shrimp species (*Penaeus monodon)* as stated by Kian *et al.* (2004).

The potential of the eggs to produce viable fry is determined by several physical, chemical, biological and genetic parameters (Kjørsvik *et al.,* 1990; Morehead *et al.,* 2001), and by physiological processes occurring initially in the eggs (Kjørsvik *et al*., 1990). If one of these parameters is not present, or is incomplete, the egg development will fail at some point (Kjørsvik *et al*., 1990).

## 2. Factors affecting egg quality

The reproduction of marine species in captivity depends of numerous external factors and carries various different risks (Devauchelle & Coves, 1988; Brooks *et al*., 1997; Dinis *et al.,* 1999). The quality of the eggs produced can be variable, and this variability can be attributed to many factors (Morehead *et al.,* 2001).

## 2.1. Factors relative to the broodstock

### 2.1.1. Broodstock nutrition

The embryonic demands for growth and nutrition should be reflected in the composition of a healthy egg (Morehead et al., 2001). The nutrients that the organism is not able to synthesize have to be present in the egg in the proper amount to satisfy biological demands (Kjørsvik *et al*., 1990). These nutrients are taken from the maternal bloodstream and stored for posterior use by the developing embryo (Carnevali *et al*., 1999). If a particular compound or the appropriate amount of a compound is not present, the egg may not be able to sustain the development of a viable embryo (Kjørsvik *et al.,* 1990; Brooks *et al*., 1997).

There have been several studies in how the diet of the broodstock can affect the egg quality (Brooks *et al.,* 1997). Food quality is generally accepted as one of the most important factors in determining spawning performance and subsequent egg quality in fish (Navas *et al.,* 2001). Kjørsvik *et al.* (1990) report that excessive feeding can lead to an increase in total egg numbers, but not to an increase in egg size. For the striped trumpeter (*Latris lineata*), Morehead *et al*. (2001) obtained differences between egg and larval parameters of eggs collected from 3 different broodstock, fed with different diets. One of the most common used parameters is the lipids ratio in diets since it has been shown to clearly influence the egg quality (Navas *et al.,* 2001). The content of highly unsaturated fatty acids (HUFAs) in broodstock feed and present in eggs has also been used because fish are not able of a "*de novo*" synthesis of them (Mourente & Toucher, 1993). In *Sparus aurata*, Rodriguez *et al*., (1998) have found that both fecundity and hatching success were reduced when fish were fed a diet poor in HUFAs.

### 2.1.2. Oogenesis

When an oocyte is still within the ovary, it must incorporate all the necessary information to direct the normal development of an embryo, and all the nutritive contents that will latter determine its quality as an egg (Brooks *et al.,* 1997; Carnevali *et al.,* 2001). In teleosts fishes, there seems to be a ubiquous basic pattern in oocyte development, independently of their reproductive strategy (Kjørsvik *et al.,* 1990; Arukwe & Gorksøyr, 2003).

The phases of the oocyte development are the oogenesis, the primary oocyte growth, the cortical alveolus stage, vitellogenesis, maturation and ovulation (Brooks *et al*., 1997). Oogenesis begins when the oogonia proliferate by mitotic division. The oogonia can remain capable of mitotic division throughout life in species that produce and repeatedly release hundreds, or thousands, of eggs (Purves *et al.,* 1998). Then comes the differentiation into primary oocytes, and the oocytes enlarge in response to the production of ribossomes, RNA, cytoplasmatic organelles and energy reserves. This phase is called vitellogenesis (Brooks *et al*., 1997; Purves *et al.,* 1998).

After vitellogenesis the oocytes face two consecutive asymmetric meiosis; in each of them is formed one large cell containing almost all of cytoplasm of the mother cell (the ootid), that continues undergoing oogenesis, and three smaller cells (the polar bodies). The polar bodies will degenerate and thus from one primary oocyte, in oogenesis, only one mature egg is produced that arrests developing. This egg is well provisioned for the cell divisions that rapidly follow fertilization. The egg may stay in this stage until ovary expulsion in ovulation. In many species the second meiotic division, which originates the maturing egg does not continue until the egg is fertilized by a sperm (Purves *et al*., 1998).

If the right progression of the phases of oogenesis is affected, it will reflect itself in the production of good-quality eggs (Brooks *et al*., 1997). Therefore it is necessary to know the optimal conditions for normal embryogenesis, in the area in question for any particular species cultivated (Devauchelle & Coves, 1988; Brooks et al., 1997).

### 2.1.3. Other factors related to broodstock

Batch spawning also influences the egg size. Multiple-spawning fish may have eggs with increasing egg size to a maximum, decreasing later in the spawning season (Kamler, 2005). The spawning time was found to have an effect on egg size, fecundity and viability (Buckley *et al.,* 1991a, b). There can be considerable variations in the quality of eggs in different batches of the same spawning period (Buckley *et al.,* 1991a, b), even when the batches are cultivated in the same apparent conditions (Brooks *et al*., 1997). This may reflect a depletion of the energy resources available by repeated

spawning (Kamler, 2005). However, in the work of Buckley *et al.* (1991b), the interaction of spawning time and female size influenced the egg size, fecundity and viability.

Larger fishes will produce larger eggs, this is a relation that seems to be universal (Kjørsvik *et al*., 1990; Kamler, 2005). In turn, larger eggs will produce larger larvae (Kjørsvik *et al.,* 1990). In the work of Buckley *et al.* (1991a) it was showed that female size affected most of the parameters examined. In general, the largest eggs will be produced by females of average length and age, smaller eggs produced by older females and the smallest eggs will be produced by young first-spawning females (Kjørsvik *et al*., 1990; Kamler, 2005).

As reported in the reviews of Kjørsvik *et al*. (1990), Brooks *et al*. (1997) and Kamler (2005) the egg size may also be a factor affecting egg quality, however Kjørsvik *et al*. (1990) adverts that the egg diameter may not be a good criterion for egg quality, because aquaculture weaning procedures tend to minimize the differences between larva produced from eggs of different sizes.

### 2.2. Factors relative to fertilization processes

The fertilization processes also can affect egg quality. As already stated above the fertilization rate has been used as an important egg quality criterion. Usually this parameter is determined in artificial fertilization tests because it is easy to perform and will indicate an approximately level of success. This is applicable to cultured species such as trout, salmon or turbot. However in fish spawning in natural conditions, such as seabream, sea bass and sole it is more difficult to collect the eggs and to evaluate the fertilizing ability always at the same time and conditions. In all teleost fishes (as reported in the review of Kjørsvik *et al*., 1990), during the fertilization and activation, the cortical reaction takes place. In this reaction, the cortical alveoli break down and release their contents starting the formation of the perivitelline space. For some species, the absence of perivitelline space after fertilization is a sign of poor quality eggs. However, the fertilization rate does not correlate always with good development in later embryonic stages for many species (Kjørsvik *et al*., 1990). Even in egg batches with high fertilization rates, abortions during embryonic development may occur. In this type

of egg batches this parameter can overestimate the quality of eggs. Other thing contributing for lower fertilization is the broodstock sperm quality. Over-ripped sperm and eggs often loose fertilization potential and, in some cases, can even be responsible for the abnormalities and abortion during embryonic development, especially if the sperm DNA presents any damage that cannot be repaired by the oocyte-repair mechanism. In this case, fertility rates would be high because first cleavages are not affected but embryonic development will be compromised at later stages. This phenomenon has been reported in fertilization tests performed with cryopreserved sperm (Cabrita *et al.,* 2005).

### 2.3. Factors related to husbandry conditions

When compared, eggs of wild fish have higher quality than those from captive stocks. This is believed to be mainly due to environmental influences (Rosety *et al.,* 1992; Brooks *et al.,* 1997), being one of the most important the water temperature (Imsland *et al.,* 2003), although in some species photoperiod can also play an important role, especially in oocyte maturation.

Temperature during oogenesis is important for successful spawning and egg viability (Kjørsvik *et al.,* 1990). Imsland *et al*. (2003) report that for *Solea solea* there is a temperature, around 20º C, that is optimal for juvenile and ongoing growth. In the studies reviewed by Lambert *et al.* (2003), temperature was one of the major factors influencing the fecundity of gadoid fish species. In the literature reviewed by Brooks *et al*. (1997) it is summarized that the water temperature is particularly important to spawning and to the incubation of eggs because it affects metabolism, the activity and structure in embryonic development.

In species with artificial reproduction, *e.g.* rainbow trout (*Oncorhynchus mykiss*), the manipulation of photoperiod can induce specific embryonic malformations linked to the reabsorption of the yolk sac (Bonnet *et al.,* 2007a;b). In this study it was also demonstrated that the post-ovulatory ageing of eggs also produced embryonic malformations. For some species, and such is the case of *Solea senegalensis* (Dinis & Reis, 1995), natural spawning of the broodstock is the only way to produce viable eggs.

Other environmental influences to egg quality in aquaculture are the mechanical damage during handling, stress and over-ripening of ovulated, but not oviposited, eggs (reviewed in Brooks *et al*., 1997; Kamler, 2005).

As reported by Kjørsvik *et al.* (1990) stressed fish spawn more irregularly and have low fertilization rates. The confinement and crowding of fish may induce stress and thus influence egg quality (Brooks *et al*., 1997). The handling of the broodstock in species with artificial fertilization such as turbot, or halibut, may also stress captive fish and influence spawning quality.

Kjørsvik *et al.* (1990) report that bacterial growth can decrease egg strength and that in *Solea solea* the eggs more susceptible to bacterial contamination seemed to be the poor quality eggs. After fertilization the dying, or the dead, eggs can be quickly colonized by fungus and bacteria. If not removed rapidly, the contact of these eggs may ease the spreading of colonizing bacterial or fungal colonies into viable eggs (Brooks *et al.,* 1997).

In oogenesis the processes that lead to the formation of mature eggs require hormonal co-ordination. During some phases of the reproductive cycle of Teleosts, under hormonal control the liver synthesizes proteins (being the vitellogenin one of these proteins) that are released into the maternal bloodstream and accumulated in the ovary in the developing oocytes (Kjesbu *et al.,* 1992; Rosety *et al.,* 1992). As already stated these processes are quite conserved in most fish species (Kjørsvik et al., 1990; Arukwe & Goksøyr, 2003). In Teleosts and other vertebrates several hormones, such as pituitary gonadotropins (GtHs) and ovarian steroids, regulate oocyte growth and maturation (Arukwe & Goksøyr, 2003). Studies reviewed in Brooks *et al.* (1997) pointed out that fish larvae are physiologically immature, with little or no capacity to produce certain enzymes, growth factors and hormones, until the end of yolk resorption. This way, developing embryos are dependent on exogenous maternal hormone sources. Since they play a role in embryonic and larval development, hormones may affect egg quality (Brooks *et al.,* 1997). There are some synthetic chemicals and some natural compounds, from both plant and animal origin, which may affect the endocrine system of various organisms. Some of the observed effects include the inhibition of oocyte development and maturation, the increase in follicular atresia in both yolked and

previtellogenic oocytes, abnormal yolk deposition and formation within oocytes, and abnormal egg maturation and production (Arukwe & Goksøyr, 2003).

## 3. How to measure egg quality?

There are several parameters to determine some aspects of egg quality, but until now it has been difficult to establish a group of parameters that could characterize and quantify the quality of natural or artificial spawns. The main problems arisen firstly because different species have different criteria of quality and secondly because it has been difficult to correlate some biological and metabolic information in order to understand the factors affecting egg quality.

### 3.1. Biological and physiological parameters

In aquaculture, the differences between eggs become apparent after their collection. The egg dispersal of many marine teleost species is due to buoyancy at spawning, given by the eggs high content in water (Carnevali *et al.*, 2001). Hatcheries cultivating marine fish species with pelagic eggs, often distinguish the "good" and the "bad" eggs through a buoyancy test in sea water; the "good" eggs will float while the "bad" eggs will sink (Kjørsvik *et al.,* 1990). Using these parameters it is easy to determine the viability percentage for each spawn in pelagic eggs. But this positive correlation is not valid for some marine species, as alerted in the review of Brooks *et al*. (1997) and cannot be used in species with benthonic eggs such as the ones from rainbow trout or winter flounder (*Pseudopleuronectes americanus*).Due to this, other commonly used criteria that can be applied in both types of eggs are the incubation of eggs, estimating the survival up to hatching and larval survival until first feeding (Nissling *et al.,* 1998). Nevertheless, parameters like egg survival and hatching rate are very crude measures of egg quality because they reveal nothing about what factors determine the quality of the eggs (Brooks *et al.,* 1997; Kjørsvik *et al*., 2003). The morphological malformations and rate of viable larvae are more reliable indicators of quality (Kjørsvik *et al*. 1990). Early cell cleavages (Nissling *et al.,* 1998; Morehead et al., 2001), appearance of the chorion, size and shape of the egg, egg transparency, distribution of oil drops (Dinis, 1992; Morehead *et al*., 2001) and larval activity (Kjørsvik *et al*., 1990) are also indicators of egg quality. The eggs exhibiting high rates of fertilization, eyeing, hatching and first feeding are considered, in aquaculture, to be "good-quality" eggs. Devauchelle & Coves (1988)

refer the egg dry weight as a possible egg quality indicator since it is correlated with both egg diameter and hatching percentage. Lahnsteiner & Patzner (2002) report that the water hardening, and consequently the eggs increase in weight, is positively correlated with egg viability, in *O. mykiss* and can also be used as a way to predict egg quality. This is especially important in species with long embryonic development (around 30 days) because hatching rate would be almost impossible to use under production conditions in order to predict egg quality.

The determination of a group of parameters that could be used easily without time consumption and the need of incubating the eggs for a long period until quality can be assessed should be the criteria for the selection of egg quality parameters.

### 3.2. Biochemical parameters

In the wild, and in captivity, egg quality is influenced by numerous factors (Kjørsvik *et al*., 1990; Kamler, 2005). As stated by Carnevali *et al.* (1999), in oviparous species the yolk is a mix of materials used for embryonic nutrition and development. Biochemical composition of eggs may be one of the most studied biomarker in egg quality for several species. The protein, total lipids (Devauchelle & Coves, 1988), carbohydrates, vitamin levels and fish feed supplements are also important in egg quality (Brooks *et al*., 1997). But these essential components may not be the same for each species, a fact that should be taken in consideration when using biomarkers that have proven to be indicators of good egg quality for one given species and apply them to others. There appears to be no universal biochemical parameter for egg quality, although there may be some species-specific indicators (Kjørsvik et al., 1990).

In the work of Lahnsteiner (2000) the amounts of protein, esterefied and non-esterefied fatty acids were considered markers of egg quality since they correlated with egg viability in *O. mykiss*. High protein concentrations and both types of fatty acids mentioned were considered as indicators of degenerative processes, indicating the presence of eggs with poor quality.

In species such as *Dicentrarchus labrax* (Devauchelle & Coves, 1988; Navas *et al*., 2001), *Cyprinus carpio* (Linhart *et al.,* 1995; Lahnsteiner *et al.,* 2001),

*Hypophthalmichthys molitrix, Ctenopharyngodon idella, Chalcalburnus chalcoides* (Lahnsteiner *et al.,* 2001), *D. dentex* (Giménez et al., 2006), *D. puntazzo (*Lahnsteiner & Patarnello, 2004b)*, S. aurata* (Rodriguez et al., 1998; Lahnsteiner & Patarnello, 2004a;b), *Latris lineata* (Morehead *et al.,* 2001), *Oreochromis niloticus* (Lu & Takeuchi, 2004) the levels of HUFAs have been analyzed. Some of these studies were dedicated to investigated the effect of different diets of the broodstock and how they could affect egg quality (Morehead et al., 2001; Lu & Takeuchi, 2004), because these fatty acids are essential to embryonic and larval development. In *S. aurata*, Rodriguez and co-workers (1998) suggested that the levels of eicosapentaenoic acid (EPA, 20:5n-3) and docosohexaenoic acid (DHA, 22:6n-3) may be implicated in egg quality. When broodstock of *D. labrax* was fed a diet rich in HUFAs, mainly EPA, DHA and arachidonic acid (AA, 20:6n-6), the high levels of these fatty acids promoted higher ratios of AA:EPA and of DHA:EPA. This was reflected in the fatty acid composition of eggs and associated to higher egg viability and hatching rates, as reported in Navas *et al*. (2001). Morehead *et al.* (2001) also point out that a severe deficiency in vitamin levels may reduce egg quality.

### 3.3. Metabolic parameters

In order for an egg or embryo to be viable it has to have metabolic processes of degradation of the yolk reserves, synthesis of new compounds and membranes and regulation of the above processes. If at some point the regular progression of metabolic processes in eggs fails, consequentially the embryo might abort. When using metabolic parameters, be them specific enzymatic activities or key metabolites presence in the eggs, it may be possible to gain a better understanding on normal egg status and to estimate potential survival of embryos and larvae, which is of most importance in aquaculture.

#### 3.3.1. Carbohydrate metabolism

The carbohydrate metabolism has proven to be essential for normal embryonic development up to the hatching stage because it provides energy, in the form of ATP, to be used in synthetic processes within the cells. The metabolism of carbohydrates

comprises some key metabolic pathways of which will be heightened the glycolysis, the gluconeogenesis and the pentose-phosphate pathway.

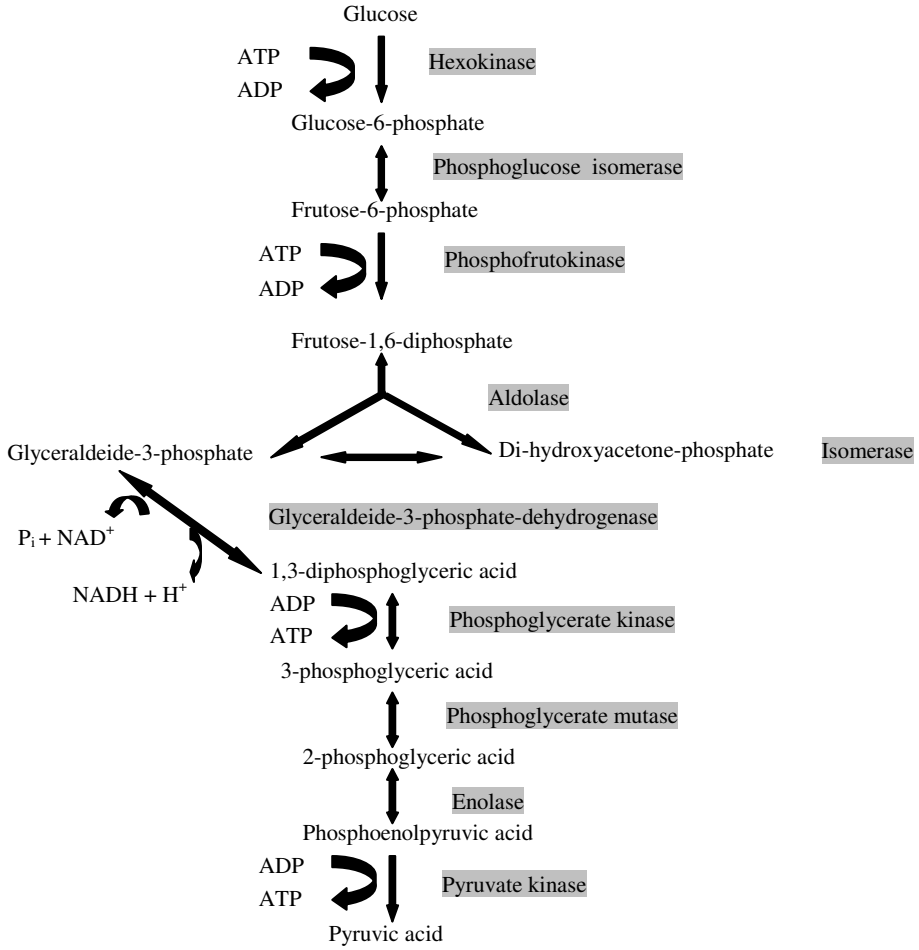


**Figure I.1** – General summarization of glycolysis (adapted from Campos, 1998).

Eggs need energy for cell division and for maintaining all the fundamental cell functions during embryo development. The energetic compound ATP has origin in the glycolytic and oxidative reactions (Linhart *et al.,* 1995). In figure I.1 it is summarized the progression of glycolysis. Whenever the energy requirements are high, there will be oxidation, as complete as possible, of glucose-6-phosphate (G6P). This occurs in two series of reaction: the first occurs in the cytosol and leads to the production of 2 molecules of pyruvic acid (or lactic acid, or ethanol, depending on the conditions in which the reaction occurs). The second series of reactions occurs inside the

mitochondria and allows the total oxidation of pyruvic acid to $CO_2$. This is the energetic part of the glucose oxidation (Campos, 1998).

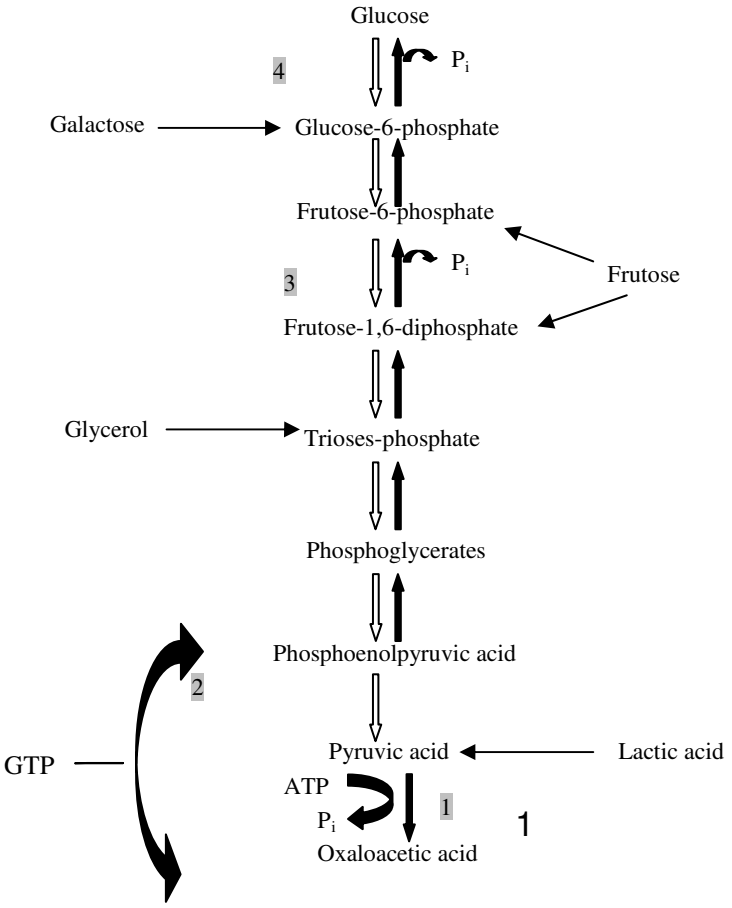


**Figure I.2** – General summarization of the gluconeogenesis. Gluconeogenesis follows the black bold arrows, while the white arrows represent the glycolysis. The numbers correspond to enzymatic activity of: 1) pyruvate carboxylase, 2) phospho-enol-pyruvate carboxykinase, 3) Frutose-1,6-diphosphatase and 4) glucose-6-phosphatase (adapted from Campos, 1998).

Figure I.2 illustrates, in a summarized way, the progression of the gluconeogenesis. It somehow reflects an inverse pathway of the glycolysis. However it is not the inverse of glycolysis since there are 3 reactions that are not reversible in the physiological conditions. They are:

- The transformation of the phospho-enol-pyruvic acid in pyruvic acid through the action of the pyruvate kinase;

- The phosphorilation of fructose-6-phosphate to fructose-1,6-diphosphate, by means of the phosphofrutokinase;
- And the phosphorilation of glucose to glucose-6-phosphate mediated by hexokinase.

The regulation of both processes (glycolysis and gluconeogenesis) is tightly connected since most of the steps occur in the same cell compartment (Campos, 1998).

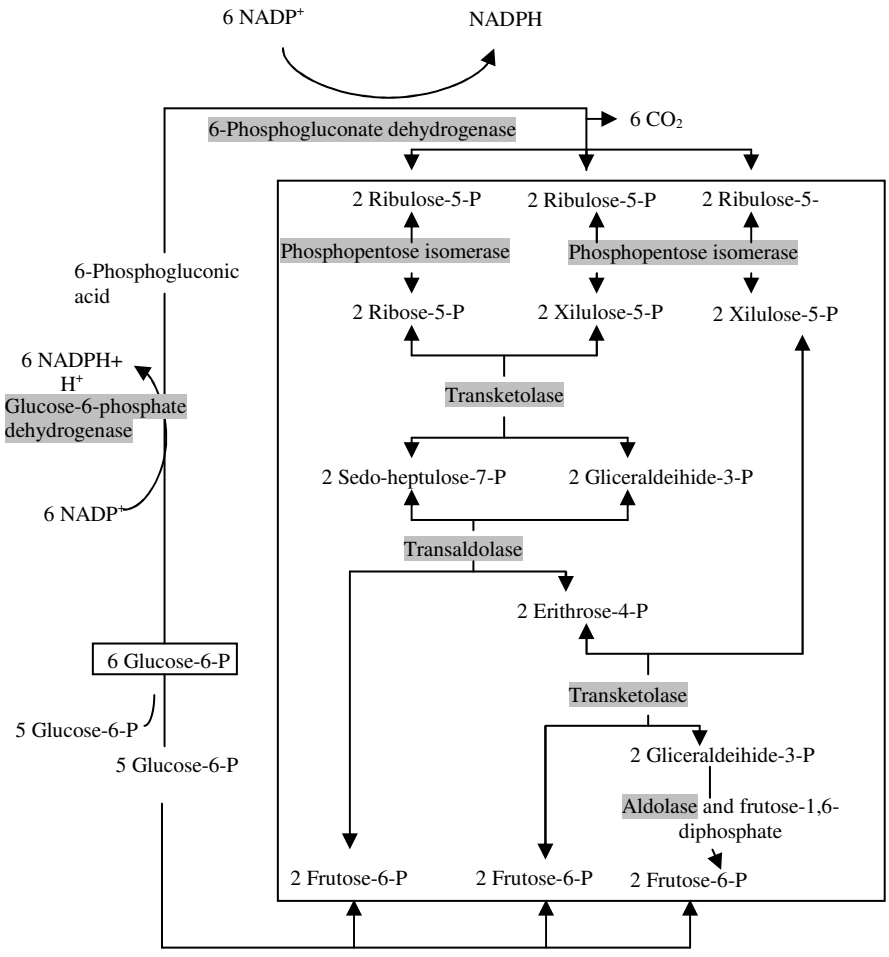


**Figure I.3** - General summarization of the pentose-phosphate pathway. P is phosphate. (adapted from Campos, 1998).

Not only the gluconeogenesis and the glycolysis are important. The pentoses-phosphate pathway (or the Dickens-Hoerecker pathway, or PPP) is also of crucial importance, because it allows the production of NADPH, which is necessary to the synthetic processes of fatty acids and other lipids. The PPP also allows cells to metabolize glucose-6-phosphate with production of ATP, without the consumption of ATP as it happens in the glycolysis; and, while working in the inverse direction, converts the glucose-6-phosphate in ribose-5-phosphate (fig. I.3); the later can be used for biosynthesis of nucleic acids (Campos, 1998). For normal carbohydrate metabolism all of the above processes should occur.

In *S. aurata* (Lahnsteiner & Patarnello, 2004a;b), *D. puntazzo* (Lahnsteiner & Patarnello, 2004b), *Coregonus* spp. (Lahnsteiner, 2005), *D. dentex* (Giménez *et al.,* 2006), *Serranus cabrilla* and *Mullus barbatus* (Lahnsteiner, 2006) the carbohydrate metabolism was studied, through carbohydrate content and metabolic enzyme activities and glycolysis, gluconeogenesis and the pentose-phosphate pathways were proven. It was demonstrated that the activities of pyruvate kinase and glucose-6-phophatase, monosaccharides concentration (Lahnsteiner & Patarnello, 2004a;b; Giménez et al., 2006) and the levels of hexoses sugars, ribose and ketoses (Lahnsteiner, 2005) were related to egg quality (hatching percentage).

### 3.3.2. Protein metabolism

Carnevali *et al*. (1999a; 1999b; 2001) works have used cathepsin D, the intra-oocytic catalyst of the cleavage of the phosphoglycoprotein vitellogenin, as potential marker for egg quality. Vitellogenin is the major constituent of the yolk (Brooks *et al.,* 1997; Purves *et al*., 1998; Carnevali *et al*., 1999a; 1999b; 2001) and its cleavage originates polypeptidic components and amino acids that will be used in the early stages of the embryogenesis of the developing embryo (Brooks *et al.,* 1997). Pelagic eggs of marine teleosts undergo a second period of proteolysis during oocyte maturation that can partly generate the osmotic gradient required for water uptake in the hydration phase (Carnevali *et al*., 1999). In *S aurata*, cathepsin L was found to be the responsible for this cleavage (Carnevali *et al.,* 2001; 2003).

According to Campos (1998) the transamination is a fundamental reaction in the amino acids metabolism. In this reaction a reversible transfer of the N-terminal occurs, from one amino acid to α-ketoacid. Aspartate aminotransferase (GOT), beyond being a cytoplasmatic enzyme which is released during cell damage (Lahnsteiner, 2000), catalyses the reaction:

L-glutamic acid + oxaloacetic ↔ α-ketoglutaric acid + L-aspartic acid → glutaric acid → $NH_3$ (Campos, 1998)

This enzyme has been used in several species such as *O. mykiss* (Lahnsteiner, 2000), *C.s carpio, H. molitrix, Cte. idella* and *Chal. chalcoides* (Lahnsteiner *et al.,* 2001).

Lahnsteiner & Patarnello (2004a;b) report the use of acid phosphatase activity and amino acids concentration as potential indicators for egg quality since they were correlated with the percentages of pre and post-hatch survival in *S. aurata (*Lahnsteiner & Patarnello, 2004a;b) and in *D. puntazzo* (Lahnsteiner & Patarnello, 2004b).

### 3.4. Molecular parameters

In recent years molecular tools have been used to identify the egg quality. Carnevali *et al.* (1999) have demonstrated that for *S. aurata* there were differences between the messenger RNA (mRNA) transcripts for cathepsin D, suggesting a different expression in nonfloating eggs. Bonnet *et al*. (2007a) have used complementar DNA (cDNA) microarrays to analyze the egg transcriptome after natural and controlled ovulations and its relationship with the eggs developmental potential. In their work, the total abundance of prohibitin 2 was negatively correlated with the eggs developmental potential.

Apoptosis is a mechanism of programmed cell death which can be initiated by a wide range of physiological stimuli. In this mechanism is involved the activation of a family of proteinases, called caspases, that targets a nuclear enzyme, the poly-(ADP-ribose)-polymerase (Carnevali *et al*., 2003). The detection of apoptotic signals as also been used as a potential biomarker for egg quality in *S. aurata*. Levels of high alkaline phosphatase were found in embryos from batches with lower hatching and higher larval mortality in *D. dentex* (Giménez *et al.,* 2006).

## 4. Why is the Senegalese sole important to aquaculture?

In the last decades, the aquaculture industry has expanded vastly in Europe. This growth may be attributed to the cultivation of few marine species that already show some signs of market saturation (Dinis *et al*., 1999; Imsland *et al.,* 2003) and as a consequence of the worldwide decline in the fisheries stocks (Naylor *et al.,* 2000).

Given the present saturation of the market for aquaculture products new species are required to be introduced in the market, not only to generate new market opportunities, but also to ease the referred market saturation (Dinis *et al.,* 1999). These species should have a biological cycle that can be reproduced with the breeding techniques currently used (Imsland *et al.,* 2003).

Investigations since the early eighties (as reported in Dinis, 1986; Dinis *et al.,* 1999) have pointed out the Senegalese sole (*Solea senegalensis*, Kaup 1858) as one of the species with potential for fish farming in Europe (Dinis & Reis, 1995; Dinis *et al*., 1999; Imsland *et al*., 2003). *S. senegalensis* is a member of the Soleidae Family with a vast distribution in Atlantic waters (from the Bay of Biscay, in the north, to Senegal, in the south) and also in the Mediterranean Sea. The Senegalese sole can be found from the shore line until 80m deep (Dinis, 1986) and is well adapted to warm climates (Dinis *et al*., 1999). This species has a high market price (Dinis & Reis, 1995), refined flavor and has been commonly exploited in semi-intensive polyculture with *Sparus aurata* and *Dicentrarchus labrax*, in the southern coast of Portugal and Spain, since the early 1980s, in earthen ponds (Dinis, 1986; Dinis *et al*., 1999).

The reproduction in captivity of *S. senegalensis* has been studied in Portugal and Spain since almost thirty years ago (as reviewed in Dinis *et al.,* 1999; Imsland *et al.,* 2003). However due to bad results in weaning and growth of juveniles until a marketable size the importance and potential of this species to aquaculture decreased, with a subsequent decrease in research studies (Dinis *et al.,* 1999). Only recently some problems related with aspects of technology and disease, such as the black patch necrosis, have been solved (Imsland *et al.,* 2003), thus renewing the interest in this species for aquaculture. Nevertheless, some aspects related with egg quality and reproduction, in this species, are still a bottleneck in the worldwide commercialization of *S. senegalensis*.

## 5. Justification of the present work

Several studies support the idea that one constrain to the expansion of the aquaculture of marine species is the production of good quality eggs (Kjørsvik *et al.,* 1990; Brooks *et al.*, 1997; Kamler, 2005). The production of eggs of good quality is important to the aquaculture industry in order to optimize the production process, allowing a prediction of the larval rearing performances (Devauchelle & Coves, 1988).

Bromage *et al.* (1994) refer that one of the biggest obstacles in the study of egg quality for different fish species is the difficulty in establishing quality parameters that imply good results. Such test parameters should be easy to reproduce without requiring sophisticated laboratory techniques and should be of quick applicability to obtain results as soon as possible in the development of the eggs. This way, the early prediction of failure, or success, in larval rearing based on egg properties, could avoid the unnecessary occupation of personnel and incubation facilities in the hatcheries (Bromage *et al*., 1994; Kamler, 2005). Nevertheless, there is still little agreement about the methods for assessment of egg quality in marine fish (Brooks *et al*., 1997). Investigations in egg quality parameters should also try to involve comparative tests that already showed results in other species whenever possible, like the fertilization rate, buoyancy of pelagic eggs, morphological features and hatching and survival rates (Kjørsvik *et al*., 1990).

As already stated the Senegalese sole has been pointed as one of the species with potential for fish farming in Europe (Dinis & Reis, 1995; Dinis *et al*., 1999). Up to now no previous work found in literature applied the methodology of enzyme activity and metabolite concentration assays described in Lahnsteiner & Patarnello (2004a) to *S. senegalensis* eggs. The knowledge of the metabolic pathways progress, nutrients requirements in embryogenesis may bring new light in the definition of good egg quality for this species and may allow the leap will permit high production of *S. senegalensis* in aquaculture.

## 6. Aim of the present work

The present study aims to find biomarkers of egg quality in *Solea senegalensis* eggs using information related to morphologic and physiological status of the eggs and some selected enzymatic and metabolic parameters related to the eggs energetic status, tricarboxilic acid cycle, carbohydrates metabolism, catabolism of amino acids and autolytic processes, both in viable and nonviable eggs. The selected enzymatic and metabolic parameters have already proven to be biomarkers for egg quality in others species (Lahnsteiner & Patarnello, 2004a; b; Giménez *et al.,* 2006).

# CHAPTER II

# MATERIAL & METHODS

## 1. Broodstock management

*Solea senegalensis* broodstock was kept in captivity at the Ramalhete Experimental Station, of the University of Algarve. The broodstock was maintained in four circular fiber glass tanks (figure 2.1), with a flat bottom, mildly covered with sand to prevent the incrustation of fouling organisms, such as barnacles. These tanks had a water column of 50cm in a 2.5$m^3$ capacity and operated in an open circuit, with a water flow rate of 360 $l \cdot hour^{-1}$. The broodstock was kept at salinity of approximately of 35‰, under the natural photoperiod conditions and the natural variations of water temperature for the geographic area in question. Physical parameters such as water temperature (WT), salinity and oxygen levels were measured daily and registered.

**Figure II.1** – *Solea senegalensis* husbandry tanks at Ramalhete station.

The fish were fed *ad libitum* with squid and mussels, following the feeding plan, as described in table II.1 bellow. This feeding plan was generally the same for each broodstock tank, with the addition of a vitamin complex to the amount of mussels given, from February onward.

**Table II.1** – Weekly feeding plan for *S. senegalensis* brood stock

| Week day | Type of food |
|---|---|
| Monday | Squid |
| Tuesday | Squid |
| Wednesday | Mussel |
| Thursday | Squid |
| Friday | Mussel |
| Saturday | Squid |
| Sunday | No food distributed |

## 2. Egg collection

The eggs were laid in the natural breeding period, occurring between late March and June. The 39 samples were collected between 2006/03/29 and 2006/06/25. The eggs were collected through an air-lift surface collector inside the tank (figure II.2), and another external collector placed outside the tank captured the eggs that escaped the interior egg collector (figure not shown). The eggs were then weighted and sampled for measurements and sorted by buoyancy in salt water, with salinity ≥ 35‰, for 15min. The eggs with positive buoyancy, *i.e.,* that remained at the surface, were then classified as "viable eggs" and the eggs that sank were classified as "nonviable eggs". After this separation the eggs were sampled as described below.

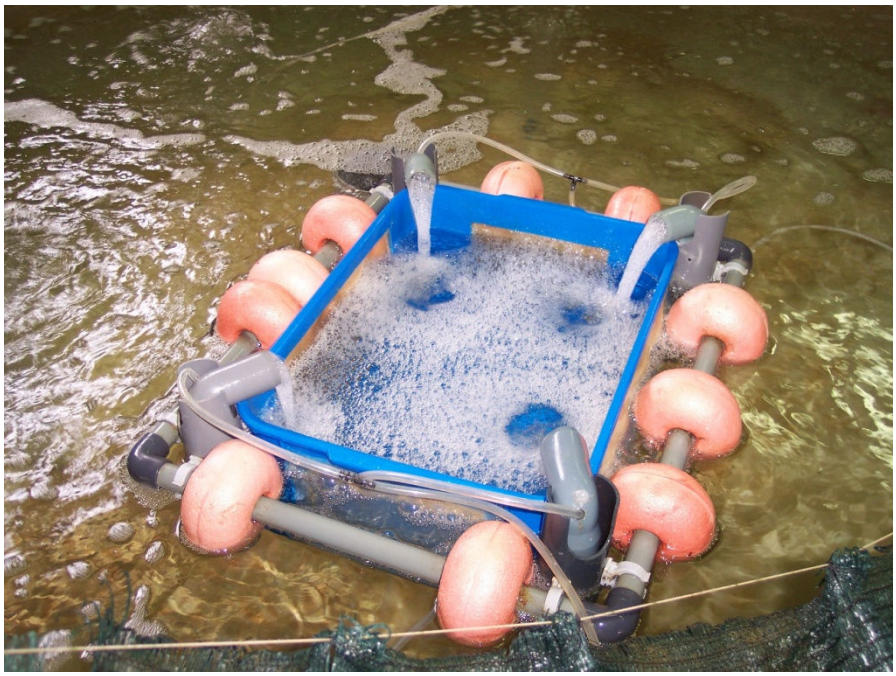

**Figure II.2 -** Air-lift surface collector used in broodstock tanks.

## 3. Egg Sampling

### 3.1. Diameter measurements and fertility

Prior sorting the eggs by their buoyancy, morphological parameters were measured and registered. The diameter of 20 eggs, from each of the floating and non-floating batch, was measured in a graduated binocular stereomicroscope. While measuring the diameter, the eggs were also checked for fertilization (existence of embryo, developmental stage, egg opacity) in order to register the percentage of fertilization (FP), according to the expression bellow:

**FP (%) = (Nr. Eggs fecundated/ total of eggs analyzed) x 100**

This procedure was performed in triplicate for each batch of eggs.

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### 3.2. Incubation and hatching percentage

Thirty floating eggs were incubated in small incubators design for the purpose as shown in figure II.3.

**Figure II.3.** Structure used to incubate the eggs in the present study

These structures were composed by modified falcon tubes with 150µm nylon mesh at the bottom to allow water and oxygen exchange with the surrounding medium. Buoyancy was given by a styrofoam structure attached to the tubes. During incubation period (approximately 48h), the eggs remained in the same conditions of the broodstock, and in 3 replicates. Hatching percentage (HP) was calculated according to:

**HP (%) = (Nr. of larvae hatched/ Total egg Nr incubated) x 100 %**

Eggs were transported to the laboratory and were washed with distilled water, in a 150µm net. One hundred and fifty gram of eggs were weighted and stored in 1.5ml eppendorf tubes, with one of 2 solutions; eggs used for enzyme activity tests were stored in 600µl tris buffer solution (0,121g of tris/ 10ml of DDW, pH set to 7.5 with NaOH/ HCl), eggs used for the metabolites tests were preserved in 600µl perchloric acid solution (3mol/l). This procedure was done in triplicate both for the viable and the nonviable eggs. Immediately after weighting each eppendorf tube was stored at -80ºC.

**4. Biochemical quality assays**

The present study investigated some selected metabolic parameters in viable and nonviable eggs of the Senegalese sole (*Solea senegalensis*). Samples collection and preparation were performed at the facilities of the University of Algarve, while biochemical analysis was performed both at the University of Algarve and at the Institute for Zoology, University of Salzburg, Austria.

Egg energy status was determined by assaying adenylate kinase activity and the energetic compound, ATP). Malate dehydrogenase was investigated as tricarboxylic acid cycle, because of their part in the membrane-driven ion transport for oxidative phosphorylation. Carbohydrates (total levels of carbohydrates, monosaccharides) were analyzed as well as the enzymes related to their metabolism (glycolysis: pyruvate kinase; gluconeogenesis: glucose-6-phosphate dehydrogenase; pentose phosphate way: transaldolase). Enzymes related to autolytic processes (acid and alkaline phosphatases) and enzymes involved in catabolism of amino acids (aspartate aminotransferase: transamination) were studied.

These enzymes and metabolites were chosen according to the results published for *Sparus aurata* (Lahnsteiner & Patarnello, 2004a; b) and for *Puntazzo (Diplodus) puntazzo* (Lahnsteiner & Patarnello, 2004b) eggs.

### 4.1. Sample preparation

The eggs kept at -80º C were thawed on ice and maintained there during homogenization. The samples were homogenized in a potter and centrifuged at 800g, 4º C during 10min (SIGMA 4K 15). After centrifugation the supernatant was pipetted into eppendorf tubes and was stored at -80º C.

The handling of the samples during homogenization was the same both for enzymes and metabolites except for the use of pipettes. For metabolites glass Pasteur pipettes must be used because the acid that preserves the samples may corrode other pipette tips.

### Chemicals

All chemicals were from reagent grade or higher. Unless indicated, they were purchase in Sigma-Aldrich, Austria and Portugal.

### 4.2. Protein determination (Lowry *et al*., 1951)

The amount of total protein present in each sample was determined to establish the total amount of biological material present in the samples.

A solution was prepared by dissolving 0.5g NaOH in 50mlDDW; after total dissolution of the NaOH, 5g of sodium carbonate ($Na_2CO_3$). This was named solution A for simplicity. The working solution for this assay consisted in a mix of 45ml of solution A, 2.25ml of solution B (1g $CuSO_4$/ 100ml DDW), 2.25ml of solution C (2g of potassium tartarate/100ml of DDW) and 43.5ml of DDW. A Folin reagent solution (FRS) was prepared by mixing 1ml of stock Folin reagent in 10ml of DDW.

This assay was carried out by mixing in a cuvette (C5416; SIGMA; 10x4x45mm) 20µl of sample and 300µl of the working solution. This was left to incubate for 15 minutes at room temperature. After this incubation period 480µl of FRS were added to the cuvette

and immediately thoroughly mixed in order to obtain a reliable color reaction. Another incubation period followed, from 15-30 minutes, until the color developed totally. Then the absorvance of each sample was measured in a spectrophotometer (Hitachi U-2000) at 600nm of wavelength.

In order to determine the concentration of the protein a BSA (bovine serum albumin) standard curve was prepared. To make this curve, a standard solution of BSA (10mg/ml in DDW) was prepared, as well as its dilution series, summarized in the table bellow (table II.2).

**Table II.2** – General proportions of the dilution series prepared for most of the standard solutions used in the present work

| Dilution | Volume of stock solution (µl) | Volume of DDW (µl) |
|---|---|---|
| **0/1** | 0 | 100 |
| **1/10** | 50 | 500 |
| **1/8** | 50 | 400 |
| **1/6** | 50 | 300 |
| **¼** | 50 | 200 |
| **½** | 50 | 100 |
| **1/1** | 50 | 50 |
| **1/0** | 100 | 0 |

### 4.3. Enzymes

The set of assays was designed to determine the presence, or the activity of the following enzymes: transaldolase, pyruvate kinase, acid and alkaline phosphatases, malate dehydrogenase, glucose-6-phosphatase, glucose-6-phosphate dehydrogenase, and aspartate aminotransferase and adenylate kinase. However, the assays of transaldolase, succinate dehydrogenase, pyruvate kinase, and adenylate kinase were sent to appendix II since no enzyme activity was shown. The unit used to express the enzyme activity is µmol/min/l. This unit will be referred as U/l, where U is µmol/min.

#### 4.3.1. Acid and Alkaline Phosphatase (Lahnsteiner & Patarnello, 2004a)

A buffer solution of sodium citrate (2.94g/ 100ml of DDW) was prepared; adjusting pH to 4.5 by adding HCl. Solutions of phosphatase substrate (18mg/ ml of DDW; S0942: SIGMA) and of NaOH (0.4g/ 100ml of DDW) were prepared as well. These solutions were used in the acid phosphatase assay. For the alkaline phosphatase assay 2-amino-2-methyl-1-propanol (A65182: SIGMA) was used as a buffer, and solutions of phosphatase substrate (13.8mg/ ml DDW; S0942: SIGMA) and NaOH (0.2g/ 100ml of DDW) were prepared.

Both assays were performed the same way, adding in a cuvette (C5416: SIGMA) 80.6 µl of substrate, 80.6µl of working solution and 32.3µl of sample. This was then left to incubate for 60min, at room temperature. After the incubation period, 806.5µl of the corresponding NaOH solution was added. A yellowish color developed and the absorvance was measured. A standard curve was made, using p-nitrophenyl (250µl/ 50ml of a NaOH solution; 0.08g of NaOH/100ml of DDW), and a dilution series as summarized on table II.3 below. This allowed the calculation of the amount of enzyme activity present in the analyzed samples.

**Table II.3** – p-nitrophenyl standard solution (StaSol.) dilution series used to perform the standard curve

| Dilution | $V_{StaSol}$ (µl) | $V_{NaOH}$ (µl) |
|---|---|---|
| **1** | **100** | **1000** |
| **2** | **200** | **900** |
| **3** | **400** | **700** |
| **4** | **600** | **500** |
| **5** | **800** | **300** |
| **6** | **1000** | **100** |

### 4.3.2. Malate Dehydrogenase (Lahnsteiner & Patarnello, 2004a)

A buffer was prepared by mixing 0.95ml diethanolamine (398179: SIGMA) in 99ml DDW. The pH of this solution was adjusted to 9.2, by adding 0.1M NaOH. Solutions of NAD; 20mg/ 17ml buffer; N1511: SIGMA) and malic acid (117mg/ 3ml buffer) were prepared. The assay was performed by adding, per sample, in a cuvette 560µl NAD solution, 50µl DDW and 20µl of sample. After an incubation period of 30min, 70µl malic acid solution was added. After 15 seconds, Abs1 was measured at the wavelength of 340nm. After measuring, the samples were incubated for 5min at room temperature. Then Abs2 was measured in the same conditions as above. The activity of the enzyme

was determined by an increase in absorvance, and quantified in the same way as described below.

$$\mathbf{conc} = \frac{\mathbf{\Delta A \times V \times 1000}}{\mathbf{\varepsilon \times d \times \Delta t \times v}}$$

In this expression, **ΔA** is the change in absorbance in the measured time interval, **V** is total assay volume (µl), **ε** is the absorption coefficient for NADH/NADPH, which is 6.3 x $10^3$**,** **Δt** is the measured time interval in minutes, **v** is the sample volume (µl) and **d** is distance of the light path through the cuvette (=1cm). The units of the concentration obtained are µmol/min/l (=U/l).

### 4.3.3. Glucose-6-Phosphatase (Lahnsteiner & Patarnello, 2004a)

This assay is very easy to contaminate due to the presence of phosphates in tap water (Lahnsteiner, F., 2007, *pers. comm.*). To avoid phosphate contamination, all glass material used was washed in 10% HCl solution (10ml of HCl/ 100ml DDW) and rinsed 3 times in DDW.

A dimethylarsinic acid buffer was prepared by dissolving 0.69g dimethylarsinic acid in 50ml DDW. The pH of this solution was adjusted to 6.5 by adding 0.1M NaOH. The working solution for this assay consisted in a glucose-6-phosphatase solution (G6Pase; 3.3mg/ 1ml buffer). The phosphate determination reagent was a mix of solutions: 1/5 ascorbic acid solution (10g/100ml DDW), 1/5 ammonium molybdate solution (2.5g/100ml DDW), 1/5 diluted sulphuric acid solution (18ml concentrated $H_2SO_4$/90ml DDW) and 2/5 distilled de-ionized water. This reagent was used within 30 minutes of being prepared, because otherwise it would become unstable (Lahnsteiner, F., 2007, *pers. comm.*).

In a cuvette 100µl working solution and 20µl sample were mixed and left to incubate for 30min. After ending the incubation time, 700µl phosphate determination reagent was added and the absorvance measured at 700nm.

In order to determine the concentration of the G6Pase, a phosphate standard curve was prepared. To make this curve, a standard solution $K_2HPO_4$ (10mg/100ml DDW) was prepared, as well as its dilution series, summarized in the table II.2.

In a cuvette 110µl standards were mixed with 700µl working solution and incubated until color developed. After this, the absorvance of the standards was measured at 700nm. Standards and samples were measured at the same time.

### 4.3.4. Glucose-6-Phosphate Dehydrogenase (Bergmeyer, 1985)

A Tris buffer was prepared by dissolving 1.2g Tris (252859: SIGMA) in 20ml DDW. The pH of this solution was adjusted to 7.5, by adding NaOH (0.1M). Solutions of $MgCl_2$ (1.28mg/ 100ml DDW), nicotinamide adenine dinucleotide phosphate (NADP; 10mg/ 3.5ml of DDW; N5755: SIGMA), glucose-6-phosphate (G6P, G7772: SIGMA; 32mg/ 3.5ml DDW) and a maleimide solution (12958: SIGMA; 16mg/ 3.5ml DDW) were prepared. Working solution was prepared by mixing the following solutions in the proportion calculated according to the relation described below, for the total of samples to be analyzed:

For one sample mix:

- 330µl of DDW;
- 70µl Tris buffer;
- 70µl NADP solution;
- 70µl G6P solution
- 70µl maleimide solution

In a cuvette were mixed 680µl working solution and 20µl sample. After 15 seconds, Abs1 was measured at 340nm wavelength. After measuring, the samples were incubated 20min because sometimes the activity of the enzyme may be low, at room temperature, and Abs2 was measured in the same conditions as above.
The activity of the enzyme was determined by an increase in absorvance (*i.e.* Abs2-Abs1>0) and was quantified in the same way as described in 4.3.2.

### 4.3.5. Aspartate Aminotransferase (GOT) (Bergmeyer, 1985)

The working solution of this assay was a mixture of 10ml aspartic acid tris solution, 5µl malate dehydrogenase (or malic enzyme, MDH; M9004: SIGMA), 5µl lactic dehydrogenase (LDH; L2625: SIGMA), 0.25ml pyridoxal-5-phosphate (144mg/ 1ml DDW; P9255: SIGMA) and 1mg NADH (N4505: SIGMA). The aspartic acid tris solution was prepared by dissolving 3.9g of aspartic acid and 1.2g Tris in 80ml DDW. The pH of this solution was adjusted to 7.8 by adding NaOH (0.1M) and then the solution was filled up to 100ml with DDW.

A solution of ketoglutaric acid was prepared by dissolving 2.1g of ketoglutaric acid and 1.21g Tris in 80ml DDW. Concentrated NaOH was added until all was dissolved. The pH was then adjusted to 7.8 by addition of HCl (0.1M).

In a cuvette 666µl working solution, 46µl DDW and 20µl sample were added. After incubation for 15 minutes, 66µl ketoglutaric solution was added. After 15 seconds the Abs1 was measured at 340nm wavelength. The samples were incubated for 1-5min, at room temperature, and Abs2 was measured in the same conditions as above. The activity of the enzyme was determined by a decrease in absorvances and quantified in the way described in 4.3.2.

## 4.4. Metabolites

The set of tests was designed to determine if the metabolites hexose, 6-deoxyhexose, heptose, glucose-6-phosphate, fructose-6-phosphate, ATP, total carbohydrates, sialic acids, ketoses and free and bound ribose were present in the eggs of *S. senegalensis*. Metabolite concentration is expressed in µmol/l. The assays used in the progress of this work are described bellow.

### 4.4.1. Hexose, 6-Deoxyhexose, Heptose Assay (Lahnsteiner & Patarnello, 2004a)

This assay required two solutions: one of diluted sulphuric acid and another of cystein. For the preparation of the first solution, 8ml DDW were added to 48ml of sulphuric acid, and for the preparation of the second solution 0.15g cystein were diluted in 5ml DDW. Since the cystein readily dissolved in water, there was no need to add NaOH or HCl to

help dissolution. The assay was performed in 2ml glass tubes; otherwise the plastic cuvettes would have been destroyed. In these tubes were mixed 40µl sample and 945µl of diluted sulphuric acid, that then were shaken, covered with tin foil and incubated at 100º C, for 20min. After the glass tubes were cooled to room temperature, 18µl cystein were added and left overnight to incubate in the dark. On the next day the samples were poured into the cuvettes and the absorvance of each cuvette was measured in each of the following wavelengths: 380nm, 396nm, 415nm, 427nm, 500nm and 545nm.

To establish the amounts of hexoses, heptoses and 6-deoxyhexoses, three standards were used. Glucose (G8270: SIGMA) was used as a standard for hexoses, rhamnose (R3875: SIGMA) was the standard for 6-deoxyhexoses and glucoheptose (G4375: SIGMA) was the standard for heptoses. Each of the sugars was diluted in DDW (5mg of each sugar/ 1ml DDW) and a dilution series was prepared, following the proportions described in table II.2 and was handled in the same way as the samples. The standard curve for glucose was calculated using the difference between absorvances measured at 415nm and 380nm. For rhamnose it was used the difference between absorvances measured at 396nm and 427nm, and for glucoheptose the difference between absorvances measured at 500nm and 545nm.

**4.4.2. ATP (Bergmeyer, 1985)**

A triethanolamine buffer was prepared by diluting 5,6g/ 50ml of distilled and de-ionized water. The pH of this solution was adjusted to 7.6. Solutions of magnesium chloride ($MgCl_2$; 1 g/ 50ml DDW), NADP (N5755: SIGMA; 10mg/ 1.4 ml DDW) and G6PDH (G7879: SIGMA; 400 units in 400µl of a mixture of buffer and DDW, in 1:1 proportion). Working solution was prepared per sample, by adding in a cuvette 500µl buffer, 23.3µl NADP, 46.7µl $MgCl_2$ and 5µl G6PDH. To this 25µl sample was added and left to incubate for 10 minutes at room temperature. After the incubation, 70µl glucose (4,95g/ 50ml DDW) solution was added, and 30 seconds later the first absorvance (Abs1) was measured at 340nm. Then 5µl of hexokinase solution (10µl in 400µl of a mixture of buffer and DDW, in 1:1 proportion) was added. After the addition of the hexokinase solution to samples, the absorvance increases. Samples were checked in 5 minutes intervals to establish when the increase in absorvance ceased (after 20-40min, depending of the samples). After this, absorvance 2 (Abs2) was measured at

340nm. The presence of ATP was determined through the difference between Abs2 and Abs1.

### 4.4.3. Total Free Carbohydrates Assay (Lahnsteiner & Patarnello, 2004a)

This assay was performed in 2 ml glass tubes because otherwise the plastic cuvettes d have been destroyed given the extreme causticity of the solutions used (concentrated sulphuric acid and a phenol solution).

The phenol used in this assay was solid at room, so it was warmed, in the bottle, to 60º C until it dissolved. Then, in a pre-warmed glass, 8g phenol was weighted quickly, and immediately 2ml DDW, at 45º C, were added. The solution was then shaken and mixed well. This turned the phenol stable and liquid at room temperature. The phenol reagent was prepared by dissolving 5g phenol stable solution in 100ml DDW.

In a 2ml glass tube 140µl phenol reagent, 90µl DDW and 50µl sample were mixed. After 15 seconds, 700µl sulphuric acid ($H_2SO_4$; stock solution) were added to each glass tube, which were shaken to make the assay reliable. This was incubated for 60min, at room temperature, until a brown-reddish color developed.

The content of each glass was poured into a cuvette and the absorvance was measured at 500nm. The activity of the enzyme was determined by a glucose standard curve.

To make the glucose standard curve, a standard solution of glucose (5mg/ ml DDW) was prepared and a dilution series was prepared as well, as described in table II.2. The absorvance of the standard and its dilutions was measured in the same conditions as the samples.

### 4.4.4. Sialic Acids Assay, (Fukuda and Kobata, 1993)

This assay only required the preparation of one solution: the bial reagent. This reagent was prepared by dissolving 0.1g orcinol in 40.7ml HCl (stock solution, 37%) and then was added 2ml a 1% ferric (ion) chloride ($FeCl_3$; 20mg/ 2ml DDW).

In a 2ml glass tube, 160µl water, 40µl sample and 200µl bial reagent were mixed and incubated at 90º C, for 20 minutes. The samples were then cooled to room temperature. After cooling, 1500µl of 1-pentanol(138975: SIGMA) were added and the samples were well mixed. Two phases formed; on the bottom, a layer of water and above this, a layer of 1-pentanol. The amylalchol was then collected to eppendorf tubes and centrifuged at 250 g, 4º C for 4min. All water droplets were removed in order to avoid turbidity in the samples. The samples were then transferred to a cuvette, one by one, and their absorvance measured at 570nm.

To determine the amount of sialic acids present in the samples an n-acetylneuraminic acid (A2388; SIGMA 1mg/100µl DDW) standard curve was elaborated. To produce such a curve the n-acetyl neuraminic acid standard solution was diluted in a dilution series as described in table II.3. All standards and dilutions were treated in the same conditions as the samples.

### 4.4.5. Determination of Ketoses with a Phenol-Acetone-Boric Reagent (PABR; Chaplin & Kennedy, 1986)

This assay was made in glass tubes until samples were transferred into cuvettes for measurement of absorvance, due to the same reasons described before.

The phenol-acetone-boric reagent (PABR) solution was prepared in constant mixing of 50ml phenol solution (5g/45ml DDW), 2ml acetone and 50ml boric acid solution (4g/50ml DDW). The phenol solution was prepared in the same way as described in 4.4.3.

In 2ml glass tubes, 40µl sample, 250µl PABR and 700µl $H_2SO_4$ (stock solution) were mixed, in this precise order. The samples were incubated at 37º C for 60min. After the incubation, the samples were poured, one by one, in cuvettes, and the absorvance measured at 568nm.

To determine the amount of ketoses, a fructose solution (5mg/ml DDW) was prepared, as well as a dilution series as described in table II.3, and were treated in the same conditions as the samples.

### 4.4.6. Free and Bound Ribose assay (Hotzhauer, 1988)

This assay was also performed in glass tubes. The same solutions were used to determine both the free and the bound ribose, being the protocols very similar, differing only in the incubation time and the way of mixing the reagents.

The reagents used were: a perchloric acid solution, a ferric chloride solution and the orcinol reagent. The perchloric acid solution was prepared by adding 4.6ml perchloric acid to 44.3ml DDW, in the described order. The addition of acid was very slow because the reaction produced heat. To prepare the ferric chloride solution 20mg $FeCl_3$ were added to 100ml HCl. The orcinol reagent was prepared by dissolving 0.5g orcinol in 5ml 96% ethanol.

To determine the amount of bound ribose, in 2ml glass tubes, 490µl perchloric acid solution and 20µl sample were mixed. The tubes were covered by a lid and incubated at 80º C, for 25min. Then, 465µl $FeCl_3$ solution was added and actively mixed and, after this, 30µl orcinol reagent was added. The samples were then incubated at 80º C, for 30 minutes.

To determine the amount of free ribose, 490µl perchloric acid solution, 465µl $FeCl_3$ solution and 20µl sample were mixed in 2ml glass tubes. The tubes were covered, well mixed and 30µl orcinol reagent was added. The samples were then well shaken and incubated at 80º C, for 40min.

After the last incubation, the content of each glass tube was poured into the cuvette and the absorvance measured at 660nm.

To quantify the amount of both types of ribose, a ribose (R4377; SIGMA) standard solution (5mg/ ml DDW), and its dilution series were prepared (as described in table

II.3) and processed in the same way as the samples, for both kinds of ribose that could be present in the samples.

**5. Statistical Analysis**

The eggs used for this project were from the first breeding period for *Solea senegalensis*, which occurred from March to June. From sample 7 no data in viability, fecundation, egg diameter and hatching was collected. This sample appears in the figure below to illustrate only the temporal frame, in the register of biological parameters.

The regular treatment of averages and standard deviation was made in the Excel worksheet where the data was registered. After that, all statistic treatment was made using the SPSS program.

The results obtained in the former assays were tested to establish some statistically significant correlation between the obtained tests results and: a) percentage of viability, b) average egg diameter, c) percentage of fecundation and d) percentage of hatching, with a $\alpha = 0.05$ and $\alpha=0.01$. This correlation analysis failed since the present set of data wasn't in accord with one of the assumptions of the Pearson bivariate correlation: the normality of the distribution of the data. The non-parametric bivariate correlation of Spearman was used using the same $\alpha$ as above. The Receiver-Operating-Characteristic (ROC) Curves procedure of SPSS software was used to separate the samples through a cutoff value. Each cutoff value has a unique pair of sensitivity and specificity. This is used to measure the analytical usefulness of a test. The ROC curve provides a view of the spectrum of sensitivity/specificity pairs, allowing the investigator to examine the test's discriminative power over all the possible cutoff values (Jensen *et al.,* 1996). To the highest pair [sensitivity; specificity] is associated a value, chosen as the cutoff value according to each the sample's test results. This enables the separation of the samples in higher quality viable eggs and lower quality eggs (which contained both nonviable eggs and viable eggs excluded by the cutoff value). Some authors consider the pair composed by the highest sensitivity and by the lowest 1-specificity correspondent (Jensen *et al.,* 1996).

After the selection of the samples through the cutoff value it was checked which samples remained as viable in each assay. Of a total of 10 assays used in the ROC curve test it was chosen the samples that were beyond the cutoff value in, at least, 7 assays. As the goal of this work is the tentative of establishment of correlations between egg biochemical parameters and the eggs morphophysiological parameters Spearman correlations were checked between the assays results of the best sample from the total of viable eggs analyzed and: a) percentage of viability, b) average egg diameter, c) percentage of fecundation and d) percentage of hatching, with an $\alpha = 0.05$ and $\alpha=0.01$.

# CHAPTER III

# RESULTS

## 1. Broodstock

Figure III.1 describes the monthly averages of water temperature, salinity and percentage of dissolved oxygen. It is easily observed that salinity was fairly constant throughout the year. The water temperature ranged between 11.72º and 20.96º C, being the lowest temperature registered in January and the highest in October. The percentage of dissolved oxygen ranged between 94.97 and 97.7, being the lowest value registered in March, and the highest in February. The decrease in the quantity of dissolved oxygen was registered when water temperature started to rise in March was more pronounced than the decrease registered there after.

In the same period of time the amount, and total amount, of food given to the broodstock was registered (figure III.2). In the winter time, December to February, the broodstock consumed less feed than in the period from March to November. It is also noticeable that there are two peaks, in April and in October, where more mollusks were consumed. This corresponded to the two periods when water temperature increased (figure III.1); in March it starts to rise and in October it reaches the highest average.

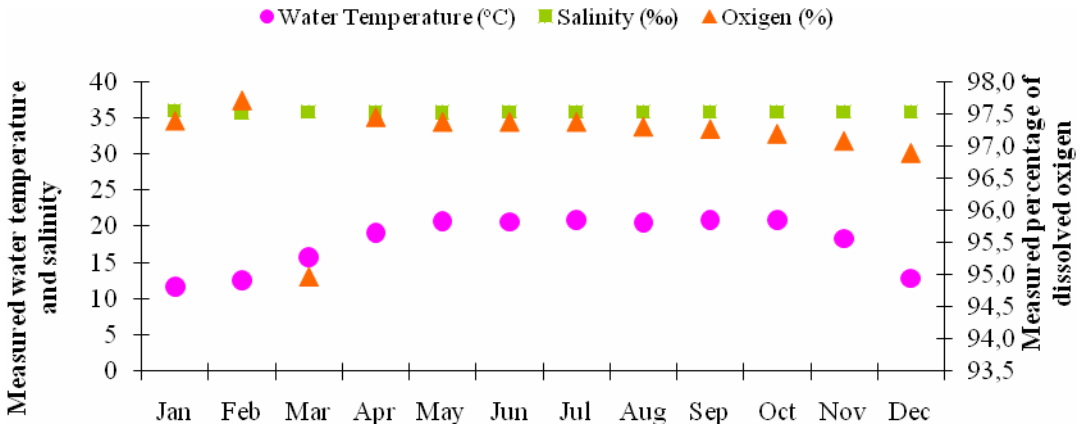


**Figure III.1** – Monthly averages of water temperature (ºC), salinity (‰), and dissolved oxygen (%), registered in the broodstock tank of *Solea senigalensis*, in 2006.

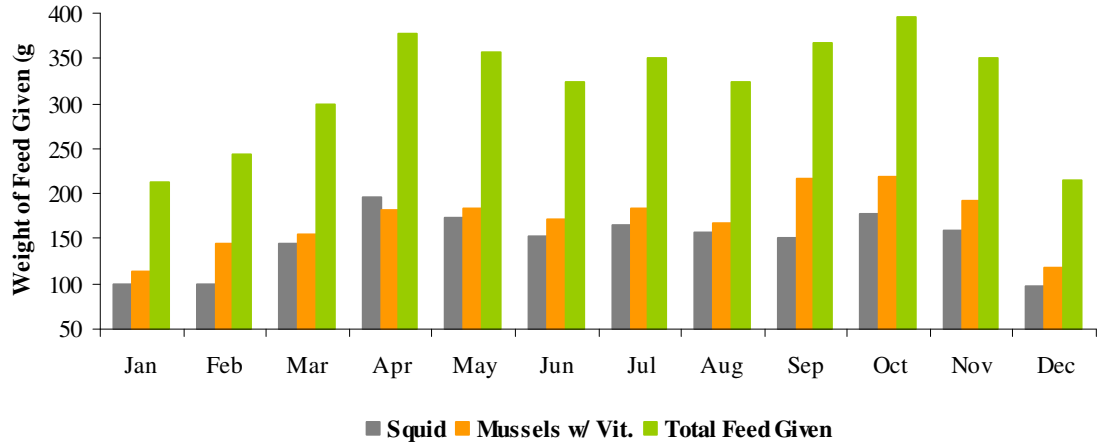


**Figure III.2 –** Feed distributed to the broodstock of *Solea senegalensis*, in 2006. The values presented correspond to a monthly average.

## 2. Morphophisiological parameters of the eggs analyzed

The results showed in figure III.3 correspond to the percentage of viability in each of the egg batches analyzed.

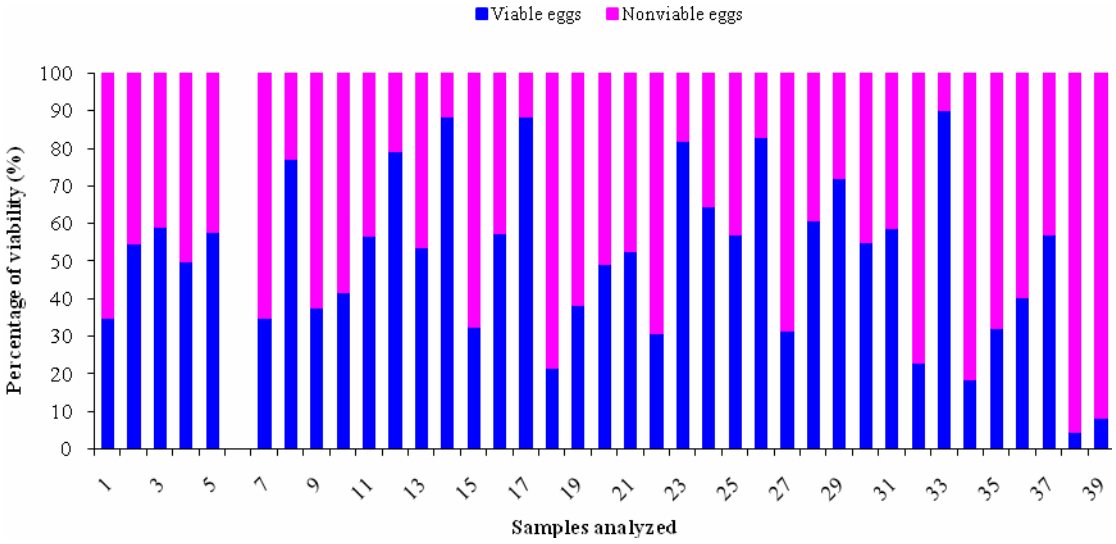


**Figure III.3** – Percentage of viable and nonviable eggs of *Solea senegalensis* harvested during the 2006 breeding period

The average percentage of viable eggs in the sampling period of 2006 was of 50.91%, which is slightly higher than those of nonviable eggs (49.09%). In this breeding season

the percentage of viable eggs varied considerably, from 4.54%, in sample 38, to 90%, in sample 33. It was also noticeable that after a batch with a high percentage of viability followed a decrease in viability in the next batch. After this, an increase in viability occurred until a new peak.

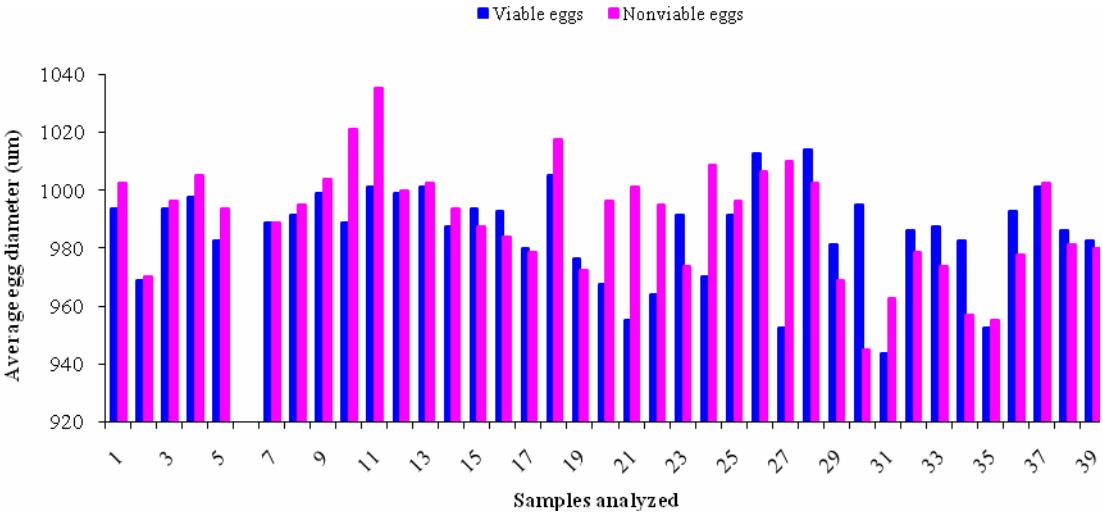


**Figure III.4** – Average diameter of viable and nonviable eggs of *Solea senegalensis* measured during the 2006 breeding period.

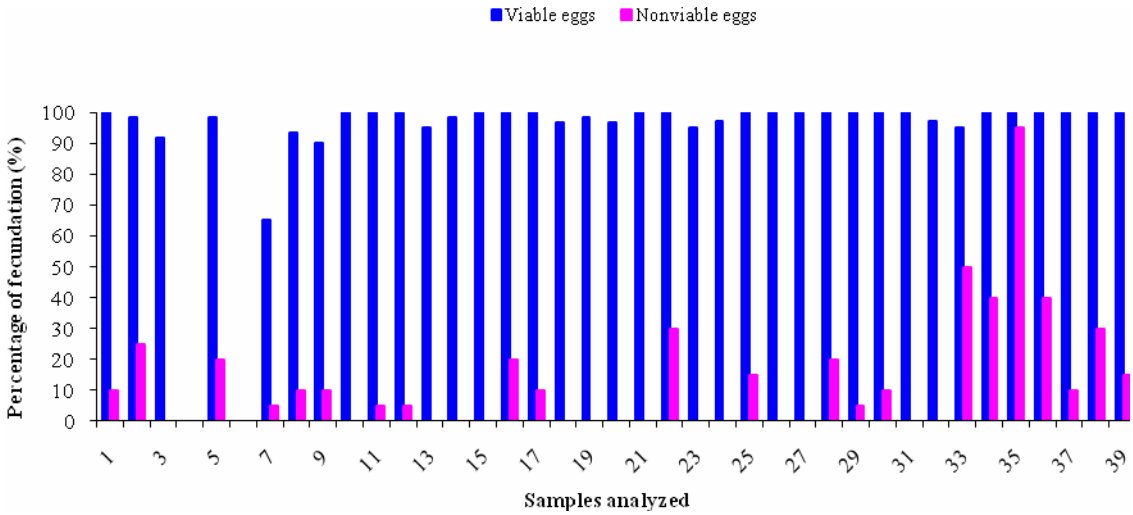


**Figure III.5** – Percentage of fertilization of viable and nonviable eggs of *Solea senegalensis* registered during the 2006 breeding period.

The last two samples, 38 and 39, which are from the end of the breeding period (appendix I), were mainly composed of nonviable eggs (≥ 90%).

Figure III.4 shows the average diameter of both viable and nonviable eggs. The values recorded for both types of eggs demonstrated that the average diameter of eggs varied along the sampling period. Throughout the 2006 breeding season, and for most of the analyzed batches, the nonviable eggs were larger (989.97µm of average diameter). However, this difference had no statistic meaning, as check by the Mann-Whitney test ($z=1.241451$, $\alpha=0.05$). The nonviable eggs have larger variation of sizes (945.0-1021.3µm). The average diameter for viable eggs was of 985.49 µm, and ranged between 952.5 and 1012.5µm. It is possible to see that in samples from 18 onward, which correspond to the end of the first month of the 2006 breeding period (appendix I) there are larger viable eggs, with an overall larger fluctuation in the viable egg diameter.

Figure III.5 demonstrates that the percentage of fertilization in the viable eggs was, in most of the days higher than 80%, being samples 4, 6 and 7 the exceptions.

It is also noteworthy that the nonviable eggs have some fertilized egg. Sample 31 registered the highest percentage of fertility in the nonviable eggs subsample (90%).

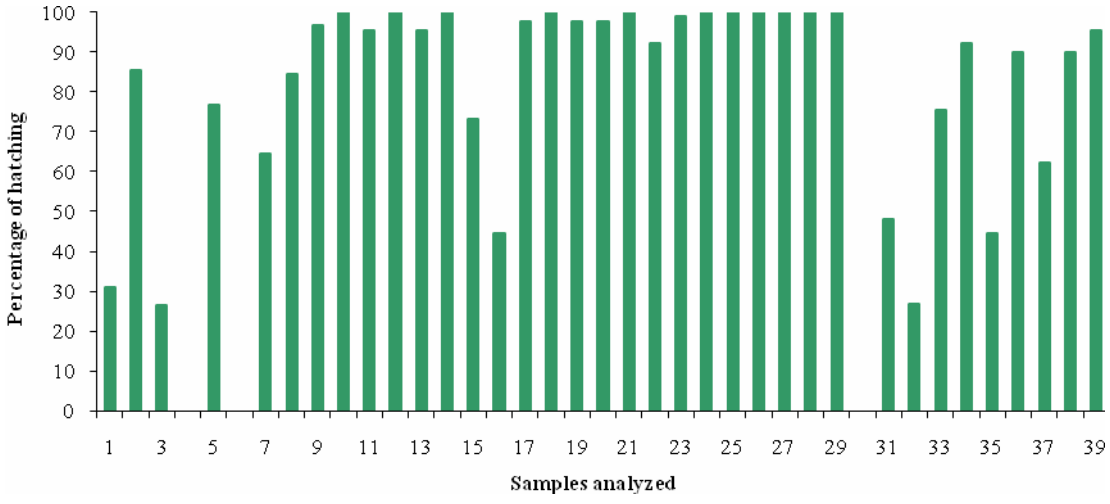


**Figure III.6 –** Percentage of hatching eggs of *Solea senegalensis* registered during the 2006 breeding period.

The percentage of hatching is recorded in figure III.6. In samples from 8 to 14, 16-26, and sample 35, the hatching percentage was above 90%. The rest of the samples showed a larger variation in the hatchings registered.

## 3. Biochemical quality assays

### 3.1. Enzymes

#### 3.1.1. Acid phosphatase

Figure III.7 describes the activity of acid phosphatase in viable and nonviable eggs. Viable eggs had higher values of enzymatic activity. Samples 15, 16, 19, 22 and 34 were exceptions with 0.038, 0.0068, 0.0224, 0.0034 and 0.0156U/l.

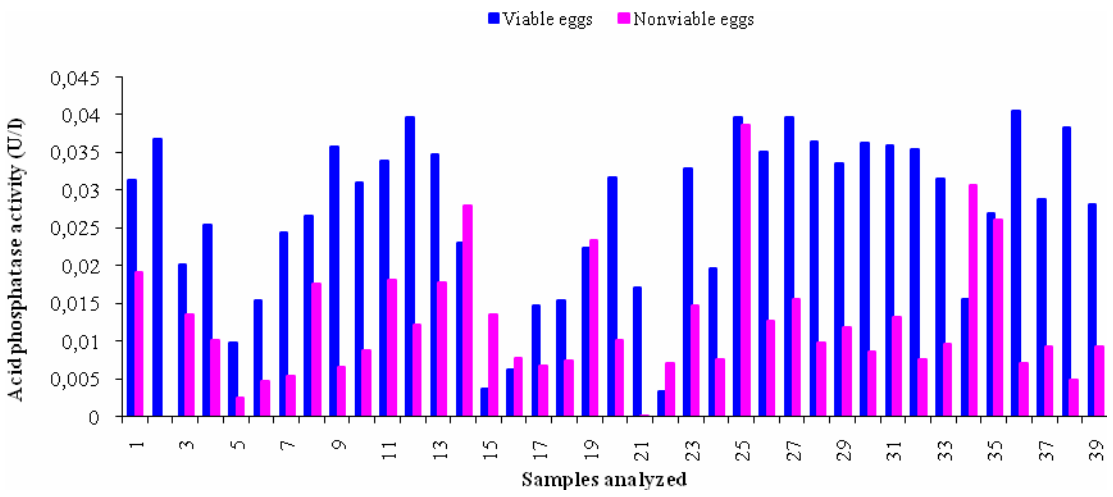


**Figure III.7 –** Acid phosphatase activity determined in viable and nonviable egg of *Solea senegalensis*s.

#### 3.1.2. Alkaline phosphatase

Alkaline phosphatase activity was not reported in all samples (Fig. III.8), in both viable and nonviable eggs. Most the samples analyzed showed results higher in viable eggs that in nonviable eggs, however this is not conclusive. Samples 13, 19 and 20 only had enzyme activity detectable in the nonviable eggs, while samples 3, 27-31, 33 and 38 only show values of enzyme activity for the viable eggs. In the nonviable eggs the

enzyme activity ranged from 0.00004 to 0.00406U/l and in the viable eggs the alkaline phosphatase ranged from 0.00001 to 0.0989U/l.

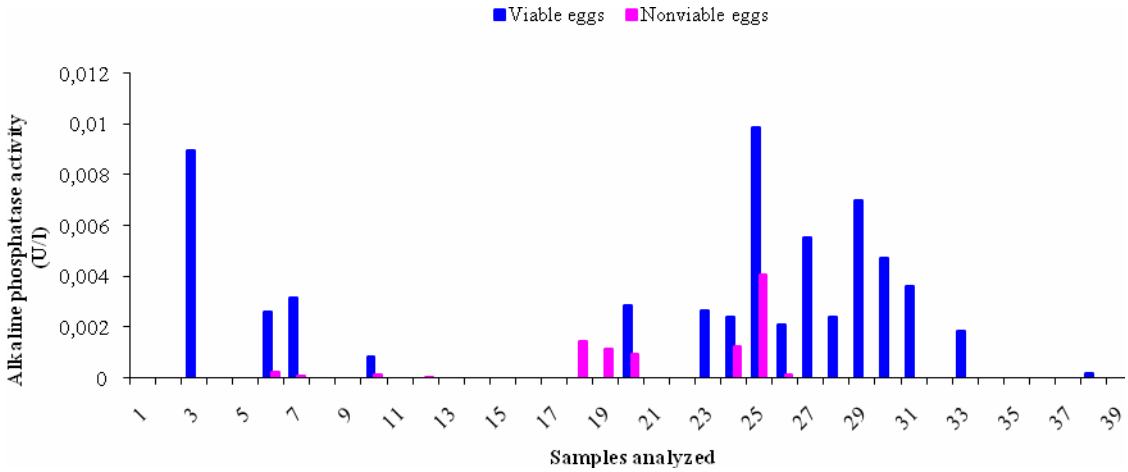


**Figure III.8 –** Alkaline phosphatase activity determined in viable and nonviable eggs of *Solea senegalensis*s.

### 3.1.3. Malate dehydrogenase (MDH)

Samples 13, 14 and 36 (figure III.9) registered the highest values of enzyme activity in viable eggs (0.309, 0.326, 0.372 U/l, respectively), and the values registered were higher than the determined for all the samples tested. In sample 22 the viable eggs expressed less enzyme activity than any other samples, both for viable and nonviable eggs. Nonviable egg sample 23 registered the highest values of MDH activity (0.553U/l), and in most samples analyzed the nonviable eggs showed higher enzymatic activity.

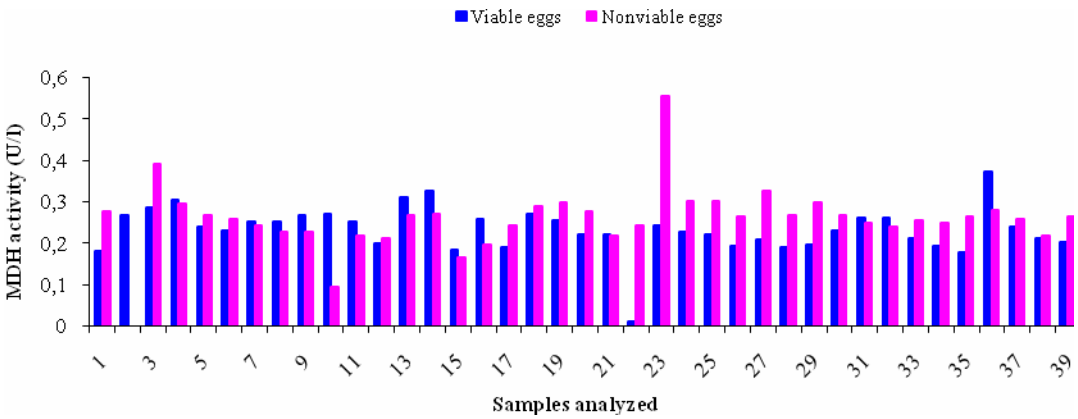


**Figure III.9 –** Malate dehydrogenase activity determined in viable and nonviable eggs of *Solea senegalensis*s.

### 3.1.4. Glucose-6-phosphatase (G6Pase)

This assay provided results for all the samples analyzed. In samples 3, 16, 30, 35 and 36 no results were obtained with nonviable eggs (figure III.10).

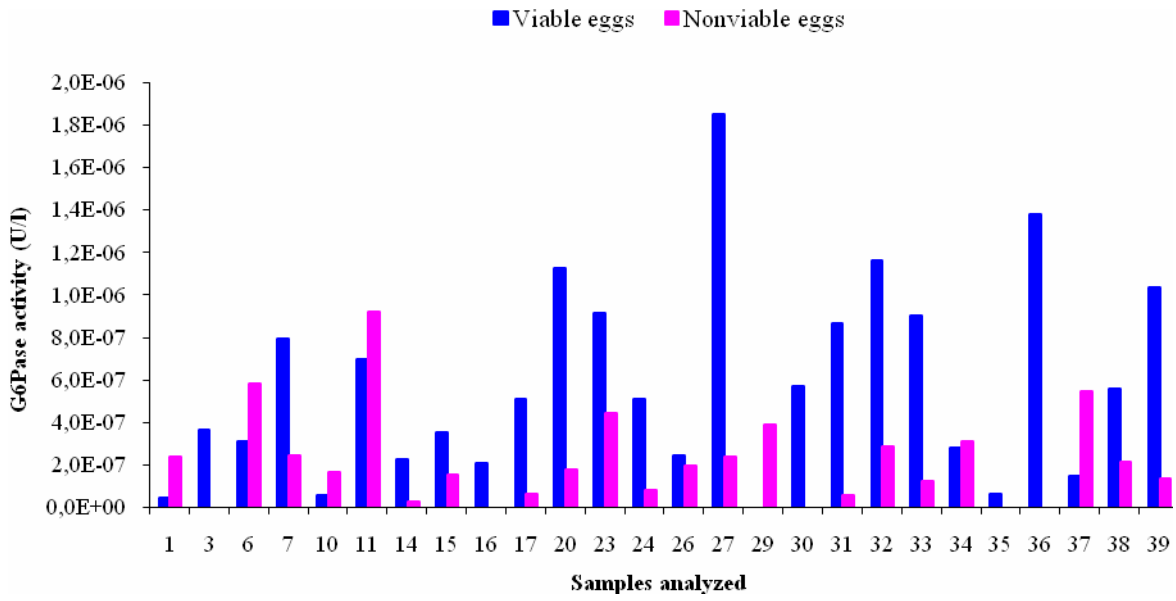


**Figure III.10** – Glucose-6-phosphatase (G6Pase) activity determined in viable and nonviable eggs of *Solea senegalensis*s.

In an overall perspective the viable eggs showed more G6Pase activity than the nonviable eggs. This is seen when comparing the ranges of G6Pase activity between

viable and nonviable eggs. Nonviable eggs G6Pase activity ranged between 2.31x10$^{-8}$U/l (sample 14) and 9.2x10$^{-7}$U/l (sample 11) and in viable eggs it ranged between 4.05x10-8U/l (sample 1) and 1.85x10-6U/l (sample 27). Sample 29 have no registered enzymatic activity for the viable eggs, due to a lack of sample.

### 3.1.5. Glucose-6-phosphate dehydrogenase (G6PDH)

Figure I.11 shows the results obtained for G6PDH activity. All samples analyzed show G6PDH activity, although the enzyme activity has high variation \during the breeding period. However, in 23 of the 39 analyzed samples the enzyme activity was higher in viable eggs.

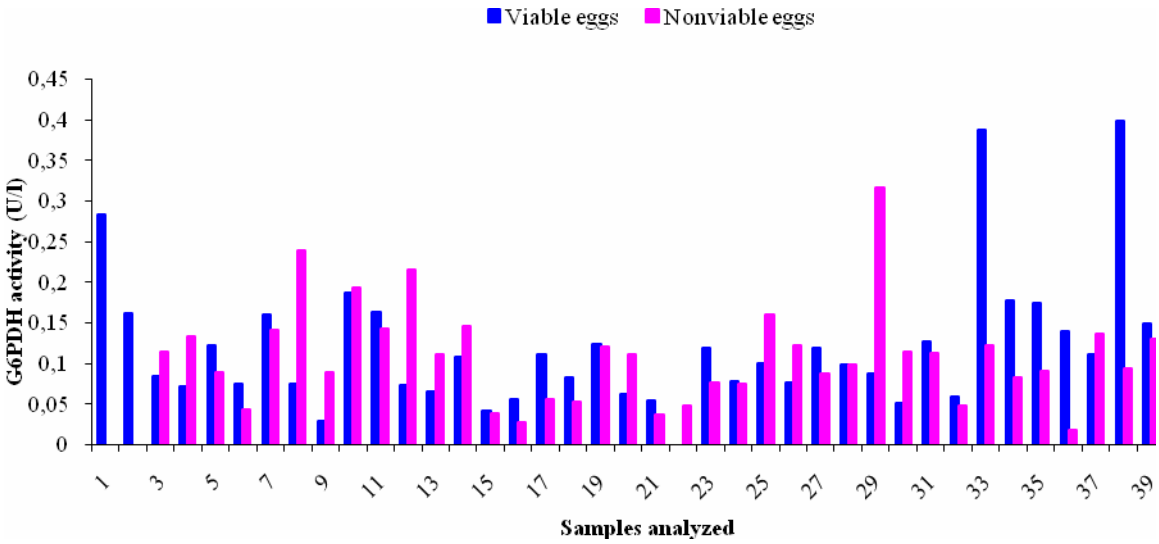


**Figure III.11**- Glucose-6-phophate dehydrogenase (G6PDH) activity determined in viable and nonviable eggs of *Solea senegalensis*s.

Viable egg G6PDH activity ranges from 0.028 to 0.398U/l, and the nonviable egg enzymatic activity ranges from 0.013 to 0.316U/l. The highest value for G6PDH activity was registered in the viable egg sample 38 (0.398U/l) and in the nonviable egg sample 29 (0.316U/l).

### 3.1.6. Aspartate aminotranseferase (GOT)

Figure III.12 shows the results of the GOT activity assay. It is possible to see that samples 5 and 36 did not show any results.

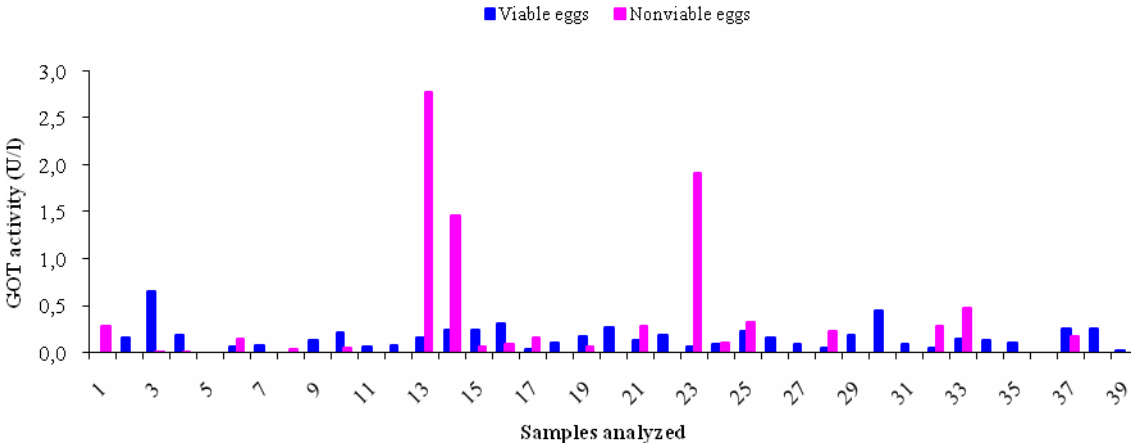


**Figure III.12 -** Aspartate aminotransferase activity determined in viable and nonviable eggs of *Solea senegalensis*s.

The enzyme activity expressed was low for most samples, below 0.5U/l. Samples 14, 15 and 24 provide the most evident exceptions whose values ascend from 1.5 to almost 3.0U/l.

In most of the analyzed samples the viable eggs had more GOT activity than the nonviable eggs.

## 3.2. Metabolites

### 3.2.1. Hexoses, 6-deoxyhexoses and heptoses

Figures III.13, III.14 and III.15 represents the concentrations of hexoses, 6-deoxyhexoses and heptoses, respectively, determined in all samples from viable and nonviable eggs.

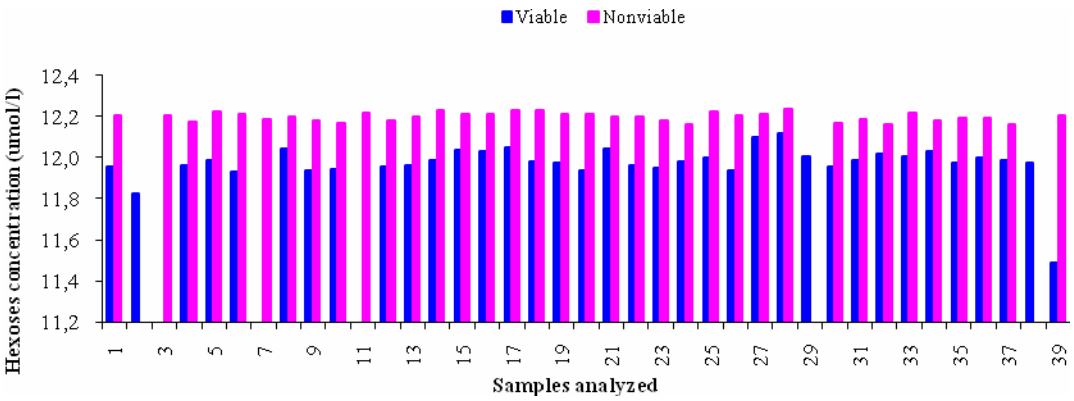


**Figure III.13 –** Hexoses concentration determined in viable and nonviable eggs of *Solea senegalensis*s.

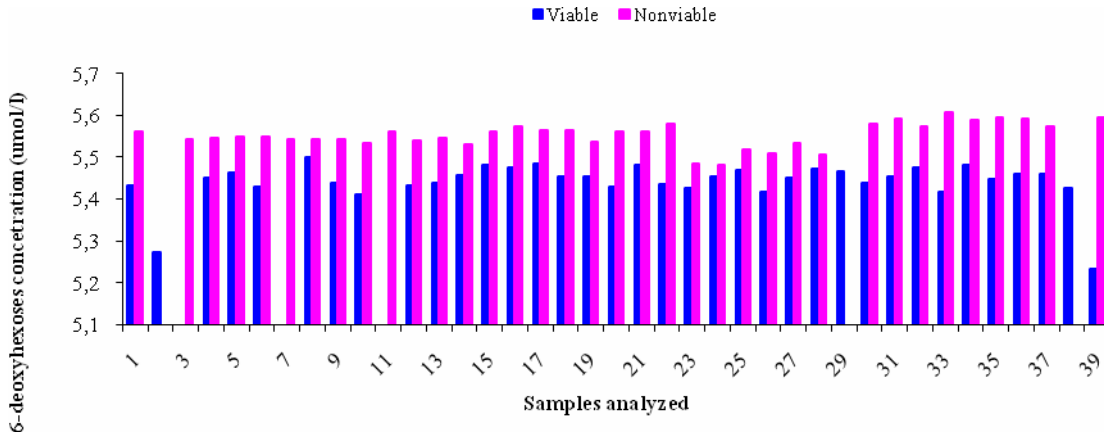


**Figure III.14** – 6-deoxyhexoses concentration determined in viable and nonviable eggs of *Solea senegalensis*s.

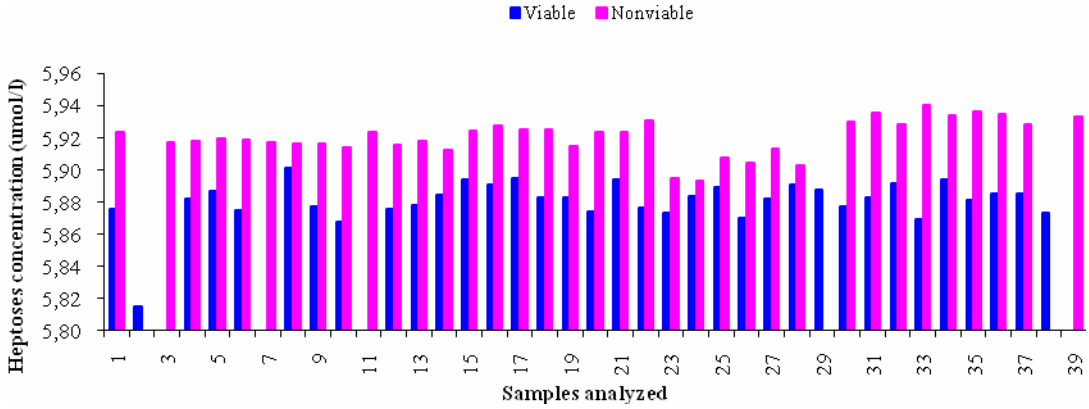


**Figure III.15** – Heptoses concentration determined in viable and nonviable eggs of *Solea senegalensis*s.

As seen in the above figure (Fig. III.13), there is a significant difference between the viable and the nonviable eggs, regarding hexoses concentration. Viable eggs had, in all the samples, lower concentrations of hexoses. The concentration of hexoses for viable eggs ranged between 11.492 to 12.119µmol/l; the nonviable eggs hexoses concentration ranged from 12.160 to 12.230µmol/l. For hexoses, heptoses and 6-deoxyhexoses the viable eggs were found to have lower concentrations of the studied metabolites than the nonviable eggs (Fig. III.13, III.14 and III.15).

Samples 2 and 39 had the lowest concentrations of both hexoses and 6-deoxyhexoses (fig. III.13, III.14)**.** These samples hexoses concentrations were, respectively, 11.828µmol/l and 11.492µmol/l. Their respective 6-deoxyhexoses concentrations were 5.274µmol/l and 5.231µmol/l.

In viable egg samples the heptoses concentration ranged between 5.875 and 5.901µmol/l, nonviable eggs, as already stated, had higher heptoses concentration, that range between 5.893 and 5.940µmol/l. In sample 39, the viable eggs show no concentration of this sugar (fig. III.15).

### 3.2.2. ATP

The ATP concentration was not constant for neither viable nor nonviable eggs, varying along the breeding season. By visual analysis of figure III.16 it is possible to see that not all of the samples have shown ATP concentration, in both viable and nonviable eggs.

When detected in both egg types (19 samples), most of the viable eggs (11 samples) had higher ATP concentration. Viable eggs although have register both the lowest ATP concentration (sample 32; 0.021µmol/l ATP) and the highest (sample 35; 1.058µmol/l ATP). The range of ATP concentrations for the nonviable eggs was somewhat smaller, from 0.043 to 0.905µmol/l.

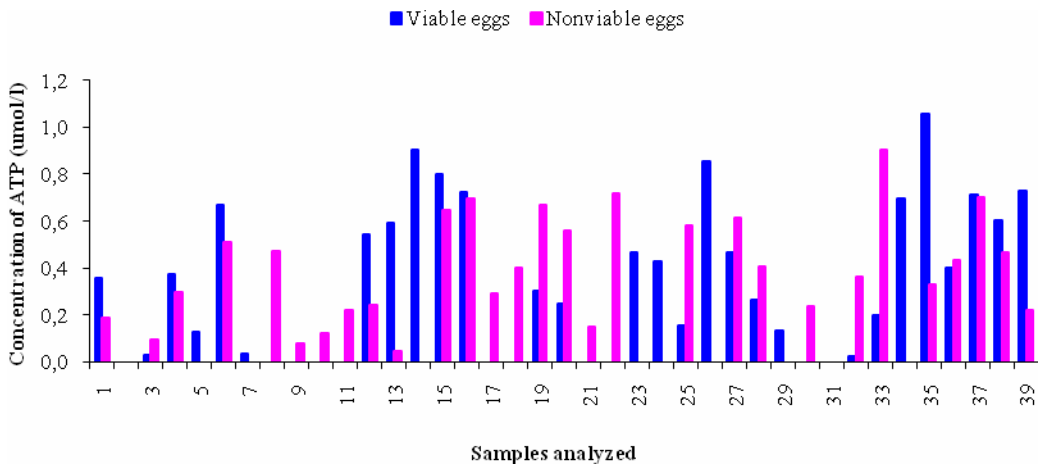


**Figure III.16** - ATP concentration determined in viable and nonviable eggs of *Solea senegalensis*s.

### 3.2.3. Sialic acids

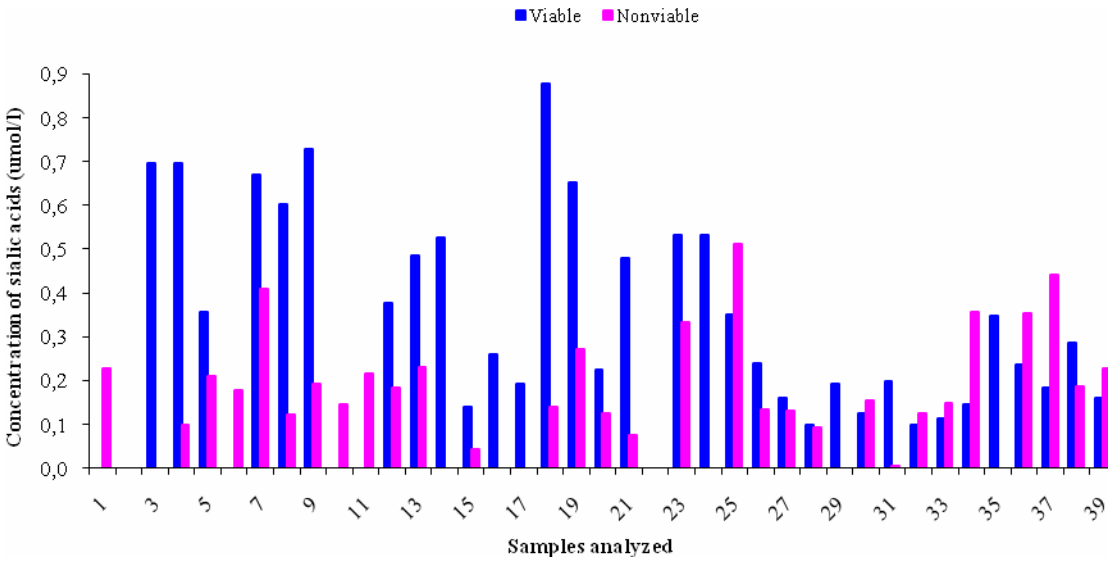


**Figure III.17** – Sialic acids concentration determined in viable and nonviable eggs of *Solea senegalensis*s.

From sample 1 to 25 viable eggs have higher concentrations of sialic acids (Fig. III.17). This change in samples collected later in the breeding period where nonviable eggs show higher concentrations of sialic acids. Nevertheless viable eggs have the highest value registered (0.878µmol/l; sample 18). The range of sialic acids in viable eggs varied between 0.099µmol/l and 0.878µmol/l. On nonviable eggs the range of this metabolite varied between 0.006µmol/l (sample 31) and 0.511µmol/l (sample25).

### 3.2.4. Total free carbohydrates

Figure III.18 shows the amount of total free carbohydrates detected in the samples analyzed. As this assay measures the total amounts of free monosaccharides (FM), from now on the results obtained will be referred only as free monosaccharides, which will also ease the comparison with other studies. Concentration of the FM was relatively similar for both viable and nonviable eggs.

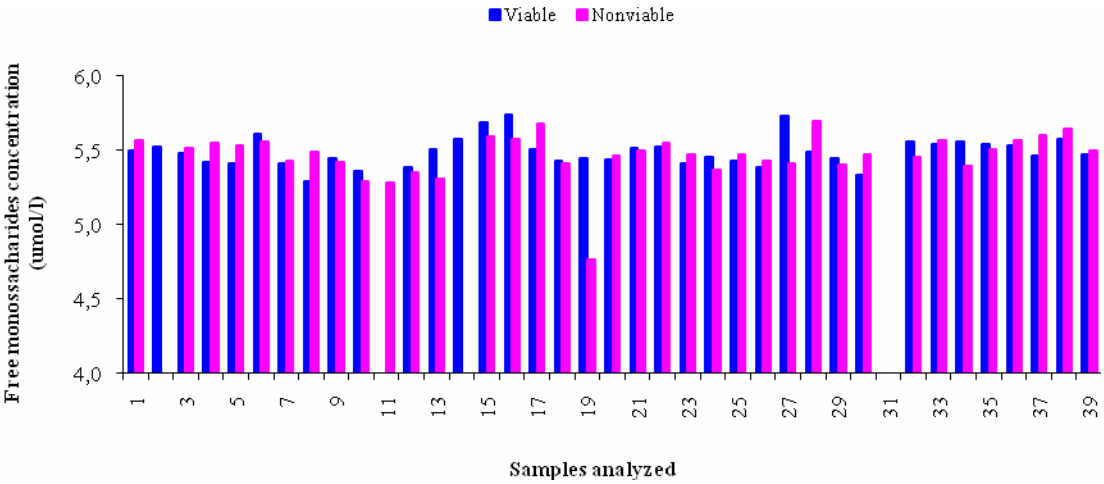


**Figure III.18** – Free monossacharides concentration determined in viable and nonviable eggs of *Solea senegalensis*s.

All viable eggs analyzed have concentrations of FM above 5.0µmol/l. The lowest value registered for viable eggs was of 5.295µmol/l of free monossacharides, in sample 8, while for nonviable eggs it was of 4.765µmol/l, in sample 19. The highest values registered were, for viable eggs, 5.742µmol/l, in sample 16, and 5.694µmol/l, in sample 15, for the nonviable eggs.

### 3.2.5. Ketoses

As illustrated in figure III.19, the concentration of ketoses varied in the samples analyzed.

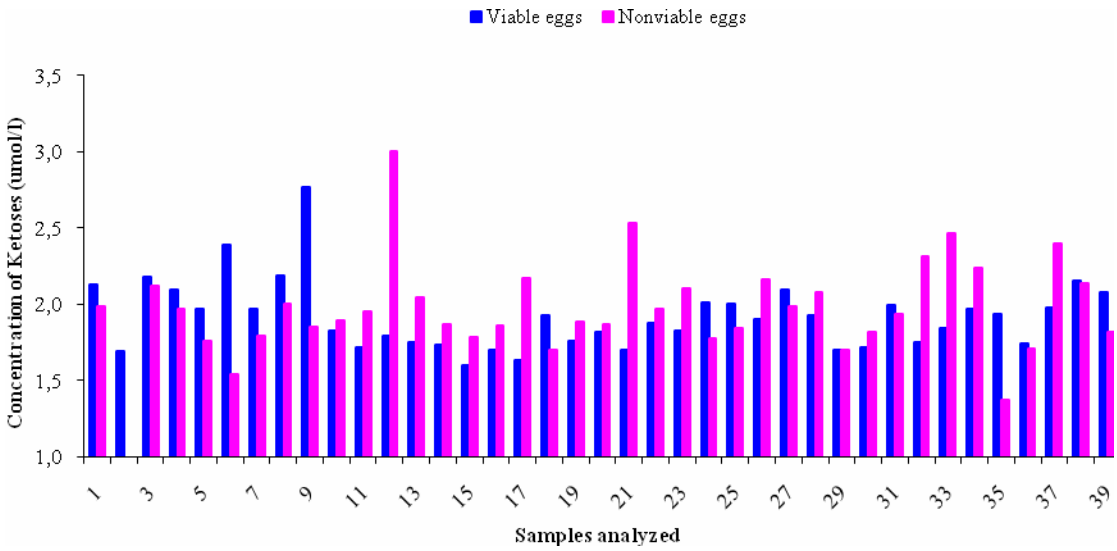


**Figure III.19 –** Ketoses concentration determined in viable and nonviable eggs of *Solea senegalensis*s.

In the beginning of the breeding period (samples 1 to 9, appendix I) viable eggs have higher concentrations of ketoses. This shifts, and in the rest of the samples nonviable eggs have, in average, higher concentrations of ketoses. Most of the viable eggs have ketoses concentrations in this range [1.5; 2.0] µmol/l. The range of concentration of ketoses for the viable eggs was from 1.598 to 2.770µmol/l, while in nonviable eggs the range was different from 1.370 to 3.001µmol/l.

### 3.2.6. Bound & free ribose

Most of the analyzed samples showed values of free ribose higher in viable eggs than in nonviable eggs. This difference is more evident in samples collected at the end of the breeding period (Fig. III.20, appendix I). In these sample the viable eggs had higher concentration of free ribose than the nonviable eggs. The range of concentration of free ribose, for nonviable eggs, is form 24.145 to 25.684µmol/l.

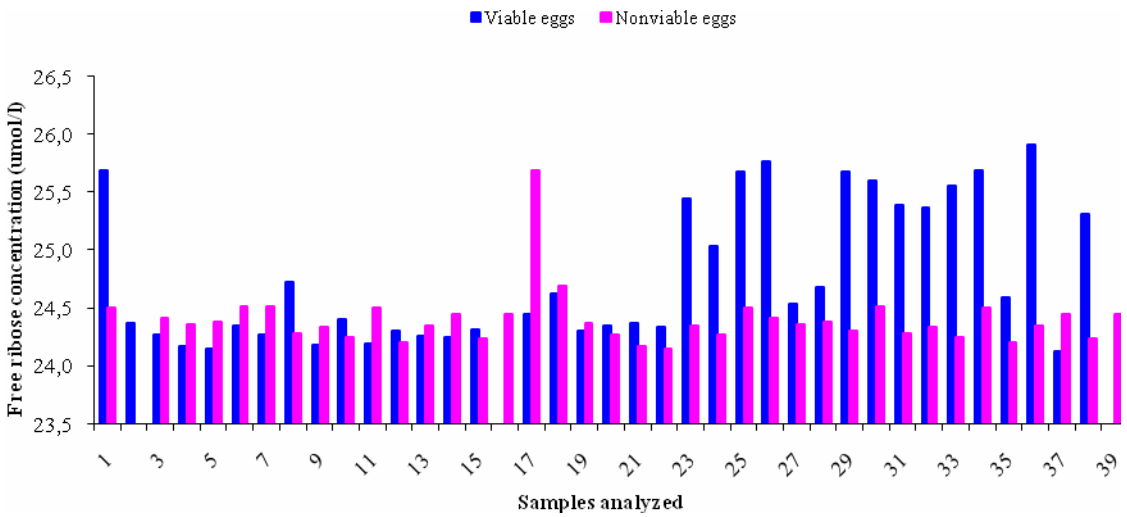


**Figure III.20** – Free ribose concentration determined in viable and nonviable eggs of *Solea senegalensis*s.

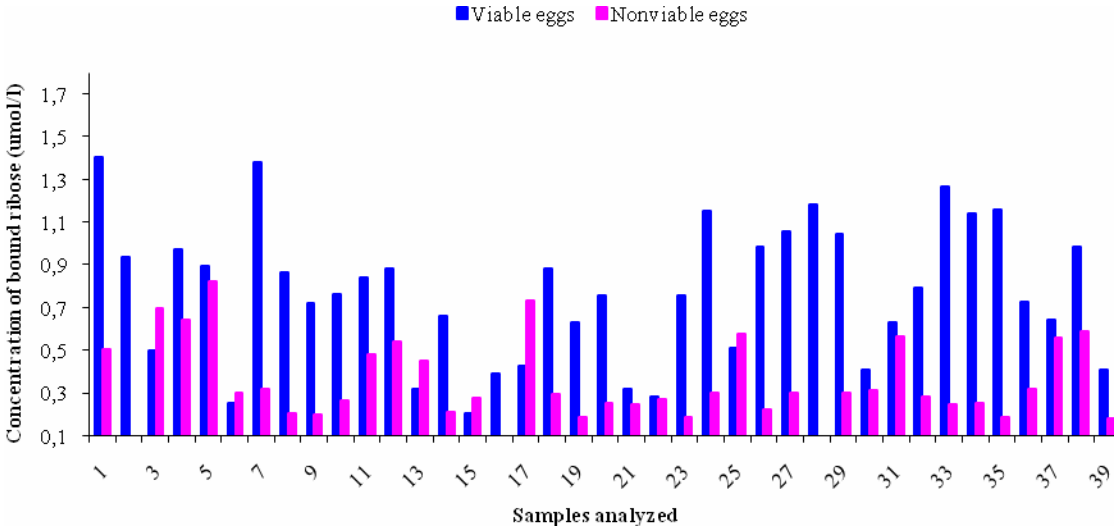


**Figure III.21 -** Bound ribose concentration determined in viable and nonviable eggs of *Solea senegalensis*s.

When compared with the values obtained for the bound ribose, showed in figure III.21, it is noticeable that they are of different magnitudes, being the values of free ribose higher. The free ribose concentrations range from 24.123 to 25.909µmol/l (which also corresponds to the range of free ribose concentration of the viable eggs analyzed), while the bound ribose concentrations ranges from 0.077µmol/l (in nonviable eggs) to

1.406µmol/l (in viable eggs). The ranges of concentration of bound ribose are from 0.209 to 1.406µmol/l, in viable eggs, and from 0.077 to 0.824µmol/l, in nonviable eggs.

### 3.3 – Statistic treatment of the collected data

The Spearman's correlation test results between egg parameters are showed in table III.1. It is possible to see that no statistical significant correlation was obtained between the egg parameters. When correlated with the egg parameters only sialic acids show correlation with the percentage of fecundation. This negative correlation had, however, no explanatory power since the significance was of 0.000 (Table III.2).

**Table III.1 -** Spearman correlation test results between morphophysiological parameters measured in the viable eggs of *Solea senegalensis*s.. [a)] Correlation is significant at the 0.05 level; [b)] correlation is significant at the 0.01 level (in both cases, 2-tailed).

| | | **Dependent variables** | | | |
|---|---|---|---|---|---|
| **Independent Variables** | **Spearman Correlation test** | **% viability** | **Average egg diameter** | **% fecundation** | **% hatching** |
| **% viability** | Correlation Coefficient | 1.000 | 0.175 | -0.124 | 0.029 |
| | Sig. (2-tailed) | , | 0.295 | 0.458 | 0.867 |
| | N | 38 | 38 | 38 | 36 |
| **Average egg diameter** | Correlation Coefficient | 0.175 | 1.000 | -0.124 | 0.231 |
| | Sig. (2-tailed) | 0.295 | , | 0.457 | 0.176 |
| | N | 38 | 38 | 38 | 36 |
| **% fecundation** | Correlation Coefficient | -0.124 | -0.124 | 1.000 | 0.031 |
| | Sig. (2-tailed) | 0.458 | 0.457 | , | 0.857 |
| | N | 38 | 38 | 38 | 36 |

With the ROC-Curves procedure of SPSS software the assay results were separated through a cutoff value, showed in table III.3. To the highest pair [sensitivity; specificity] is associated a value, chosen as the cutoff value according to each the sample's test results. Some authors consider the pair composed by the highest sensitivity and by the lowest 1-specificity correspondent (Jensen *et al.,* 1996), reason why this column is showed in table III.3. According to the statistic of the test samples were separated in

higher quality viable eggs and in lower quality eggs (which contained both nonviable eggs and viable eggs excluded by the cutoff value).

**Table III.2 -** Spearman correlation test results between biochemical test results and morphophysiological parameters measured in the viable eggs of *Solea senegalensis*s. [a)] Correlation is significant at the 0.05 level; [b]) correlation is significant at the 0.01 level (in both cases, 2-tailed).

| | | Dependent variables | | | |
|---|---|---|---|---|---|
| **Independent Variables** | **Spearman Correlation test** | **% viability** | **Average egg diameter** | **% fecundation** | **% hatching** |
| | Correlation Coefficient | -0.122 | -0.148 | 0.285 | 0.084 |
| **G6PDH** | Sig. (2-tailed) | 0.498 | 0.411 | 0.108 | 0.654 |
| | N | 33 | 33 | 33 | 31 |
| | Correlation Coefficient | -0.016 | -0.040 | 0.117 | -0.225 |
| **GOT** | Sig. (2-tailed) | 0.928 | 0.825 | 0.518 | 0.224 |
| | N | 33 | 33 | 33 | 31 |
| **G6Pase** | Correlation Coefficient | -0.129 | -0.240 | -0.002 | -0.005 |
| | Sig. (2-tailed) | 0.473 | 0.179 | 0.993 | 0.978 |
| | N | 33 | 33 | 33 | 31 |
| **MDH** | Correlation Coefficient | 0.258 | 0.128 | -0.097 | -0.199 |
| | Sig. (2-tailed) | 0.148 | 0.477 | 0.593 | 0.284 |
| | N | 33 | 33 | 33 | 31 |
| **Acid Phosphatase** | Correlation Coefficient | 0.144 | 0.058 | 0.226 | -0.100 |
| | Sig. (2-tailed) | 0.424 | 0.747 | 0.205 | 0.592 |
| | N | 33 | 33 | 33 | 31 |
| **Hexoses** | Correlation Coefficient | 0.144 | -0.107 | 0.212 | 0.249 |
| | Sig. (2-tailed) | 0.408 | 0.542 | 0.221 | 0.177 |
| | N | 35 | 35 | 35 | 31 |
| **6-Deoxyhexoses** | Correlation Coefficient | 0.098 | -0.016 | 0.110 | -0.041 |
| | Sig. (2-tailed) | 0.574 | 0.928 | 0.530 | 0.821 |
| | N | 35 | 35 | 35 | 33 |
| **Heptoses** | Correlation Coefficient | 0.098 | -0.016 | 0.110 | -0.068 |
| | Sig. (2-tailed) | 0.574 | 0.928 | 0.530 | 0.706 |
| | N | 35 | 35 | 35 | 33 |
| **ATP** | Correlation Coefficient | -0.129 | 0.169 | 0.251 | -0.068 |
| | Sig. (2-tailed) | 0.483 | 0.356 | 0.166 | 0.706 |
| | N | 32 | 32 | 32 | 33 |
| **Free monossacharides** | Correlation Coefficient | -0.313 | -0.324 | 0.266 | 0.044 |
| | Sig. (2-tailed) | 0.063 | 0.054 | 0.116 | 0.815 |
| | N | 36 | 36 | 36 | 30 |
| **Sialic acids** | Correlation Coefficient | 0.049 | 0.145 | -0.621[b] | -0.330 |
| | Sig. (2-tailed) | 0.787 | 0.420 | 0.000 | 0.057 |
| | N | 33 | 33 | 33 | 34 |
| **Ketoses** | Correlation Coefficient | -0.210 | 0.050 | -0.261 | 0.143 |
| | Sig. (2-tailed) | 0.205 | 0.766 | 0.114 | 0.441 |
| | N | 38 | 38 | 38 | 31 |
| **Free ribose** | Correlation Coefficient | -0.079 | -0.179 | 0.235 | -0.150 |
| | Sig. (2-tailed) | 0.643 | 0.289 | 0.161 | 0.381 |
| | N | 37 | 37 | 37 | 36 |
| **Bound ribose** | Correlation Coefficient | 0.011 | -0.002 | -0.139 | -0.102 |
| | Sig. (2-tailed) | 0.947 | 0.989 | 0.404 | 0.560 |
| | N | 38 | 38 | 38 | 35 |

After the selection of the samples through the cutoff value it was checked which samples remained as viable in each test. As the goal of this work is the tentative of establishment of correlations between egg biochemical parameters and the eggs morphophysiological parameters, the best samples were selected. The best samples were considered those that ranked at least 7, or more, best results after the ROC curves establishment of the cutoff value in the 10 biochemical assays performed. The list can be seen in table III.4.

**Table III.3 –** Results of the ROC-curve test from the assays tested in eggs of *Solea senegalensis*s.

| Tests | Statistic of the test | Cutoff value | units | Sensitivity | Specificity | 1 - Specificity |
|---|---|---|---|---|---|---|
| **G6PDH** | Smaller values of enzyme activity indicate stronger evidence for better viable eggs | 2.92E-02 | U/l | 0.706 | 0.639 | 0.361 |
| **GOT** | Larger values of enzyme activity indicate stronger evidence for better viable eggs | 2.11E-02 | U/l | 0.647 | 0.611 | 0.389 |
| **MDH** | Smaller values of enzyme activity indicate stronger evidence for better viable eggs | 7.61E-02 | U/l | 0.853 | 0.750 | 0.250 |
| **Acid Phosphatase** | Larger values of enzyme activity indicate stronger evidence for better viable eggs | 4.02E-03 | U/l | 0.853 | 0.639 | 0.361 |
| **Hexoses** | Smaller values of metabolite concentration indicate stronger evidence for better viable eggs | 1.21E+01 | µmol/l | 1.000 | 1.000 | 0.000 |
| **6-Deoxyhexoses** | Smaller values of metabolite concentration indicate stronger evidence for better viable eggs | 5.50E+00 | µmol/l | 1.000 | 0.944 | 0.056 |
| **Heptoses** | Smaller values of metabolite concentration indicate stronger evidence for better viable eggs | 5.89E+00 | µmol/l | 0.861 | 1.000 | 0.000 |
| **Sialic Acids** | Larger values of metabolite concentration indicate stronger evidence for better viable eggs | 2.21E-01 | µmol/l | 0.636 | 0.697 | 0.303 |
| **Free Ribose** | Larger values of metabolite concentration indicate stronger evidence for better viable eggs | 2.44E+01 | µmol/l | 0.553 | 0.605 | 0.395 |
| **Bound Ribose** | Larger values of metabolite concentration indicate stronger evidence for better viable eggs | 3.23E-01 | µmol/l | 0.897 | 0.684 | 0.316 |

**Table III.4 –** Samples selected after the establishment of the cutoff value for each test.

| Tests | Samples with values above/below the cutoff value |
|---|---|
| G6PDH | 1 11 38 |
| GOT | 6 3 16 22 26 29 33 |
| MDH | 3 15 |
| AciPho | 2 3 11 14 17 21 28 35 36 38 39 |
| Hex | 2 4 12 15 16 18 22 28 27 31 32 33 35 37 38 39 |
| 6Deoxy | 2 4 13 18 19 22 26 32 33 36 37 38 |
| Hep | 2 4 14 19 20 23 27 32 33 36 37 38 |
| SiaAcid | 3 5 14 19 20 23 35 |
| FreRib | 2 7 26 29 30 32 36 |
| BouRib | 2 3 10 13 14 17 21 28 29 31 32 33 35 37 38 39 |

**Table III.5** – Spearman correlation test results between biochemical test results sorted after the ROC-curves tests and morphophysiological parameters measured in the viable eggs of *Solea senegalensis*. [a)] Correlation is significant at the 0.05 level; [b]) correlation is significant at the 0.01 level (in both cases, 2-tailed).

| Assay | | %Viability | Average egg diameter | % Fecundation | % Hatching |
|---|---|---|---|---|---|
| **G6PDH** | Correlation Coefficient | -0.549[a] | -0.383 | 0.335 | -0.283 |
| | Sig. (2-tailed) | 0.015 | 0.105 | 0.160 | 0.271 |
| | N | 19 | 19 | 19 | 17 |
| **GOT** | Correlation Coefficient | 0.305 | 0.189 | 0.026 | 0.219 |
| | Sig. (2-tailed) | 0.205 | 0.439 | 0.917 | 0.398 |
| | N | 19 | 19 | 19 | 17 |
| **MDH** | Correlation Coefficient | 0.423 | 0.308 | -0.144 | 0.247 |
| | Sig. (2-tailed) | 0.071 | 0.200 | 0.557 | 0.575 |
| | N | 19 | 19 | 19 | 17 |
| **Acid** | Correlation Coefficient | -0.104 | 0.159 | 0.278 | 0.348 |
| **Phosphatase** | Sig. (2-tailed) | 0.673 | 0.515 | 0.249 | 0.172 |
| | N | 19 | 19 | 19 | 17 |
| **Hexoses** | Correlation Coefficient | 0.060 | -0.105 | 0.150 | -0.067 |
| | Sig. (2-tailed) | 0.808 | 0.670 | 0.540 | 0.798 |
| | N | 19 | 19 | 19 | 17 |
| **6-** | Correlation Coefficient | 0.465[a] | 0.048 | 0.188 | -0.143 |
| **Deoxyhexoses** | Sig. (2-tailed) | 0.045 | 0.844 | 0.440 | 0.585 |
| | N | 19 | 19 | 19 | 17 |
| **Heptoses** | Correlation Coefficient | 0.465[a] | 0.048 | 0.188 | -0.143 |
| | Sig. (2-tailed) | 0.045 | 0.844 | 0.440 | 0.585 |
| | N | 19 | 19 | 19 | 17 |
| **Sialic Acids** | Correlation Coefficient | 0.278 | 0.215 | -0.335 | -0.012 |
| | Sig. (2-tailed) | 0.297 | 0.424 | 0.206 | 0.967 |
| | N | 16 | 16 | 16 | 14 |
| **Free Ribose** | Correlation Coefficient | 0.033 | -0.138 | 0.312 | -0.280 |
| | Sig. (2-tailed) | 0.892 | 0.575 | 0.194 | 0.277 |
| | N | 19 | 19 | 19 | 17 |
| **Bound** | Correlation Coefficient | -0.411 | -0.243 | 0.022 | -0.026 |
| **Ribose** | Sig. (2-tailed) | 0.081 | 0.315 | 0.928 | 0.921 |
| | N | 19 | 19 | 19 | 17 |

Spearman correlations were checked between the tests results of the best sample from the total of viable eggs analyzed and their parameters (Table III.5). Only 3 correlations with statistical significance, at 0.05 level emerged: G6PDH/ % viability; Heptoses/ % viability; 6-Deoxyhexoses/% viability (Table III.3). The previously reported correlation between the sialic acids and the percentage of viability was not supported by this new analysis.

# CHAPTER IV

# DISCUSSION

Since the Senegalese sole started to be studied as a new potential species for the European aquaculture its mass production was never achieved. Problems such as the Black Patch Necrosis, water temperature control or with the reproduction in captivity of broodstocks hampered sole production. In recent times new developments in feeding and in water recirculation technologies resolved some of these problems (Imsland *et al.,* 2003). At the present time, Senegalese sole aquaculture is limited due to poor reproduction of the captive broodstocks in many facilities (Anguis & Cañavate, 2005; Gárcia-Lopez *et al.,* 2006).

So far, reproduction in captivity has been obtained with wild captured animals because the F1 (or first descendents) generation commonly fail to reproduce (Cabrita *et al.,* 2006); which could be partially cased by the males. The work of Cabrita and co-workers (2006) demonstrated that the Senegalese sole have a very low production of semen, but nevertheless, males captured from the wild produced more semen than those from the F1 generation.

As stated earlier in this work, no literature was found that proposed some quality criteria for the eggs of *Solea senegalensis*. The study of egg quality is of great importance to aquaculture. Earlier works with other species have showed that hatching rate, larval survival to mouth opening or to the point of no return could be predicted through their relationships with both morphological and biochemical parameters (*e.g*. Lahnsteiner *et al*., 2001; Kjørsvik *et al*., 2003; Lahnsteiner & Patarnello, 2004a; b; Giménez *et al*., 2006)

**1. Morphophysiological parameters of the eggs analyzed**

The natural spawning of *S. senegalensis* broodstock, which is considered the only way to obtaining viable eggs (Dinis & Reis, 1995; Dinis *et al.,* 1999; Imsland *et al.*, 2003), started in late March and lasted until June, after the rise of water temperature above 18º C. This is in concordance with the findings of Dinis (1986) and Andrade (1990) and the reviews of Dinis *et al.* (1999) and Imsland *et al.* (2003).

Egg buoyancy can affect the spawning success in marine Teleosts because the eggs must stay buoyant in the top layer of the water column in order to avoid low oxygen conditions that might decrease the development and hatchability of eggs (Guisande *et al.*, 1994). The average percentage of viable (floating) eggs of *S. senegalensis* in the sampling period of 2006 was of 50.91%, which is lower than the reported in Dinis & Reis (1995; 87-98% viable eggs), Dinis *et al.* (1999) and Imsland *et al.* (2003; 72.1±26.5% viable eggs). Although the average is lower than in other years, the percentage of viable egg varied from 4.54% to 90%. The lowest of these values was obtained in the end of the breeding period where the samples collected were mainly composed of nonviable eggs. It is also noticeable that there is a tendency for a decrease in egg viability when approaching the end of the spawning season. This is partially supported by Kjørsvik *et al.* (1994) who report in *Gadus morhua* higher viability in the peak of spawning period. The last two samples from the spawning season (appendix I) were mainly composed of nonviable eggs (≥ 90%). After a batch with high percentage of viability followed a decrease in viability in the next batch which was followed by an increase in viability occurred until a new peak. This may be linked to the maturation of the females of the broodstock. It is known that females of the same broodstock may mature at different times during the spawning season (Lambert *et al.,* 2003). Moreover it could also be attributed to some eggs that were not released in the previous day of spawning and stay being liberated in the next day/days already over-ripped. This phenomenon has been reported in several species and has been stated in turbot in spawns obtained by stripping (Chereguini, pers. comment).

Throughout the 2006 breeding season there seems to be a tendency in most of the analyzed batches regarding the size of the eggs; nonviable eggs were larger (989.97µm of average diameter in a range of 945.0-1021.3µm) than viable eggs. However this difference has no statistic support (Mann-Whitney, $z=1.2415$; $\alpha=0.05$, two-tailed). The average diameter for viable eggs was of 985.49µm, which is higher that those reported by Dinis *et al.* (1999): 929.6±0.01µm in 1996 and 960.6±0.03µm in 1997. The range of viable eggs was between 952.5 and 1012.5µm which encompass the range reported by Dinis & Reis (1995; 975-1013µm). Although there is an apparent decline in egg size toward the end of the spawning season, there are some batches with larger eggs than in the beginning of the spawning season; leading, consequently, to an overall larger fluctuation in the viable egg diameter. This decline in egg size toward the end of the

spawning period is also reported in *S. senegalensis* and in *Solea vulgaris* (Dinis *et al.,* 1999), *Scophthalmus maxinus* (McEvoy & McEvoy, 1991) and in *G. morhua* (Kjesbu *et al.*, 1992). The size variations observed in the analyzed batches may be due to depletion of energetic resources of the spawning females near the end of the spawning period, or due to asynchronous spawning activity of larger and smaller females (Kamler, 2005).

The percentage of fecundity in the eggs of 2006 analyzed was, in most samples, higher than 80%. Dinis & Reis (1995) found 100% of fertilization in *S. senegalensis*, while Dinis *et al.* (1999) and Imsland *et al.* (2003) report fecundity ranging from 20% to 100%. It is also noteworthy that the nonviable eggs have some fertilized eggs and that, by the end of the spawning season, the nonviable eggs have higher percentage of fertilized eggs. This may suggest that a larger number of fertilized eggs abort at first cleavages, becoming nonviable by some reason, given the decrease in viability observed.

The percentage of hatching varied throughout the spawning season, being more constant in the peak of spawning, where it ranges from 90 to 100% of hatching. Giménez *et al.* (2006) report that, for *D. dentex*, both in the beginning and in the end of the spawning season the quality of the eggs is lower. This may mean a depletion of the resources dedicated in the vitellogenesis.

**2. Biochemical quality assays**

According to Giménez *et al*. (2006) hatchery production can be optimized by starting production cycle with high-quality eggs. These eggs will have higher hatching rates and more robust larva with better growth, survival and stress resistance. In order to establish egg quality parameters the analysis of enzymatic activity and of the concentrations of metabolites can be useful since they mirror the metabolic status of developing embryos.

**2.1. Enzymes**

According to Livni (1971) and Rosety *et al.* (1992) the alkaline phosphatase (ALP) and the acid phosphatase (ACP) take part, respectively, in absoption and transport of macromolecules across membranes, phagocyting and resopting the atretic residual oocytes, especially after spawning. Both of these enzymes are also reported to be involved in the catabolism of phospholipids; ALP is involved as well in the

dephosphorilation of phosphovitin in the yolk (Giménez *et al*., 2006). Giménez *et al.* (2006) report that in *D. dentex,* higher activity of ALP was found in eggs with reduced viability and higher mortalities. In *S. aurata*, high activity of ACP was associated with low viability eggs (Lahnsteiner & Patarnello, 2004a). However, in the present study, viable eggs had higher values of enzymatic activity for both ALP and ACP. This may be due to a higher catabolic activity of phospholipids in the developing embryo, since the yolk phospholipids provide most of the structural components for building cell membranes.

The malate dehydrogenase (MDH) is an enzyme associated with energy metabolism (Lahnsteiner, 2000) due to its participation in the gluconeogenesis. This enzyme mediates the transformation of oxaloacetic acid to malic acid in the mitochondria, because oxaloacetic acid is unable to cross the mitochondria membrane, and, once in the cytoplasm, mediates the reoxidation of the malic acid into oxaloacetic acid. In the first case there is the oxidation of NADH to $NAD^+$, while on the second case occurs the reduction of $NAD^+$ to NADH (Campos, 1998). Both $NAD^+$ and NADH are used in the respiratory chain as co-substrates (Lahnsteiner *et al.*, 2001). The MDH activity was, in the present study, higher in nonviable eggs. Lahnsteiner *et al.* (2001) found a similar relation because MDH activity was correlated with reduced egg viability and alterations in metabolism in the cyprinid fishes *Cyprinus carpio, Hypophthalmus molitrix, Ctenepharyngodon idella* and *Chalcalburnus chalcoides*.

The glucose-6-phosphatase (G6Pase) is a regulatory enzyme of gluconeogenesis catalyzing the reaction glucose-6-phosphate (G6P) into glucose (Campos, 1998; Lahnsteiner & Patarnello, 2004a, Lahnsteiner, 2005). In the present study, G6Pase activity was low (from $10^{-8}$ to $10^{-6}$µmol/min/l) and was, in general, higher in viable eggs. This seems to be in concordance with the findings of Lahnsteiner & Patarnello (2004a) which report that in *S. aurata* reduced G6Pase activity is indicative for low quality eggs. It was later found out that other Sparidae, such as *in Puntazzo puntazzo* (Lahnsteiner & Patarnello, 2004b), *D. dentex* (Giménez *et al.,* 2006), and *Coregonus* spp. (Lahnsteiner, 2005) showed similar results.

The glucose-6-phosphate dehydrogenase (G6PDH) is an enzyme also involved in the gluconeogenesis (Lahnsteiner & Patarnello, 2004a) and in the oxidative part of the

pentose-phosphate pathway where it catalyses the reaction G6P into 6-phosphogluconate (Campos, 1998). Lahnsteiner (2005) reports the activity of this enzyme in *Coregonus* spp, thus proving the occurrence of the pentose-phosphate pathway in the eggs and developing embryos. In the comparative study of vitellogenic follicles and eggs of *Serranus cabrilla* (Serranidae), *Mullus barbatus* (Mullidae) and *S. aurata* (Sparidae; Lahnsteiner, 2006) it was found that although different in size, the eggs of these species are quite similar, both in enzymatic activity and in metabolite concentration, in terms of the carbohydrate metabolism. In *Solea senegalensis* and in most of the analyzed samples G6PDH activity was higher in viable eggs, with a high variability throughout the spawning season. In *S. aurata* (Lahnsteiner & Patarnello, 2004a; b), *P. puntazzo* (Lahnsteiner & Patarnello, 2004b) and *in D. dentex* (Giménez *et al.,* 2006) G6PDH was found to be related to egg quality.

According to Campos (1998) the aspartate aminotransferase (GOT) is involved in the transfer of reduction power from mitochondria to the cytoplasm, thus allowing the cytoplasmic formation of NADH. In the cytoplasm the NADH may be used to reduce nitrates or to reduce the hydroxipyruvic acid. According to Lahnsteiner (2000) GOT may also be an indicator for the integrity of the plasma membrane and has been used as an integrity assay in sperm physiology (Cabrita, E., *pers. comm.*). In most of the analyzed samples the viable eggs had more GOT activity than the nonviable eggs. As an enzyme involved in the amino acids metabolism, the higher presence in viable eggs may be due to the metabolic needs of the developing embryos, as the amino acids are "the building blocks" of proteins and membranes. However the high amounts present in nonviable eggs may be due to lytic activity of phospholipids in egg membranes, as reported in *Oncorhyncus mykiss* ovarian fluid in over-ripen eggs (Lahnsteiner, 2000)

**2.2. Metabolites**

From the existing hexoses the most important in animal tissues are galactose, fructose and glucose; the later can be directly catabolized in glycolysis or transformed in ribose via the pentose-phosphate pathway. In teleosts the 6-deoxyhexose fucose, present in the oocytes, is considered to intervene in the organogenesis of developing embryos (Lahnsteiner, 2005; 2006). The heptoses measured may be representative of sedo-heptulose an intermediate of the pentose-phosphate pathway (Lahnsteiner, 2005). For

hexoses, heptoses and 6-deoxyhexoses the viable eggs were found to have lower concentrations of the studied metabolites than the nonviable eggs. Giménez and co-workers (2006) found that low quality *D. dentex* eggs had higher amounts of glucose (the standard for hexoses, see material & methods chapter) and 6-deoxyhexoses; which may indicate an impaired glycolysis for energy production, with the consequent accumulation of glycolysis metabolites. According to these authors, this may indicate that the low-quality eggs are using other sources for energy production, most probably lipids. In *Coregonus spp* the heptoses concentration increased from the unfertilized egg stage to the epiboly stage (Lahnsteiner, 2005). The present data seems contradictory with this study because the nonviable eggs had higher amount of heptoses than the viable eggs.

ATP is formed by the energy release of catabolic processes and is an energetic cellular reserve to be used when required in synthetic reactions (Campos, 1998). The ATP concentration was not constant for neither viable nor nonviable eggs, varying along the breeding season; but when detected in both egg types, most of the viable eggs had higher ATP concentration. This seems to be in concordance with Lahnsteiner (2005) findings in *Coregonus* spp. because the amount of ATP increased from unfertilized eggs to eyed embryo stage, considering that in most of the analyzed batches there was few fertilized nonviable eggs (Fig. III.5).

Sialic acids are the main components of the cortical vesicles that are released from the cortical vesicles into the perivitelline space where they generate an osmotic gradient to the environment. The water will flow in direction of this gradient until the egg chorion has hardened and an osmotic equilibrium has been restored (Lahnsteiner & Patarnello, 2004a). Lahnsteiner & Patarnello (2004a) report that there is a critical minimal level of sialic acids necessary for normal egg development and that values above that level have no influence in egg quality. In the beginning of the spawning season viable eggs have higher concentrations of sialic acids, but these changes in samples collected later in the breeding period, whereas nonviable eggs show higher concentrations of sialic acids (fig. III.17). As sialic acids are constituents of glycoproteins and of glycolipids, which in turn are part of biological membranes (Campos, 1998), the initial high levels of sialic acids in viable eggs may be associated with the membranes synthesis processes in the developing embryo. In the nonviable eggs the high amounts of sialic acids may be due

to the degradation of the yolk that, without a developing embryo, accumulates in the egg.

The monosaccharides are essential to all anabolic processes, and are required for synthetic processes of nucleic acids (RNA, DNA; Campos, 1998) which, according to Carnevali *et al.* (2001), increase during embryogenesis. But monosaccharides may be as well related to the egg buoyancy since they are osmotic active compounds (Lahnsteiner & Patarnello, 2004a; Giménez *et al.,* 2006). Concentration of the FM (free monosaccharides) was, in the present work, relatively similar for both viable and nonviable eggs, although in most of the samples nonviable eggs had higher concentration of FM. Given the essential part that FM has in all anabolic processes it is not surprising that both viable, and nonviable, eggs have similar FM concentrations. Lahnsteiner & Patarnello (2004a; b) and Giménez *et al.* (2006) have related the FM amount with egg quality. Giménez *et al.* (2006) report that a minimum quantity of monosaccharides is required for normal embryogenesis and that high level of glucose (the standard for the monosaccharides assay, as described in the material & methods chapter) decreased the viability parameters due to interruption or low activity of the pathways of carbohydrate metabolism.

Ketoses are sugars with a functional keto-group, thus with a high reductor potential (Campos, 1998) that accumulates in egg during oogenesis in *S. cabrilla, M. barbatus* and *S. aurata* (Lahnsteiner, 2006). In the beginning of the breeding period (samples 1 to 9, appendix I), viable eggs have higher concentrations of ketoses. This shifts and in the rest of the samples nonviable eggs have, in average, higher concentrations of ketoses. This may mean that a minimal amount of ketoses is needed for normal embryogenesis to occur, similarly to monosaccharides.

Ribose can be found in RNA and ATP molecules (1 adenine, 1 ribose, 3 phosphate groups; Campos, 1998). As such it has an important part in the development of embryos not only because of the production of new cells as the embryo grows and differentiates, but also because of the part of ATP as the energy source for several metabolic processes. In most of the analyzed samples, free and bound ribose amounts were higher in viable eggs than in nonviable eggs. This difference is more evident in samples collected at the end of the breeding period for free ribose (fig.3.20, appendix I). This seems to by in

concordance with several studies performed in other species that have demonstrated increasing ribose levels during embryogenesis (Carnevali *et al.,* 2001; Lahnsteiner, 2005; 2006).

**3. Relations between analyzed parameters and egg quality**

As stated earlier in the present work egg quality is currently defined as "the egg's potential to produce viable fry" (Kjørsvik *et al.*, 1990; Nissling *et al.,* 1998). As such, egg size, percentage of viability and of fecundation has been used to correlate between them and with the percentage of hatching because egg viability, percentage of fertilization and hatching rate have been used as important egg quality criteria in most of the egg quality studies (Kjørsvik *et al.*, 1990; Brooks *et al.*, 1997 -reviews; Buckley *et al.,* 1991a;b; Nissling *et al.*, 1998; Navas *et al.*, 2001; Lahnsteiner *et al.,* 2001; Lahnsteiner & Patzner 2002; Kjørsvik et al., 2003; Kian *et al.,* 2004; Mylonas *et al.,* 2004; Lahnsteiner & Patarnello, 2004a; b ; 2005, Lahnsteiner, 2005 Giménez *et al.,* 2006). However, in the present study, no statistically significant correlations were found. As reported by Brooks *et al.* (1997) these parameters may be the ultimate measure of quality in egg in a crude way, since they give no information about what are the factors that determine egg quality.

When correlated the analyzed parameters, discussed in chapter 4.2, with the egg parameters through the Spearman's non-parametric bivariate correlation only sialic acids show correlation with the percentage of fertility. This negative correlation had, however, no explanatory power since the significance was of 0.000 (Table 3.2). Sialic acids were correlated with the percentage of developing embryos (Lahnsteiner & Patarnello, 2004a). Since for some of the biochemical parameters analyzed there was no distinct separation between viable and nonviable eggs, samples were subjected to the ROC curves test.

ROC curves are particularly useful because they provide a view of all the possible sensitivity/specificity pairs, allowing the examination of the test(s) discriminative power over all the possible cutoff values. ROC curves are independent of the quality of the eggs because sensitivity and specificity are calculated from two independent subgroups of assay results. Having calculated sensitivity and specificity for the range of cutoff

values chose, the ROC curve graphic is constructed with the sensitivity, which is the true positive rate (in the present study good quality viable eggs), in the ordinate as a function of 1-specificity (the false positive rate; low quality eggs) for the range of cutoff values (Jensen *et al.,* 1996). The selected cutoff value allowed the separation between high quality eggs, composed of viable eggs, and low quality eggs, which contained both nonviable eggs and viable eggs excluded by the cutoff value, for each biochemical assay result (Table 3.3).

Nineteen samples were selected after the ROC curve procedures since they scored beyond the cutoff value for most of the assays performed. This selection was then examined for Spearman's correlation with the previously reported egg parameters (table 3.5). Only 3 correlations with statistical significance, at 0.05 level emerged: G6PDH/ % viability; heptoses/ % viability; 6-deoxyhexoses/% viability. The previously reported correlation between the sialic acids and the percentage of viability was not supported by this new analysis. The possible correlations obtained suggest the importance of gluconeogenesis and of the pentose-phosphate pathway in egg viability, and thus in egg quality for *S. senegalensis.*

# CHAPTER V

# CONCLUSIONS AND FINAL CONSIDERATIONS

In the 2006 breeding period, the collected eggs of *Solea senegalensis,* had an average percentage of viability of 50.91% (in the range of [4.54; 90.00]%), diameter of 985.49 µm (in the range [952.5; 1012.5] µm), percentage of fecundation of 94.89% (range [0; 100]%) and an average percentage of hatching of 82.88% (range [26.67; 100.0]%).

When compared with the nonviable eggs, the viable eggs had more enzymatic activity of the following enzymes: acid phosphatase, alkaline phosphatase, glucose-6-phosphatase, glucose-6-phosphate dehydrogenase, aspartate aminotranseferase and higher levels of ATP and ribose. Nonviable eggs showed higher activity malate dehydrogenase and higher levels of monosaccharides, sialic acids and ketoses.

As a result of the present work it is proposed the use of glucose-6-phosphate dehydrogenase activity, heptoses and 6-deoxyhexoses concentrations as bio-markers for egg quality determination in *Solea senegalensis*.

The present work also leave some questions for future investigations in egg quality of the Senegalese sole, since the reasons why some of the assays of the original set did not produce any results, which have worked in other different species, still remain unknown. This suggests that there may be species-specific adjustments that need to be met in the future. Unveiling this information might increase the knowledge of the mechanisms involved in oocyte growth and development, and of how these mechanisms are coordinated, which is essential to fully understand the factors that affect egg quality.

Several studies have demonstrated the influence of female age and size in egg quality; however, no data related to female age was studied. Since the eggs were collected from a communal broodstock that naturally spawned it was not possible to determinate the parental contribution in the hatching larvae, nor was it possible to collect the eggs right after spawning which prevented analytic procedures to be performed in the same stage of development. This could be masking other possible relations between the analyzed biochemical parameters and egg quality. Different egg collection and sampling design could be formulated to minimize the differences in developmental embryonic stages. Microsatellite identification of broodstock and of egg batches may be used to identify the parental contribution into the offspring, the maternal contribution to egg quality and

composition, and for the discrimination between egg batches, which might provide further useful information.

Finally, and considering that in the present work the analyzed eggs came from a spawning season of lower viability when compared to what is reported in previous works, analyzing different spawning seasons may confirm, or refute the findings in the present work, since parameters may vary.

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