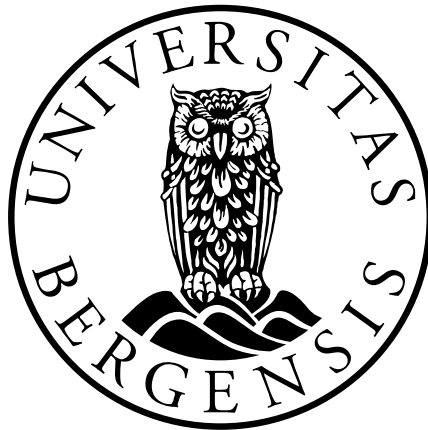


**Male reproductive biology of**  
**European hake**  
***Merluccius merluccius***

Anne-Laure Groison



Dissertation for the degree philosophiae doctor (PhD)  
at the University of Bergen

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**“My grandfather once told me that there are two kinds of people:  
those who work and those who take the credit.  
He told me to try to be in the first group; there was less competition there.”**

Indira Gandhi



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*Ce travail est dédié à Théo, Tibouts, Mazarine, Abel et Léon*



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## Scientific environment

This study was undertaken at the Department of Biology, University of Bergen (UoB) with Principal Scientist Dr. Olav Sigurd Kjesbu, Institute of Marine Research, Bergen as the principal supervisor and Professor Dr. Ian Mayer, UoB as secondary supervisor from 2008 until 2010.

Professor Dr. Audrey Geffen, UoB, was my main supervisor from October 2004 to 2007, and Dr. Anne Christine Utne-Palm, UoB was my secondary supervisor from mid-2006 until 2007.

Sampling conducted in French waters were undertaken in collaboration with Ifremer, Plouzané, France. The resulting studies on sperm motility analysis were performed in collaboration with Marc Suquet (Ifremer, Argenton, France); the computer assisted sperm analysis with Christian Fauvel (Ifremer, Palavas, France); the flagellar wave characteristics analysis with Jacky Cosson (CNRS, Université P. & M. Curie, Villefranche-sur-Mer, France) and the determination of adenylate compounds content with Jean-René Le Coz (Ifremer, Plouzané, France).

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## List of publications

**PAPER I - Groison, A.L.**, Suquet, M., Cosson, J., Le Coz, J.R., Jolivet, A. & Garren, F. (2008): “Biological characteristics of European hake (*Merluccius merluccius*) sperm”, *Cybium*, Vol. 32: 178.

**PAPER II - Cosson, J., Groison, A.L.**, Suquet, M. & Fauvel, C. (2008): “Motility characteristics of spermatozoa in cod (*Gadus morhua*) and hake (*Merluccius merluccius*)”, *Cybium*, Vol. 32: 176-177.

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**PAPER IV - Groison, A.L.**, Fauvel, C., Suquet, M., Kjesbu, O.S., Le Coz, J.R., Mayer, I. & Cosson, J. “Some characteristics of sperm motility in European hake (*Merluccius merluccius*, L., 1758)” Conditionally accepted to the *Journal of Applied Ichthyology*.

**PAPER V - Cosson, J., Groison, A.L.**, Fauvel, C. & Suquet, M. “Description of hake (*Merluccius merluccius*) spermatozoa : flagellar wave characteristics and motility parameters in various situations” MS submitted.

**PAPER VI - Groison, A.L.**, Kjesbu, O.S. & Suquet, M. “Sexual dimorphism of drumming muscles in European hake (*Merluccius merluccius*)” MS submitted.



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## Abstract

The reproductive biology of European hake (*Merluccius merluccius*) has been studied extensively in the field, but mainly focusing on fecundity regulation in females and its implications for the fishery. The European hake is highly important commercially throughout its geographical range. Because catches have been decreasing since the 1960s, interest in hake as a potential aquaculture species has recently increased. However, for successful domestication of hake, a better understanding of its reproductive biology, including sperm biology, is needed for purposes of broodstock management and also for the development of sperm storage techniques, including cryopreservation.

The objectives of this thesis were to assess hake sperm quality, including sperm production characteristics and energetics, and to characterize sperm movement parameters such as the percentage of motile cells, sperm velocity and flagellar beats. Changes of these parameters over time following activation were evaluated qualitatively for fresh hake sperm. The effects of i) salinity of the activation medium, ii) survival in relation to short term storage duration at 4°C, and iii) sperm cryopreservation on sperm motility characteristics, as well as the reliance on the sperm's energetic content, were evaluated.

In many other Gadoid species, drumming muscles are an important component of reproductive behaviour in spawning males: the contraction of these muscles associated to the swim-bladder results in an audible 'drumming' sound during the courtship of the females. However, the presence of drumming muscles has never been reported for hake. Mature hake collected for the sperm analyses, as well as mature females and immature individuals were thus dissected to investigate the presence of drumming muscles and the existence in mature males of potential correlations between their morphological characteristics and the sperm motility parameters.

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Hake sperm were collected from mature males caught during the summer-early autumn waters off western Norway and during the winter-early spring in the Bay of Biscay (France). Sperm quality characteristics were assessed after storage at 4°C for  $25 \pm 14$  h for transportation. The total ATP, ADP and AMP concentrations were measured using high-performance liquid chromatography followed by calculation of the Adenylate Energy Charge (AEC). Computer Assisted Sperm Analysis (CASA) was used to measure a series of head parameters characterizing the sperm swimming performances. The flagellar characteristics of spermatozoa were explored by high resolution video images. Dissections of mature and non-mature European hake males and females were conducted to investigate the presence of drumming muscles.

Sperm production characteristics were evaluated for both Norwegian (Nw) and French (Fr) sperm samples: sperm volume in ml (Nw:  $3.9 \pm 4.0$ ; Fr:  $2.6 \pm 4.0$ ), spermatozoa concentration (in  $\times 10^9$  spermatozoa / ml) (Nw:  $6.6 \pm 3.2$ ; Fr:  $13.9 \pm 5.1$ ), spermatocrit in % (Nw:  $80.2 \pm 3.3$ ; Fr:  $81.8 \pm 10.7$ ) and total number of spermatozoa (in  $\times 10^9$ ) (Nw:  $23.5 \pm 30.0$ ; Fr:  $35.1 \pm 36.2$ ). Osmolality ( $349 \pm 28$  mOsmol / kg) and pH ( $7.6 \pm 0.1$ ) of French samples were also measured.

When sperm was activated with 100% filtrated sea water (100 SW), the percentage of motile sperm, the velocity, the straightness of the movement, the flagellar beat frequency, the wave amplitude, the number of flagellar waves and the linearity of flagellar waves shape were initially at maximum but decreased sharply later. As a result, active sperm motility sufficient to allow the sperm to reach and fertilize the egg is limited to only a brief time period post-activation ( $< 100$  sec). When transferred into 50 % sea water diluted with distilled water (50 SW), the percentage of motile sperm and the velocity of the movement increased initially but subsequently reached a maximum followed by a decline. Sperm were motile for a longer duration (up to *ca.* 1600 s) when activated with an activating medium of lower salinity (50 SW: 498 mOsmol / kg) compared to 100 SW (998 mOsmol / kg) (*ca.* 450 s).

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Sperm storage: initial percentage of motile sperm in 100 SW, velocity and straightness of the movement were at maximum after 0.5 - 1 day storage duration and then decreased gradually to reach their minima after about four days. Further, both the Adenylate Energy Charge ( $AEC = 0.78 \pm 0.07$ ) and the Adenosine triphosphate ( $ATP = 85 \pm 80 \text{ nmoles} \times 10^9 \text{ spermatozoa}$ ) content decreased with storage duration (minima reached after *ca.* two days:  $0.20 \pm 0.09$  and  $5 \pm 4$  respectively).

Cryopreservation significantly negatively affected the percentage of motile sperm, with 0 - 76.4 % motile sperm following thawing.

Dissections of hake fish demonstrated for the first time that this gadoid species contains drumming muscles. The results indicated that sound production by adult hake males was much more frequent during the spawning season than in the rest of the year, i.e., formed a central component of the mating system in this species.

The present thesis represents a first step towards a better comprehension of the male reproductive biology of European hake, for which no studies were published. Basic knowledge on sperm biology, including movement characteristics, cell energy but also the effects of salinity on sperm movement were recorded. Based on these descriptive elements, sperm management methods were tested such as short term storage and cryopreservation techniques.

While hake exhibited only low sperm production, this was compensated by the fact that hake sperm were actively motile for a relatively long time. While this thesis has increased our knowledge on the sperm characteristics of hake further research is needed on determining the relationship between sperm quality and fertilization success, including after cryopreservation procedures. The availability of a hake broodstock facility would be beneficial for future studies in determining which sexual characteristics (drumming muscles, sperm quality) influence male reproductive success.



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## 1. INTRODUCTION

### 1.1. Reproductive biology studies: males are also important

The subject of reproductive biology of fish populations is generally dominated by studies on the female gender, and the stock reproductive potential (SRP) (Trippel, 1999) is therefore largely measured by ‘total egg production’ (e.g. Tomkiewicz et al., 2003). It is generally assumed that there is always an excess of sperm compared to the availability of eggs and this is one of the many reasons why male state-variables have not been included in estimating the SRP (Nash et al., 2008). However, whether the SRP is adequately represented by egg production alone requires further testing (Marshall et al., 1998).

Considerable effort has been made in recent years to measure maternal factors affecting fecundity, egg size and fertilization success of gadoids that spawn freely in captivity (Hislop et al., 1978; Kjesbu et al., 1996; Trippel, 1998; Thorsen et al., 2003). In these studies, paternal factors may have partly influenced the results (especially related to fertilization) and thus affecting the SRP, such as variations in male number and sperm production (Trippel, 1999, 2003; Tomkiewicz et al., 2003). According to the genetic incompatibility hypothesis (e.g. Curtsinger, 1991; Jennions & Petrie, 2000; Zeh & Zeh, 2003), when a particular male fertilizes eggs from a particular female this is expected to do well, whereas sperm from other competing males fertilizing the same batch of eggs do poorly. Such sire-dam interactions have been demonstrated empirically. As this appears to be a general phenomenon, according to Nordeide (2007) this might have major implications for the commercial aquaculture industry, concerning fertilization method, methods to preserve gametes, spawning protocols, and breeding programs. Likewise, indications of sperm quality related to fish size may affect the reproductive potential in exploited fish stocks. The quality of

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both gametes may affect fertilization success and larval survival (Rurangwa et al., 2004).

Despite this, studies on male reproduction in teleost fishes are relatively few, although the number of publications has increased during the last decade. Trippel (2003) reviewed a number of experimental protocols that have been employed over the years to assess male fertility. The review is broad in nature, and includes references to a number of marine fishes. It concentrated on exploited species that occur in the North Atlantic and Baltic Sea within the taxonomic groups gadidae, pleuronectidae and clupeidae.

Interactions between sexes are consistent with the hypothesis that females, and possibly males, undertake mate choice (Hutchings et al., 1999). Mating competition in fishes usually occurs among males and may take the form of either competition for access to females or sperm competition for the fertilization of eggs (Rakitin et al., 1999a). Mating strategies of species differ and these questions may be inter-related with spawning behaviour (Trippel, 2003). Selection process involves mechanisms by which the quality of the fertilizing spermatozoon is controlled, thereby ensuring that females and their offspring receive high quality genetic material.

## **1.2. Sperm competition for the fertilization of eggs. Sperm quality and associated definitions**

A sperm, from the ancient Greek word “seed” and “living being” and more commonly known as a sperm cell, is the haploid cell that is the male gamete.



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In externally fertilizing teleosts, including all gadoids, sexual selection can also occur following spawning, and is termed post-copulatory sexual selection. This selection can be achieved by either sperm competition, where sperm from different males compete to fertilize a given set of eggs (Parker, 1970), or by cryptic female choice, where females bias reproductive success towards sperm from particular males (Eberhard, 1996). Currently, there is intense interest in this field, as both processes are potential agents for directional sexual selection (Birkhead & Møller, 1998; Birkhead & Pizzari, 2002). To date, little is known of the relative importance of these two processes on post-copulatory sexual selection in externally fertilizing fish. The current assumptions are that the primary determinants of sperm competition is sperm motility (Lahnsteiner et al. 1998; Gage et al., 2004; Casselman et al., 2006; Rudolfson et al., 2008) although sperm quantity can also be important (Trippel, 2003), and that cryptic female choice is driven by modification of sperm motility in female ovarian fluid (Urbach et al., 2005; Nordeide, 2007; Rosengrave et al., 2008).

The most common factors employed in studying sperm biology are the spermatozoa structure and the sperm motility parameters, the biochemistry of the sperm cells and of the seminal plasma, and the metabolism of spermatozoa (respiration and energetics of motility) (Billard & Cosson, 1992; Billard et al., 1994). The sensitivity of sperm to storage and cryopreservation is important information for broodstock management programs. This can be assessed by studying the activation, motility, and fertility of stored sperm including freezability for cryopreservation (Billard & Cosson, 1992; Billard et al., 1994).

### 1.2.1. Sperm formation

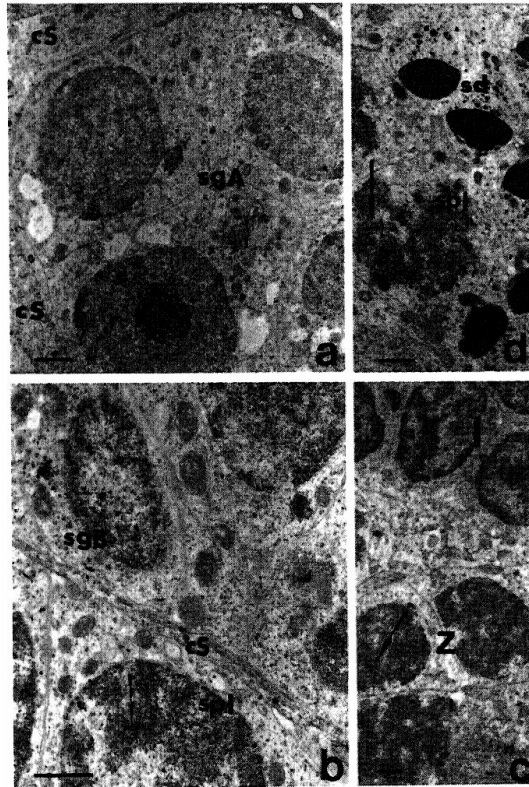


Fig. 1.1. Electron micrographs of testis of hake, *Merluccius merluccius* L.

a) Type A spermatogonia enclosed by Sertoli cell processes; b) Type B spermatogonia and primary spermatocytes separated by a Sertoli cell; c) spermatocytes in leptotene and zygotene; d) primary spermatocytes and spermatids. *cS*, Sertoli cell; *l*, spermatocytes in leptotene; *sd*, spermatids; *sgA*, type A spermatogonia; *sgB*, type B spermatogonia; *spI*, primary spermatocytes; *z*, spermatocytes in zygotene; arrow, synaptonemal complex. Uranyl acetate – lead citrate. Bar, 1 µm. (from Desantis et al., 2000).

Spermatogenesis is a sequence of differentiating events taking place in male germ cells. It begins in proliferating and differentiating spermatogonial stem cells (i.e. cells characterized by the ability to renew themselves through mitotic cell division and differentiating into a diverse range of specialized cell types). The population of spermatogonia divides into two groups: one is that of spermatogonia in an

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undifferentiated state and the other is that of spermatogonia in a differentiated state; these are designated type A spermatogonia (primary spermatogonia) and type B spermatogonia (secondary spermatogonia), respectively (Fig. 1.1). Type B spermatogonia undergo several mitotic divisions and differentiate into primary spermatocytes (Fig. 1.1). After meiosis, they differentiate into spermatids (Fig. 1.1). In the spermatids, multiple events, such as nuclear remodeling, organelle assembly and flagellum formation, etc., take place; together, these events are called spermiogenesis. The completion of spermiogenesis results in the formation of viable spermatozoa capable of fertilization.

### *1.2.2. Sperm morphology and ultrastructure*

Sperm is defined as spermatozoa (Fig. 1.2) plus seminal plasma. Details and functions of the various morphological structures are presented in Figure 1.2. Centrioles are involved in organizing microtubules in the cytoplasm. The position of the centriole determines the position of the nucleus and plays a crucial role in the spatial arrangement of the cell. Sperm of teleost fish can be divided into two groups characterized by either internal or external fertilization. Sperm of teleost species with external fertilization are termed aquasperm. They mostly have an ovoid or spherical nucleus which measures  $< 5 \mu\text{m}$  along its maximal extension, the midpiece is also small and measures 2-4  $\mu\text{m}$  in length and contains only few mitochondria (mostly 1-6) (mitochondria have their own independent genome, generate most of the cell's supply of adenosine triphosphate (ATP), used as a source of chemical energy). Such spermatozoa can be monoflagellate or biflagellate. The flagellum measures circa 30-40  $\mu\text{m}$ .



Fig. 1.2. Schematic reconstruction of teleost spermatozoon. cc = cytoplasmic canal, dc = distal centriole, fl = flagellum, hr = head region, lr = lateral ribbon of the flagellum, mi = mitochondrion, mp = midpiece, nu = nucleus, pc = proximal centriole, pm = plasma membrane, tr = tail region (from Lahnsteiner & Patzner, 2008).

### *1.2.3. Biochemistry of the sperm cells and of the seminal plasma*

The seminal plasma has a unique composition: some components support the spermatozoa, while others reflect the functions of the reproductive system and the spermatozoa (Ciereszko et al., 2000). Studies on sperm characteristics are necessary to understand the basic biochemical processes that affect sperm motility and fertilization (Linhart et al., 1991; Coward et al., 2002; Ingermann et al., 2002; Wojtczak et al., 2003), to evaluate the reproductive abilities of different fish species (Billard, 1986; Coward et al., 2002; Rurangwa et al., 2004; Alavi & Cosson, 2005, 2006) and to improve methods for short- and long-term storage of fish sperm (Piros et al., 2002).

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The main role of seminal plasma is to create an optimal environment for the storage of spermatozoa, very often for a prolonged time (Ciereszko, 2008). During storage, sperm fertilizing ability, sperm motility, and viability must be protected. Damage to any of these functions is likely to result in failure of fertilization. In most of the fish species studied so far, spermatozoa are immotile in the testis and seminal plasma (Stoss, 1983; Billard, 1986), in contrast to the situation in reptiles or mammals (Krasznai et al., 1995). The osmolality and composition of the seminal plasma usually prevent sperm motility in the fish sperm duct (Billard, 1986). The seminal fluid not only immobilizes the spermatozoa, but also protects them (see Methods in Cosson et al., 1997). In marine species, activation is generally triggered when sperm is diluted in media of high osmotic pressure (OP) (Morisawa, 1985). Thus, sperm are normally activated only following release into the marine environment; there are clear relationships between seminal plasma composition and osmolality and the duration of fish sperm motility.

Spermatocrit is a measure of the percentage of the total volume of sperm occupied by spermatozoa; high spermatocrit should yield high fertilization rates because of the higher density of spermatozoa per unit volume of sperm (Bouck & Jacobson, 1976). Concentration of spermatozoa is especially an important factor determining fertilization success in aquatic animals with external fertilization. Rakitin et al. (1999b) state that sperm density is a principal factor contributing to male fertilization success.

#### *1.2.4. Energy metabolism in fish spermatozoa*

Fish reproduction is dependent upon successful fertilization of eggs which, in turn, is dependent on the metabolism and appropriate functioning of spermatozoa. Spermatozoa remain quiescent for relatively long periods in the testes and reproductive tract and during *in vitro* storage. In contrast, they are generally highly

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active and motile for very short durations after discharge from the male reproductive tract into the surrounding aqueous medium (seawater or freshwater for marine and freshwater species respectively), or into an appropriate activating medium (AM). Fish spermatozoa exhibit a hypermotile behaviour (high but brief beat frequencies, BF) (Cosson et al., 2008b) remarkably similar to the hyperactivated motility exhibited by mammalian spermatozoa in the vicinity of eggs before fertilization. The hypermotility of fish spermatozoa is demonstrated by a high velocity and a fast consumption of energy, which was accumulated during spermatogenesis (Cosson et al., 2008b). Motility is initiated and maintained by the hydrolysis of ATP catalyzed by dynein ATPase that is coupled to the sliding of adjacent microtubules leading to the generation of flagellar movement (Tibbs, 1959; Gibbons, 1968; Gatti et al., 1989; Omoto, 1991; Ingermann, 2008). *In vitro* observations combined with ATP measurements have led to the explanation that changes in the internal ionic concentration occurring in response to external osmolality could control fish sperm motility (Cosson et al., 2008b).

### *1.2.5. Sperm motility and the methods to analyse the movements of fish spermatozoa and their flagella*

#### **Sperm motility**

Sperm motility is the most commonly used parameter for evaluating sperm quality and is regarded as the most reliable sperm characteristic to be used as an indicator of male reproductive potency (Daye & Glebe, 1984; Munkittrick & Moccia, 1987). This estimation is important as sperm must generally be fully motile to achieve egg penetration through the micropyle (Chauvaud et al., 1995). Motility is induced after the sperm are released into the aqueous environment (marine or freshwater environment depending on the species) during natural reproduction or into the AM

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during artificial reproduction. Media that are hyper-osmotic relative to seminal fluid trigger sperm motility in marine fishes (Morisawa, 1985; Cosson et al., 2008b). Sperm motility characteristics have been documented in a number of marine fish species (for review see Stoss, 1983; Billard et al., 1994, 1995; Inaba, 2003; Alavi & Cosson, 2005, 2006; Lahnsteiner & Patzner, 2008; and a book, Fish Spermatology Alavi et al., 2008) and in particular the influence of sperm motility on egg fertilization rates (e.g. Okada & Ito, 1955; Billard & Cosson, 1992; Trippel & Neilson, 1992). According to Trippel (2003) high sperm motility and its associated greater fertilization range should be positively correlated with fertilization success. While most studies on Northwest Atlantic cod (*Gadus morhua*) and several other species have failed to show a positive correlation between sperm motility and fertilization success (Scott & Baynes, 1980; Trippel & Neilson, 1992) a recent study by Skjæraasen et al. (2009) demonstrated that fertilization rates in Atlantic cod was positively correlated to sperm motility (curvilinear velocity). Fast swimming sperm are more likely to encounter an egg's micropyle than slow-swimming sperm (Trippel & Neilson, 1992). The percentage of motile sperm and the sperm velocity have been correlated to reproductive success in other species as e.g. boque (*Boops boops*) or striped mullet (*Mullus barbatus*) (Lahnsteiner & Patzner, 1998; Rurangwa et al., 2004). The short duration of sperm motility is consistent with the short duration over which eggs are capable of being fertilized (Hoysak & Liley, 2001; Cosson, 2004). Osmotic damage is the basis of, or contributes to the generally brief duration of sperm motility in most teleost species (Cosson, 2008, 2010). Although sperm motility has been commonly measured in water, a sperm cell shortly after being released from the testis may encounter ovarian fluid (OF) and (or) be in close proximity to an egg's surface (Litvak & Trippel, 1998). Maternal factors such as the presence of OF have been shown to prolong the period of sperm motility in a number of teleost species (Elofsson et al., 2003). The chemical characteristics of the motility-inducing medium essentially determines the duration of sperm motility even though other factors such as the sperm's energy stores will also

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have a strong influence on sperm velocity and other motility parameters (Cosson, 2004).

Just after activation by the surrounding medium, sperm flagella of most fish species beat at a high frequency, up to 100 Hz, for only a very short period of time, i.e. thirty seconds to a few minutes depending of fish species, although sperm can remain motile for much longer periods in some fish species, such as the Atlantic cod. The initial velocity of sperm cells is very high (up to 300  $\mu\text{m/s}$ ), which makes observations and records difficult (Cosson, 2008). To study the motility characteristics (sperm velocity, percentage of motile sperm and flagellar wave shape and its propagation) of these fast moving cells and to visualize their flagella, several types of methods have been developed allowing obtaining images of the tracks followed by the sperm head, or alternatively high speed images of moving flagella *in vivo*. Nowadays, these methods use video techniques, and in some cases allow to record frames thanks to stroboscopic illumination.

### **Methods to analyse the movements of fish spermatozoa and their flagella**

The computer assisted processing of the head tracks (computer assisted sperm analysis, CASA) is a useful tool to achieve reliable results to analyze sperm quality, as this system can track and analyse the movement of up to 200 sperm simultaneously using an automatic process. The CASA system allows for the quantification of different sperm motility parameters over time, and represents the most objective and comprehensive method currently available for the evaluation of sperm quality parameters (Wilson-Leedy & Ingermann, 2007). Sperm tracking systems such as CASA were initially developed to examine sperm quality in mammals and birds, but in recent years has been applied on fish sperm as well (Kime et al., 2001; Rurangwa et al., 2004; Van Look & Kime, 2004). CASA (Hobson sperm tracker) facilitates rapid assessment and has been successfully used to estimate different parameters of



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the movement of fish sperm (Kime et al., 2001): forward velocity, mean angular head displacement, beat-cross frequency, amplitude of lateral head displacement and straightness of the spermatozoa head trajectory. Also, CASA has been employed in the determination of individual variation of sperm quality (Lahnsteiner & Patzner, 1998).

By use of high resolution video microscopy combined with stroboscopic illumination (Cosson et al, 1997; Cosson, 2008), various parameters describing the flagellar behaviour during the motility period can be also investigated, including the measurement of the percentage of motile sperm, the beat frequency, and the straightness of the sperm tracks. Details on flagellar shape, wave length and amplitude can be obtained as a function of time elapsed in the motility period, and in addition, measurement of flagellar beat frequency at different temperatures can allow for a better understanding of energetic constraints involved in the sperm movement.

### *1.2.6. Sperm cryopreservation*

As fish farming expands and harvesting of wild stocks becomes more intense, there is a growing need for techniques of gamete storage to facilitate artificial reproduction procedures. Further knowledge on sperm characteristics is necessary to improve methods for short- and long-term storage of fish sperm (Piros et al., 2002). Sperm cryopreservation is a process where cells are preserved by cooling to low sub-zero temperatures, such as (typically)  $-196^{\circ}\text{C}$  (the boiling point of liquid nitrogen). At these low temperatures, any biological activity, including the biochemical reactions that would ultimately lead to cell death, is effectively stopped. Phenomena which can cause damage to cells during cryopreservation are mainly occurring during the freezing stage but can be significantly reduced by cryoprotectants such as dimethyl sulfoxide (DMSO), egg yolk, glycerol or sucrose. Cryopreserved gametes can

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theoretically be stored for between 200 and 32 000 years without deleterious effect (Ashwood-Smith, 1980). Sperm cryopreservation allows for the availability of gametes at any time and the selection of the best broodstock to establish sperm banks, avoiding also the risk of infection transmission (Asturiano et al., 2004). Sperm cryopreservation has been established for a number of teleosts (Scott & Baynes, 1980; Stoss, 1983; Billard et al., 1995; Rana, 1995; Glogowski et al., 1999; Suquet et al., 2000). Most publications are devoted to salmonids, tilapias and carps. In marine fish, the first report was by Blaxter (1953) in herring (*Clupea harengus*). Despite the need for further cryopreservation research, technology currently exists for the long-term storage of male gametes from only a selected number marine species (Mounib, 1978; Leung & Jamieson, 1991; Suquet et al., 2000; Ohta et al., 2001; Rideout et al., 2003).

While the development of cryopreservation techniques will be mostly used for aquaculture purposes these preservation methods may have a significant role in future conservation programmes. An increasing number of teleost species are threatened with extinction. The cryopreservation of the gametes of these species will be an important strategy in helping to maintain these threatened fish species.

### **1.3. Competition for the access to females : drumming muscles and associated definitions**

The gas filled swim-bladder is a characteristic feature of the viscera of most teleost fish. Its primary function is as a buoyancy regulator. In many teleosts there are, external to the swim-bladder muscles themselves, a special group of muscles attached to the ventral wall of the swim-bladder. These special muscles, composed of striated muscle fibres, are called “drumming” muscles as their rapid contractions cause the

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swim-bladder wall to vibrate or “drum” (Jones & Marshall, 1953) (Fig. 1.3). Fishes have evolved the largest diversity of sound-generating organs among vertebrates (Ladich & Fine, 2006). The main group of sound-producing mechanisms is based on swim-bladders and their associated drumming muscles.

Similar to other vertebrates, bony fishes vocalize in numerous behavioural contexts. They produce distress sounds when they are disturbed, caught, or hand held. Representatives of more than 30 families produce sounds during intraspecific aggressive interactions, in particular when defending their territories or feeding sites (Ladich & Myrberg, 2006). In the reproductive context, representatives of more than 20 families produce sounds to advertise their nest sites, attract females, and promote courtship and spawning (Myrberg & Lugli, 2006).

Conspicuous song and other acoustic displays produced by animals during the breeding season may vary between females and males, across size or age classes, and among individuals in ways that reveal much about their function. In many species, including a neotropical frog (*Physalaemus pustulosus*) and the bicolour damselfish (*Stegastes partitus*), experimental evidence has demonstrated that song and other acoustic displays by males may be favoured by sexual selection because females prefer to mate with males that have the most exaggerated features, such as the largest song repertoire or the loudest, longest, or lowest pitch acoustic displays (Ryan, 1983; Myrberg et al., 1986; Andersson, 1994). Song and other acoustic displays may also be favoured by sexual selection through their role in intrasexual contests for access to mates. For instance, possession of a good territory is crucial for male mating success in many animals (Andersson, 1994), and song or other acoustic displays may aid in territory defense, as demonstrated in red-winged blackbirds (*Agelaius phoeniceus*) (Peek, 1972; Smith, 1976, 1979). Such differences in the force of sexual selection on males versus females often make sexual dimorphism an important aspect of display trait variation.

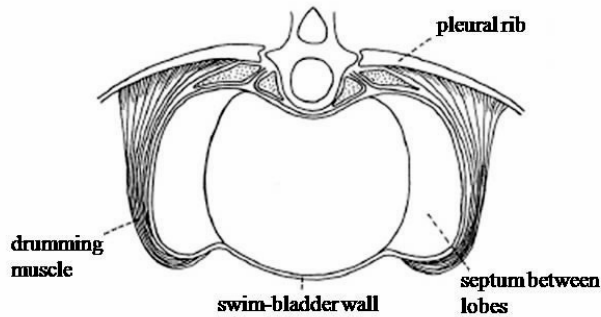


Fig. 1.3. Diagrammatic transverse section of swimbladder at the level of the drumming muscles in cod (*Gadus morhua*) (from Brawn, 1961).

#### 1.4. The European hake *Merluccius merluccius*

At present 13 species of the genus *Merluccius* (the ‘hakes’) have been described worldwide (Lloris et al., 2003) and are distributed mainly in the Atlantic Ocean and the east coast of the Pacific Ocean (Fig. 1.4). The hakes are of major commercial importance throughout their geographical distribution range. According to the Food and Agriculture Organization (FAO) statistics, the world catch of hake increased during the 1960s and reached a maximum of 2.1 million tonnes in 1973. Catches then decreased to 1.1 - 1.2 million tonnes during the first half of the 1980s, then reached another peak between 1987 and 1989, before declining again to 1.2 million tonnes in 1991 (Pitcher & Alheit, 1995). Compared to other species of the same genus, the European hake (*Merluccius merluccius*, Linnaeus, 1758) is far from being the most important in terms of catches. However, it is one of the most studied of all the *Merluccius* species (Alheit & Pitcher, 1995).

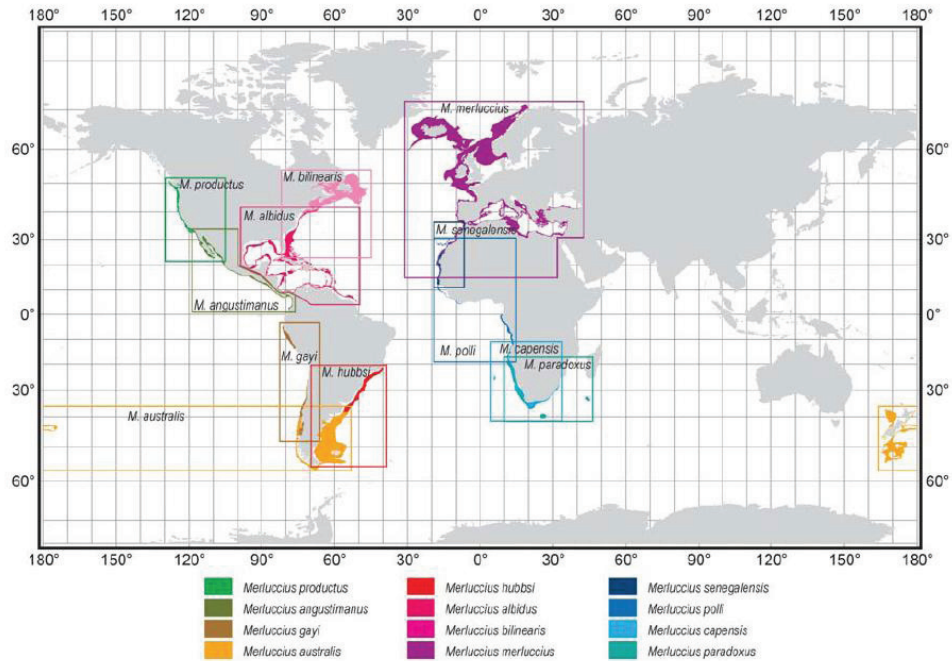


Fig. 1.4. Geographic distribution of the species of the genus *Merluccius* (from Lloris et al., 2003).

### 1.4.1. Taxonomy and morphological description

Subphylum: *Vertebrata*

Superclass: *Gnathosmata*

Class: *Actinopterygii*

Division: *Teleostei*

Subdivision: *Euteleostei*

Superorder: *Paracanthopterygii*

Order: *Gadiformes*

Family: *Merluccidae*

Genus: *Merluccius*

Species: *merluccius*



Fig. 1.5. A) European hake (*Merluccius merluccius*, Linnaeus, 1758) (from Lloris et al., 2003). B) The author with a 72 cm (total length) and 3 kg (total weight) female European hake caught in Norwegian waters in August 2006.

European hake, from now on simply referred to as hake, is a gadiform from the class Actinopterygii (ray-finned fishes). As in all hake species, it has a fusiform symmetric body covered by small scales (Fig. 1.5. A, B). Detailed descriptions of the anatomical features can be found in the literature (e.g. Inada, 1981; Svetovidov, 1986; Lloris et al., 2003). Maximum recorded size in the Atlantic Ocean is 140 cm and 15 kg, although rarely exceeding 100 cm and 10 kg (Cohen et al., 1990).

#### 1.4.2. Distribution

The **bathymetric distribution** of hake covers both the deep shelf and the upper part of the continental slope, but may also occur from inshore waters (30 m) to deep-bathyal waters (1000 m) (Stefanescu et al., 1992). As a consequence, the hake is regarded as a demersal and benthopelagic species. In the Mediterranean Sea, hake are generally found between 70 and 370 m depth, with greatest abundances of individuals commonly found between 100 - 200 m (Recasens et al., 1998; Abella et al., 2005). However, the bathymetric range at each area depends on two aspects and their

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interaction: the ontogenic change in experienced habitat (cf following part 1.4.3) and the geomorphology of the bottom (Hidalgo et al., 2008).

European hake is *geographically distributed* throughout the North-east Atlantic from Norway in the North (around 70°N) to the Guinea Gulf in the South (around 40°S) (Fig. 1.4) (Casey & Pereiro, 1995; FAO, 2006). Longitudinally, the European hake can be found as far west as Iceland (around 27°W), and as far east as the Black Sea (40°E), having first colonized the whole Mediterranean Sea (Fig. 1.4). European hake occupies the widest latitudinal and longitudinal ranges of all the *Merluccius* species (Fig. 1.4) and it is one of the most widely distributed of the hake family (family Merlucciidae). Although the European hake is commonly defined as a temperate waters species, the high heterogeneity of areas with different temperatures, salinity, productivity conditions and trophic resources indicates the high adaptative capacity and phenotypic plasticity of this species.

For management purposes it is assumed that there are two stock units of European hake within its distribution range in the Atlantic which are defined and assessed separately: the so-called Northern stock (from Portugal to Norway: ICES Subareas IV, VI and VII and Divisions IIIa and VIIIa, b, d), and the Southern stock (from the northwest coast of Spain to Morocco: ICES divisions VIIIc and IXa) (Fig. 1.6.A). This separation into two stocks is based on two main criteria. The first concerns the identification of a geographical barrier, the Cape Breton Canyon, a 4000 m deep trench situated along the wide French continental shelf (Fig.1.6.A) which separates the waters of Spain from those of France in the extreme south-eastern corner of the Bay of Biscay. This feature has been traditionally considered as a geographical boundary limiting any exchange between the two supposed populations. The second is based on observations relating to the spawning behaviour and distribution of the juveniles of the two populations. Two main spawning areas have been identified: one area is located off the French coast in the Bay of Biscay mainly concentrated on the shelf break (Álvarez et al., 2001, 2004) and to a lesser extent, in the Celtic Sea; the

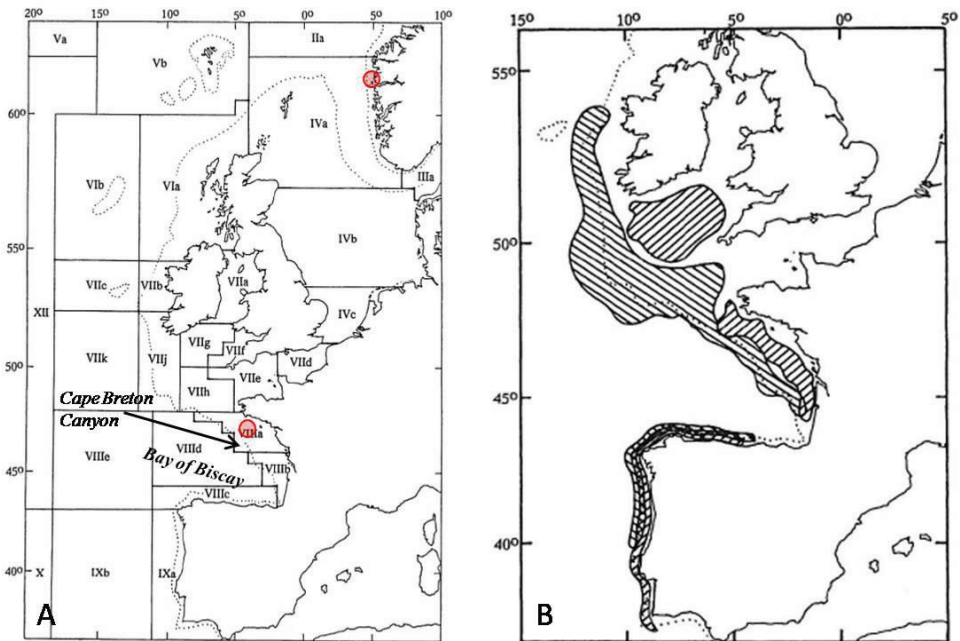


Fig. 1.6. A) Statistical zones used for the collection of fishery statistics by the International Council for the Exploration of the Sea (ICES) (from Casey & Pereiro, 1995). Red circles: sampling stations of European hake gametes. The Cape Breton Canyon is indicated by the arrow and is superimposed with the continental shelf (dotted line). B) European hake main spawning and nursery areas. Hatching sloping left to right, spawning areas; hatching sloping right to left, main nursery areas (from Casey & Pereiro, 1995).

second spawning area occurs off the north-west coast of the Iberian Peninsula (Fig.1.6.B). It is postulated that fish recruiting to the Northern stock originate from the Celtic Sea / Bay of Biscay spawning, whereas those recruiting to the Southern stock originate from the spawning to the north-west of the Iberian Peninsula (Casey & Pereiro, 1995). However, some controversy exists about the stock structure of European hake in the Atlantic. Some authors consider that there is no separation between the traditional Northern and Southern stocks because of intense gene flow related to the current systems of this area (Balado et al., 2003). Moreover, recent studies based on genetic and biological parameters indicate that the Cape Breton



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Canyon is not an effective barrier to migration within the Bay of Biscay (Lundy et al., 2000). Recently, in the north Atlantic area, at least two different population units of hake were shown to be present (Mattiucci et al., 2004; Lo Brutto et al., 2004; Castillo et al., 2005). These results are relevant for management purpose but the boundary between these stocks should likely be reconsidered based on biological evidence.

### *1.4.3. Life cycle*

Over its life-cycle, the different life-history stages of the hake are found at different bathymetric ranges in the water column. This pattern of ontogenic shifts in habitat is similar in both the Atlantic Ocean and the Mediterranean Sea: hake adapts its life cycle to the seasonality of optimal environmental conditions in each area.

It is generally believed that mature hake aggregate and spawn at depths ranging between 75 to 150 m along its Atlantic distribution range (Hickling, 1927; Coombs & Mitchell, 1982; Álvarez et al., 2001, 2005) at water temperatures of 10-13°C (Arbault & Boutin, 1968; Coombs & Mitchell, 1982; Álvarez et al., 2001). The location of the hake at the time of spawning seems to vary according to locality. Hickling (1930) and Belloc (1935) observed that non-spawning concentrations of ripe adults can occur in deep water, but that spawning takes place in the shallower waters of the continental shelf. However, Pérez & Pereiro (1985) observed spawning in the deep waters at the edge of the continental shelf in the region of the Iberian Peninsula. Literature shows that *Merluccius merluccius* exhibits an indeterminate fecundity: oocyte development is supported by food intake (i.e. income breeder) during spawning season rather than from reserves, which allows for the fine-tuning of egg production in response to energy surplus (Murua et al., 1998; Murua & Motos 2006; Domínguez-Petit et al., 2009). Many other related marine species are determinate capital spawners including most gadoids (Rideout & Burton, 2000; Murua & Saborido-Rey, 2003). Hake, in common with most gadoids, have a high fecundity, and spawns successive egg

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batches with females releasing up to 165 small pelagic eggs per gram of gutted female weight (Murua et al., 1998) (egg size:  $1.06 \pm 0.10$  mm; Groison, unpublished data), with a batch interval of 5 - 12 days (Murua & Motos, 2006). As comparison, mature wild cod female produce 250 eggs per gram of gutted weight (egg size: 1-2 mm in diameter) (Walden, 2000). Hake spawns over a prolonged spawning period (e.g. Sarano, 1986; Murua et al., 1998, 2006). In the Bay of Biscay, hake spawn from January to May with a defined spawning peak between February and March (Lucio et al., 2000; Álvarez et al., 2004). Casey & Pereiro (1995) showed that the peak spawning time for hake occurs later with increased latitude; Kvenseth et al. (1996) recorded ripening hake as late as August in Norwegian waters. This strategy of having a prolonged spawning period may increase the survival success of the offspring under fluctuating environment conditions (Domínguez-Petit et al., 2008, 2009).

Hatching takes place between 4 and 5 days after fertilization, at least under experimental conditions (Bjelland & Skiftesvik, 2006; Groison, unpublished data) (Fig. 1.7). The early life stages are pelagic. Eggs (Fig. 1.7) and larvae (Fig. 1.8) are usually concentrated over the continental shelf. The vertical distribution of *M. merluccius* eggs in ichthyoplankton surveys has been reported to range from 0 to 150 m depth (Coombs & Mitchell, 1982; Álvarez et al., 2001) while hake larvae were found from the subsurface levels (50 - 60 m) down to 150 m (Coombs & Mitchell, 1982; Motos et al., 2000). In the Bay of Biscay, the size of hake larvae increases towards the coast, indicating that the larvae migrate (whether active or passive) towards the coast as they grow bigger (Álvarez et al., 2001, 2004). This coastal migration concomitant with development has also been shown in Namibian Cape hake, *M. capensis* (Sundby et al., 2001; Stenevik et al., 2008). Around two months after hatching (*ca.* 23.5 mm total length; Bjelland & Skiftesvik, 2006) the small juvenile hake complete the settlement on the bottom. In the Atlantic Ocean, the

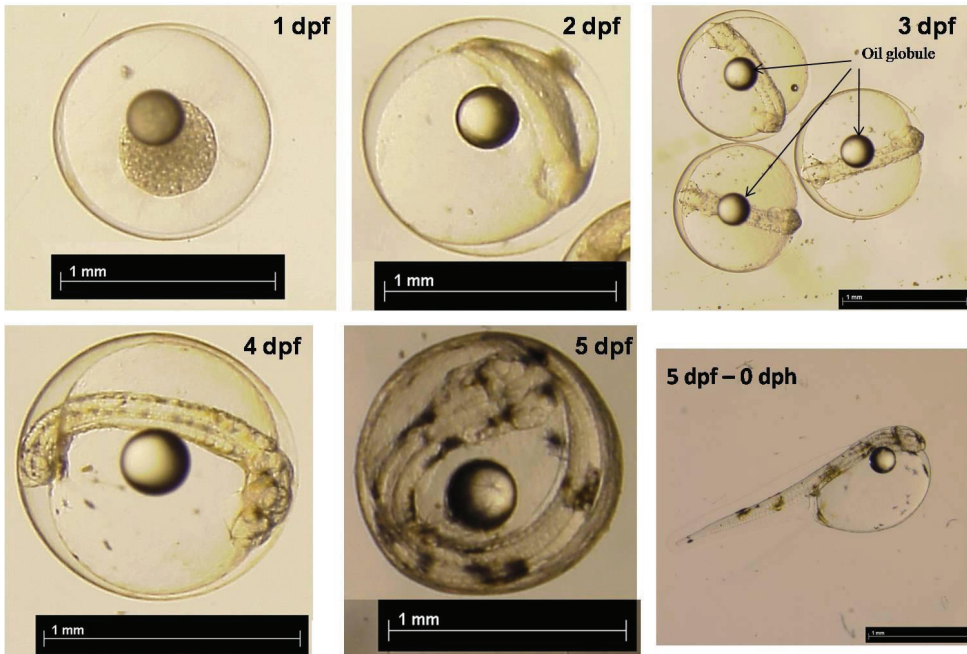


Fig. 1.7. European hake egg development under semi-intensive conditions at 12°C. From one day post fertilization (1 dpf) until hatching at 5 dpf (0 day post hatching) (from Groison, A.L., unpublished data).

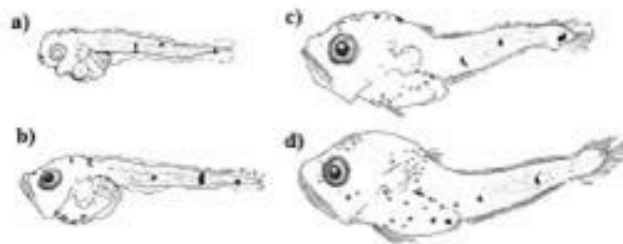


Fig. 1.8. Larval stages of European hake: a) 2.5 mm standard length (SL) larva, b) 2.8 mm SL larva, c) 6.5 mm SL larva and d) 9.1 mm SL larva (from Palomera et al., 2005; drawing by J. Corbera).

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distribution of hake recruitment varies with the year-class strength (Casey & Pereiro, 1995), contracting in less abundant years and expanding during the most abundant ones. Conversely, Sánchez & Gil (2000) reported that hake nursery areas in the Galician-Cantabrian shelf (Fig.1.6.B) remained generally stable in location and extent independently on the inter-annual density variability. In the Southern Bay of Biscay, the highest concentrations of recruits were found between 90 and 180 m depth (Sánchez & Gil, 2000) and represented the biggest hake nursery (Fig.1.6.B) (Casey & Pereiro, 1995). The juvenile recruits soon start developing a pattern nocturnal feeding migrations. Although hake are demersal fish, it does not feed on benthic or substrate-related prey, and make vertical migrations to feed in mid-water or the surface at night. The ecological position of hake is as a major predator in almost all the ecosystems where it inhabits (e.g. Atlantic systems, Sánchez & Olaso, 2004 ; Mediterranean systems, Coll et al., 2006). Hake is defined as an active, carnivorous and opportunistic predator (e.g. Olaso, 1993; Velasco & Olaso, 1998). However, the generalist (i.e. opportunistic) character can shift to a specialist depending on the life stage and on the availability and diversity of prey in the different areas. The dietary composition of hake is size dependent: larvae feed on copepod nauplii; stomach content analysis of juveniles reveals a preference for crustaceans such as copepods and euphausiids; and adults are mainly piscivorous, but also feed on squid or euphausiids; one of their main dietary items is reported to be smaller hake (Hickling, 1927; Pitcher & Alheit, 1995).

Males and females mature at different sizes and probably at different ages, males likely earlier than females. According to Sarano (1986) hake is a relatively late maturing species, with first maturation occurring at 5 and 7 years for males and females respectively. Piñeiro & Saínza (2003) give more recent estimates of 2.5 and 4.4 years for age at maturity of males and females, respectively. According to Lucio et al. (2000), first maturity is reached in European hake around 42 cm (both sexes combined). These estimates refer to hake in the Bay of Biscay; no matching estimates of age at maturity are available for Northern temperate waters. However, age

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determination in hake is uncertain, and tagging studies have shown that internationally agreed otolith reading methods result in an over estimation of fish age, by as much as a factor of two (De Pontual et al., 2006). The tagging results also provided direct evidence that hake is a fast-growing species which might mature earlier than previously concluded. The somatic growth rate of hake was estimated in the first year of life to be 2.21 cm / month for individuals captured in the Bay of Biscay (Kacher & Amara, 2005) and 1.83 cm / month for Norwegian hake (Morales-Nin et al., 2005). The overestimated hake age has raised concern also about the reliability of the age-length keys used for stock management: it causes errors in the estimated values of fishing mortality and, total and spawning stock biomass (Bertignac & de Pontual, 2007).

#### *1.4.4. Economic importance*

European hake is a highly important commercial species throughout its geographical range, especially in Spain and Italy. Hake is quite a mild-tasting fish, having a more subtle flavour than cod and easy to prepare as it has few bones. In France the hake is known as 'saumon blanc' i.e. 'white salmon'. It is almost entirely marketed fresh, whole or filleted, to specialized restaurants or retail markets (Lloris et al., 2005). Hake is utilized fresh, dried or salted and frozen; it can be prepared in every way: steamed, fried, barbecued, microwaved and baked (Frimodt, 1995). Because European hake catches have been decreasing since the 1960s (FAO, 2006) (Fig. 1.9), the commercial viability of aquaculture production and the interest in the European hake as a potential aquaculture species has recently increased (Quémener et al., 2002; Kjesbu et al., 2006). Some researchers have stated that the hake is one of the most promising new species for marine aquaculture (Engelsen et al., 2004).

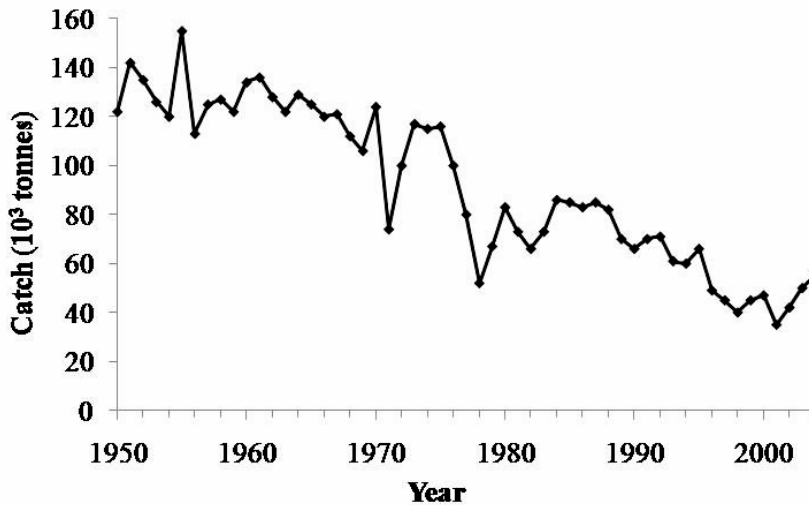


Fig. 1.9. Total catches of European hake from Atlantic populations (Northern and Southern stocks together). Data from FAO, 2006.

Quémener et al. (2002) and Kjesbu et al. (2006) highlighted its fast growth rate as a factor contributing to the high potential value for aquaculture, as well as its excellent flesh quality which gives it a very high market value when sold fresh.

To date, no report was found on European hake wild caught broodstock spawning in captivity. Only a stock of wild-caught Southern hake (*Merluccius australis* (Hutton)) has been maintained in net pens in Chile showing some success with broodstock (Anon, 1999, 2004; Carvajal, 2003). Furthermore, in an experiment with silver hake (*Merluccius bilinearis* (Mitchill)), wild caught broodstock spawned in captivity (Buckley et al., 1993). A major effort is currently put into capturing live European hake for establishing broodstocks of this species (R. Salte, Norwegian University of Life Sciences, pers. comm.; Iglesias et al., 2009) (more details in part 3).

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#### *1.4.5. Catches and evolutionary consequences*

The catch rates of hake in Western Europe seem to be much higher than elsewhere, perhaps driven by the exceptionally high economic value of this species in Europe. The status of European hake stocks, both Northern and Southern, has been described as critical in recent years due to overfishing (total annual landings declined from 120 000 to 50 000 t from the 1960s to the early 2000s (FAO, 2006)) (Fig. 1.9). As both stocks had been over exploited, and outside safe biological limits, a recovery plan was adopted for the Northern stock in 2004 and for the Southern stock in 2005.

Moreover, illegal catches of undersized hake are a common occurrence in this area (Casey & Pereiro, 1995). In many stocks of Atlantic cod, shifts towards maturation at younger ages and smaller body sizes have occurred as a consequence of overfishing of larger adult fish (Beacham, 1983; Heino et al., 2008). Such changes in size at maturity have also recently been shown in hake (Domínguez-Petit et al., 2008): a steady decline of 15 cm has been observed in the size of first maturity in hake from Bay of Biscay between 1987 - 2004. The stock reproductive potential of any stock, i.e. “the annual variation in a stock’s ability to produce viable eggs and larvae that may eventually recruit to adult population or fishery”, should take into account all factors influencing its reproductive capacity, including the age and size at spawning, maturation, condition and reproductive history of the stock (Trippel, 1999). The effectiveness of young adults as spawners and their capacity to develop gametes of comparable quality to those of older repeat spawners is therefore of current relevance when assessing the stock spawning potential (Trippel & Neilson, 1992; Solemdal 1997).

## 2. OBJECTIVES

The primary objective of this thesis was to address the current lack of knowledge on the reproductive biology of European hake, including its sperm biology in the perspective of investigating the potential of the hake being a new species for aquaculture. In particular this thesis focuses on the following three objectives:

**Objective I** to examine the hake **sperm production indices** and its sperm **biochemical characteristics**. To characterize i) the sperm **motility parameters** after short- or long **storage** duration, ii) the effect of **salinity** on sperm motility and iii) the **sperm energetics (Paper I + II + III + IV)**

**Objective II** to describe the **flagellar behaviour** of hake sperm during its motility period (**Paper II + V**)

**Objective III** to investigate the presence of **drumming muscles** and whether their morphological characteristics reflect the fertilization potential of spawning males (**Paper VI**)



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### **3. MALE REPRODUCTIVE BIOLOGY OF EUROPEAN HAKE: BACKGROUND**

Due to both its high flesh quality coupled with declining stocks, the European hake is currently regarded as a marine species with good potential for future aquaculture (Engelsen et al., 2004). However, the research devoted to its domestication is to date still in an early phase. In particular, knowledge on its reproductive biology, an important prerequisite for successful reproduction in captivity, is still scarce compared to other important commercial species. This is mainly because the hake is sensitive to handling and difficult to keep alive after capture (Hickling, 1933; Belloc, 1935). For these reasons, hake are rarely kept in captivity (Bjelland & Skiftesvik, 2006), with to my knowledge only two broodstocks currently maintained: one established in 2006 in Brekke, Norway (R. Salte, Norwegian University of Life Sciences, pers. comm.), and a second more recently established in Vigo, Spain (Iglesias et al., 2009). As a result, previous studies on the reproductive biology of the hake have generally been restricted to fish sampled in the field, mainly focusing on fecundity regulation in females and its implications for the fishery (Coombes & Mitchell, 1982; Sarano, 1986; Pérez & Pereiro, 1985; Murua et al., 1998; Lucio et al., 2000; Murua et al., 2006; Murua & Motos, 2006; Domínguez-Petit et al., 2008). For the domestication of this species, it is therefore critical to achieve a better understanding on male reproductive biology, in particular on sperm biology, including the development of sperm storage techniques to facilitate artificial reproduction procedures for aquaculture purposes.

To date, detailed information on the male reproductive biology of the European hake is largely lacking, except for two studies on the ultrastructure of hake spermatozoa by Desantis et al. (2000) (cf Fig. 1.1.) and Medina et al. (2003), who reported that hake spermatozoa possesses typical uniflagellate anacrosomal aquasperm.

## 4. GENERAL DISCUSSION

Preliminary measurements were conducted on hake sperm production indices (volume and concentration), on sperm energetics (Adenylate energy charge, AEC), and sperm motility characteristics (total motility duration, velocity, percentage of motile sperm, flagellar beat frequency and track diameter). Preliminary tests were also conducted on the effect of the swimming media, of sperm storage and of cryopreservation on sperm motility (**Paper I, II**). These studies were based on a limited number of sperm samples collected from few individuals (3 to 9) from the Bay of Biscay (France).

This thesis is based on sperm collected from mature hake from both the northern (Norway) and mid (Bay of Biscay) sections of their geographic range. In addition, a preliminary study was conducted on hake drumming muscles. In many other Gadoid species, these muscles are an important component of reproductive behaviour in spawning males: the contraction of these muscles associated to the swim-bladder results in an audible ‘drumming’ sound during the courtship of the females (Fish, 1954; Brawn, 1961; Hawkins & Chapman, 1966; Hawkins & Rasmussen, 1978; Hawkins & Myrberg, 1983; Engen & Folstad, 1999; Hawkins & Amorim, 2000; Bremner et al., 2002; Finstad & Nordeide, 2004; Ladich & Myrberg, 2006; Myrberg & Lugli, 2006; Rowe & Hutchings, 2006, 2008). However, the presence of drumming muscles has never been reported for hake. Mature hake collected for the sperm analyses, as well as immature individuals were thus used to investigate the presence of drumming muscles and the existence of potential correlations between their morphological characteristics and the sperm motility parameters.

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#### 4.1. Hake sperm biology compared to other fish sperm biology

For the European hake, sperm production characteristics (**Paper I, III**), ATP levels (**Paper IV**), and sperm motility parameters (**Paper I, II, III, IV, V**) were lower than values observed in other marine species. To date, sperm quality characteristics of marine species such as the Atlantic cod, halibut (*Hippoglossus hippoglossus*) and sea bass (*Dicentrarchus labrax*) have been well studied, due in part to the importance of these species in European aquaculture. The farming of sea bass has been a major success, and fry production of both cod and halibut has been successful on a semi-commercial scale (Engelsen et al., 2004); as a result, the reproductive biology, including sperm biology, of these species is well known (e.g. Westin & Nissling, 1991; Trippel & Morgan, 1994; Litvak & Trippel, 1998; Dreanno et al., 1999a; Rakitin et al. 1999a; Zilli et al., 2004; Babiak et al., 2006; Abascal et al., 2007; Cosson et al., 2008a; Rouxel et al., 2008).

Firstly, the volume (in ml) of Norwegian (Nw) and French (Fr) hake sperm (Nw-vol.:  $3.9 \pm 4.0$ ; Fr-vol.:  $2.6 \pm 4.0$ ; **Paper III**) was low compared to cod ( $> 10$ ; Tuset et al., 2008b) and halibut (1-60; Methven & Crim, 1991). Sperm concentration (conc.  $\times 10^9$  spermatozoa/ml) and total number of spermatozoa (tot no spz  $\times 10^9$  spermatozoa) recorded for hake (Nw-conc.:  $6.6 \pm 3.2$  and Fr-conc.:  $13.9 \pm 5.1$ ; Nw-tot no spz:  $23.5 \pm 30.0$  and Fr- tot no spz:  $35.1 \pm 36.2$ ) (**Paper III**) were lower than corresponding values measured in cod (conc.: 7.33-20.25 and tot no spz: 195; Stockley et al., 1997; Litvak & Trippel, 1998; Suquet et al., 2005). Hake sperm concentration were lower than values observed in sea bass (conc: 60; Dreanno et al., 1999a; Fauvel et al., 1999) or halibut (conc.:  $> 100$ ; Cosson et al., 2008a). The seminal fluid pH was lower in hake ( $7.6 \pm 0.1$ ; **Paper III**) than in cod (7.9-8.4; Hwang & Idler, 1969; Litvak & Trippel, 1998; Rouxel et al, 2008) or than in sea bass ( $8.21 \pm 0.45$ ; Abascal et al., 2007). The average osmolality (in mOsmol/kg) calculated for Fr-hake sperm samples ( $349 \pm 28$ ) was lower to values reported previously in cod ( $414 \pm 30$ ; Hwang & Idler, 1969; Litvak & Trippel, 1998; Trippel,

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2003; Rouxel et al., 2008) and in halibut ( $371 \pm 14$ ; Babiak et al., 2006; Ottesen et al., 2009). ATP measurements (in nmoles  $\times 10^9$  spermatozoa) in hake sperm before activation ( $85 \pm 80$ ; **Paper IV**) were low compared to values measured in sea bass ( $260 \pm 38$ ; Abascal et al., 2007; Dreanno et al., 1999a).

Hake sperm velocity is low (curvilinear velocity  $V_{CL}$  in  $\mu\text{m} / \text{s}$ : 69-102; **Paper IV**) compared to cod sperm ( $V_{CL}$ : 151.5-201.5; Tuset et al., 2008b; Skjæraasen et al., 2009), sea bass sperm ( $V_{CL}$ : 140; Abascal et al., 2007) and halibut sperm ( $V_{CL}$ : 83.4-115.7; Babiak et al., 2006). This lower sperm velocity combined with lower total hake sperm motility duration (up to ca. 450 s) (**Paper III**) compared to cod (700 - 800 s; Litvak & Trippel, 1998), lead to a lower total average distance covered by a hake sperm cell (3.4 to 6.6 mm; **Paper V**) compared to a cod sperm (14 mm; Trippel & Morgan, 1994; Litvak & Trippel, 1998; Rakitin et al., 1999b; Cosson et al., 2008b; Skjæraasen et al., 2009). On the other hand, hake sperm velocity is low (**Paper IV**) compared to sea bass sperm but showed a higher total motility duration (ca. < 50 s in sea bass; Abascal et al., 2007) which led to a higher total average distance covered by a sperm cell in hake (3.4 to 6.6 mm; **Paper V**) compared to sea bass (2.3 mm; Dreanno et al., 1999a). The total average distance covered by a sperm cell should be one of the most representative parameters of the possibility for the sperm cell to reach the egg's micropyle. Further investigations are needed to improve artificial fertilization of hake eggs including the determination of a well adapted environment and the sperm to egg ratio to be used in this species.

## 4.2. Effect of the swimming media

**Paper V** showed that sperm swimming occurs in non saline sucrose solutions, devoid of any salt and with an osmolality ranging from that of sea water. Therefore, hake sperm motility activation appears to occur through a signal generated by an osmotic

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pressure gradient between the inside and outside of the sperm cell. Similar results have been found in most fish species so far studied (Cosson et al., 2008a) with the exception of salmonids.

Changes in salinity of the swimming medium affected the hake sperm motility activity (**Paper I, III, IV, V**). When sperm was activated with 100 % filtrated sea water (100 SW), the percentage of motile sperm, the velocity and the straightness of the movement were at maximum but decreased sharply later. On the other hand, when activated with 50 % diluted with distilled water (50 SW) these parameters initially increased (with a lower percentage of motile sperm, velocity and straightness) but subsequently reached a maximum percentage of motile sperm, velocity and straightness, followed by a decline. When activated with 10 % ovarian fluid in sea water (10 OF) the percentage of motile sperm followed the same pattern as observed with 50 SW (**Paper III**). Activation of hake sperm with a lower salinity activating medium (AM) (such as 50 SW and 10 OF with osmolalities (in mOsmol / kg) of 498 and 936 respectively) compared to 100 SW (osmolality = 998 mOsmol / kg) appears to lengthen its motility period (**Paper I, III, IV, V**) in common with what has been demonstrated in other marine species such as sea bass, white seabream (*Diplodus sargus*), boque, horse mackerel (*Trachurus mediterraneus*), striped mullet or for different fresh water teleosts such as trout (*Salmo gairdneri*), pike (*Esox lucius*), or guppy (*Poecilia reticulata*) (Billard, 1978; Lahnsteiner & Patzner, 1998).

A trade-off between the mean sperm velocity and duration of sperm movement was emphasized by Burness et al. (2004): in **Paper IV**, the longer swimming duration in 50 SW is possibly due to a lower energetic expense due to the lower velocity observed; alternatively this could be due to a partial and progressive activation of spermatozoa with differential sensitivity threshold to osmolality shock, i.e. the motile cells observed as swimmers long after transfer in 50 SW or in 10 OF would probably be cells that were not activated at zero time; another hypothesis could be that the sperm cells (showing an osmolality in mOsmol / kg of  $349 \pm 28$ , **Paper III**) activated

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with an AM of lower osmolality than of 100 SW (50 SW or 10 OF) suffer of a less extreme osmotical shock and consequently less structural damage to the sperm cell; ultimately that would lead to a longer swimming duration.

Defining the optimal medium for conducting artificial fertilization of hake gametes is therefore of great interest. In hake, the optimal sperm activation was found to occur for a maximal percentage of motile cells at salinity above 70% of sea water (**Paper V**). However no fertilization trials could be conducted as ultimate measurement because no eggs could be collected. Positive correlation between sperm motility and fertilization success has previously been demonstrated in both the rainbow trout (*Oncorhynchus mykiss*) (Tuset et al., 2008a) and Atlantic cod (Skjæraasen et al., 2009). The effect of different media on hake egg fertilization and development must also be studied.

### **4.3. Changes in sperm motility parameters in relation to time post activation**

Whatever the salinity of the swimming solution, the decline of motility parameters in relation to time post activation (p.a.) (e.g. percentage of motile sperm, velocity, straightness) (**Paper I, II, III, IV**) mirrors the decline in the flagellar movement parameters (the flagellar beat frequency, the wave amplitude, the number of flagellar waves and the linearity of flagellar waves shape) (**Paper II, V**). The efficiency of sperm forward movement decreased dramatically after 100 s p.a. in 100 SW: after 100 s, sharp decreases were observed in the percentage of motile sperm, velocities, straightness of sperm tracks and amplitude of lateral head displacement. The diameter of the sperm trajectories also abruptly decreased and led to circling of spermatozoa becoming tighter restricting the effective progression of sperm cells.

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The contact of flagella with sea water provokes rapidly local osmotic damages such as flagellar blebs (**Paper II, V**), patterns seen in many other fish species such as carp (*Cyprinus carpio*), paddlefish (*Polyodon spathula*) and shovelnose sturgeon (*Scaphirhynchus platorynchus*) (Perchec et al., 1996; Cosson et al., 2000, 2008a, b), which partly disrupts the normal wave propagation from head to tip; furthermore, efficient waves become more and more restricted to the proximal part of the flagellum while the tip becomes devoid of any wave; the wave amplitude decreases as a function of time; and finally the wave envelope (surface in which the flagellum swims) becomes curved with time post activation: this curvature explains why the circularity of the sperm tracks becomes tighter over time. Such changes in the diameter of the swimming tracks have been reported in other species such as sea urchin (*Lytechinus pictus*), trout, sea bass or tuna (*Thunnus thynnus*) (Brokaw, 1991; Boitano & Omoto, 1992; Cosson et al., 1989; Dreanno et al., 1999a; Cosson et al., 2008a) and are explained by the involvement of the  $\text{Ca}^{2+}$  ions. When the intracellular  $\text{Ca}^{2+}$  ions concentration increases, the beating of flagella becomes more asymmetrical (**Paper II, V**) and therefore the circling tracks described by sperm cells become tighter (**Paper II, IV, V**), i.e. with smaller diameter. Such increase of sperm circling will decrease the volume for exploring the water space (as cells will move only locally) and reduce the probability of sperm meeting an egg, but alternatively could increase the chance of entering the egg micropyle.

Later in the swimming period (30-40 s p.a.; **Paper V**), the distal part of the flagellum stiffens: thus, the whole length of the flagellum appears straight at the end of the movement (**Paper V**) which limits the sperm motility duration. Such a rigidification process could be related to 1) the exhaustion of the sperm's energy reserves; 2) ATP, which becomes partially hydrolyzed, may be lacking in the distal part of the flagellum because of flagellar damages appearing during the motility period (Dreanno et al., 1999b); 3) Tombes & Shapiro (1985) found that the tight coupling between the energy utilization by the flagellum and the rate of energy production by the mitochondrion depends upon the transport of high energy phosphate (-P) from the

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mitochondrion to axoneme, which has been suggested to be mediated by a phosphorylcreatine shuttle in the sea urchin sperm. At the end of the sperm motility period these shuttles could be lacking, leading to the restriction of the waves in the proximal part of the flagella and therefore, limiting the sperm motility duration.

To better understand the regulation of sperm swimming, changes in energetic metabolism over the duration of motility in hake sperm needs to be investigated, and related to motility characteristics. These measurements to date refer to a restricted number of studies mostly conducted on turbot (*Psetta maxima*) (Perchec et al., 1993; Dreanno et al., 1997, 1998, 1999b, 2000) or sea bass sperm (Dreanno et al., 1999a; Abascal et al., 2007).

#### **4.4. Characterization of hake sperm energetic content**

Stored ATP values (in nmoles  $\times 10^9$  spermatozoa) measured in hake immediately after sperm collection were low ( $85 \pm 80$ ; **Paper IV**) compared to values recorded in turbot ( $260 \pm 38$ ; Dreanno et al., 1997, 1998, 1999b) or in sea bass ( $90$ ; Dreanno et al., 1999a; Abascal et al., 2007). The AEC value in hake sperm ( $0.78 \pm 0.07$ ; **Paper IV**) showed also lower values compared to turbot ( $0.90$ ; Dreanno et al., 1999a; Abascal et al., 2007). The energetic content of hake sperm could not be measured during the motility period, however its changes were measured with time of storage at  $4^\circ\text{C}$ . The AEC decreased abruptly, from  $0.78 \pm 0.07$  (right after sperm collection) to  $0.20 \pm 0.09$  (after two days of storage) as well as the ATP content (from  $85 \pm 80$  to  $5 \pm 4$  nmoles. $10^{-9}$  spermatozoa over the same time period) (**Paper IV**). Sperm transportation (approximately a day to reach the laboratories) could be one of the reasons for this abrupt decrease in AEC or ATP content. ATP, adenosine diphosphate (ADP) and adenosine monophosphate (AMP) content being highly variable, AEC was therefore also unusually variable (**Paper IV**). ATP provides the energy for



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spermatozoa movement (Inaba et al., 1999; Cosson, 2004). This abrupt ATP content decrease was reflected in the percentage of motile sperm, which decreased by more than 50 % over a 48 h period. Zilli et al. (2004) demonstrated that ATP levels are directly related to fertilization rate in the sea bass. Testing fertilization capacity of stored hake sperm should in that regard be further investigated.

#### **4.5. Preliminary study on hake sperm storage and cryopreservation**

##### *Sperm storage*

The viability of undiluted hake sperm following storage at 4°C (10% of motility after 10 days; **Paper III**) is comparable to observations made on cod ( $11.0 \pm 0.7\%$  after 10 days; De Graaf & Berlinsky, 2004) and higher than observations made on sea bass (10% after only 2 days; Sansone et al., 2001). However, when the sperm of sea bass was stored diluted (1:6 sperm / inactivator medium dilution), it could be stored for twice as long at 4°C, i.e. 10 % of motile cells after 4 days (Sansone et al., 2001). Furthermore, more than 24 h after collection, the motility of diluted sea bass sperm was always significantly greater than that of undiluted sperm kept under the same conditions (Sansone et al., 2001). For many fish species, the dilution of the sperm in a suitable medium favours the preservation for short time periods, e.g. during transportation from the place of collection to the place of employment, as it helps to keep the physico-chemical properties stable (Gwo, 1994; Ohta & Izawa, 1996; Barbato et al., 1998). These solutions, however, must induce a temporary inactivation of motility, so as to prevent exhausting the energy reserves of the spermatozoa (Villani & Catena, 1991; Gwo, 1993). To possibly improve the storage time and avoid

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spermatozoa damages, a species-specific optimal composition of diluent for hake sperm short storage should also be examined.

### *Cryopreservation*

In this thesis, it was possible to observe, for the first time, hake sperm cells motility after thawing (motility recovery index of the cells at thawing ranged from 0 to 76.4 %; **Paper I, III**). It was shown that cryopreservation had a significant negative effect on the percentage of motile sperm (**Paper III**). No conclusion could be reached regarding the effect of cryopreservation on velocity or straightness of hake sperm, as due to the difficulties in regularly obtaining sperm samples from mature hake, it was not possible to conduct such measurements on the same samples, both fresh and thawed (**Paper III**). More data must be acquired to conclude on the effect of cryopreservation on motility parameters such as velocity or straightness.

Generally, DMSO is the most commonly used and most successful cryoprotectant for sperm cryopreservation in marine fish (Stoss, 1983; Leung & Jamieson, 1991; Suquet et al., 2000). The effect of DMSO is concentration dependent, with a concentration between 5 and 20 % commonly used (Suquet et al., 2000). In agreement with this observation, our results showed that DMSO at 10 % could be used as a cryoprotectant for hake sperm. The techniques established by Dreanno et al. (1997) for turbot sperm cryopreservation appear to be suitable for European hake (**Paper I, III**). However, such protocols for cryopreservation can influence the variability of sperm quality after thawing (Kopeika & Kopeika, 2008), and therefore it would be beneficial to test different cryoprotectants such as egg yolk, glycerol or sucrose as carried out for other species such as cod (Rideout et al., 2004), haddock (*Melanogrammus aeglefinus*) (Rideout et al., 2004), turbot (Chen et al., 2004), sea bass (Sansone et al., 2002), rainbow trout (Salte et al., 2004), or European eel (*Anguilla anguilla*) (Asturiano et al., 2003, 2004), in order to develop the optimal conservation media well adapted to

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hake sperm. The measurement of sperm energetic content before and during the motility period, and before and after thawing would also be useful in defining the optimal conservation medium.

#### **4.6. Potential causes for observed differences in hake sperm quality**

For logistical reasons, the samples hake sperm of used in this thesis, were collected in French (Fr) waters most likely towards the end of the reproductive season. However, in Norwegian (Nw) waters, sperm samples could be collected from the beginning of the reproductive season and onwards. Clear significant differences were recorded for observed sperm production indices between Fr and Nw samples (**Paper III**). These differences could be due to a number of factors including the time of sperm sampling (Nw-gonadosomatic index (GSI) > Fr-GSI) and other factors such as the size or the weight of the stripped individuals (Nw-total length (TL) > Fr-TL; Nw-Total weight (TW) > Fr-TW) and the different environments where the individuals were caught (Fr vs Nw) (**Paper III**). No conclusion could therefore be given as too many of these factors interacted.

A decline in sperm motility, in sperm storage potentiality as well as in the motility recovery index following cryopreservation has been reported for sperm collected towards the end of the reproductive season in several fish species (Billard et al., 1977; Suquet et al., 1998; Rainis et al., 2003; Rideout et al., 2004; Vermeirssen et al., 2004; Babiak et al., 2006; Rouxel et al., 2008), and probably results from the ‘ageing processes’ leading to the morphological deterioration of spermatozoa, including the mid-piece regions (Suquet et al., 1998). This effect could not be demonstrated in hake as no significant differences were observed between Fr and Nw sperm samples in

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terms of the percentage of motile sperm with time post activation or with storage time; consequently, samples from both localities were pooled together. Furthermore, only one Nw sample was used for sperm cryopreservation (**Paper III**), no comparison could thus be established between Fr and Nw sperm motility recovery index.

However, our results pointed out the necessity to collect hake sperm from the same area at different dates during the reproductive season to evaluate seasonal changes in hake sperm quality.

#### **4.7. Hake reproduction**

**Paper VI** highlighted the presence of drumming muscles (DM) in hake. DM mass in spawning males was positively associated with body size and total weight, suggesting that sound production may be an indicator of the size of the mating male and may be an honest signal to the female indicating the male's fitness, a mating strategy previously demonstrated in the cod by Rowe & Hutchings (2004). In a spawning aggregate of hake, it is likely that a female would favour male's that displayed the loudest or longest drumming. Therefore, our results strongly suggest that hake produce sounds not only facilitating the synchronization of spawning but also as an important component of sexual selection, similar to what has been observed in spawning cod (Brawn, 1961; Rowe & Hutchings, 2004, 2006, 2008). The absence of these sounds may be a sign that the male has completed the annual spawnings as shown in haddock by Bremner et al. (2002). These results may be of great interest if broodstock of hake become available but also from an ecological point of view: at spawning time, sound production could help the identification of the size and location of hake and offer some help in finding the location of the spawners. An important area of future research should be on determining whether the characteristics of sound production ("drumming") such as frequency, wave length and volume in spawning

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hake change over the breeding season. Further, the possibility exists that drumming characteristics could differ between different stocks.

DM mass of spawning males was positively associated with body size and total weight, suggesting that sound production may be an indicator of the size of the signaler and may reveal information about maturation stage. For the female, consequently, the DM could be an indicator for informing on the fitness of the male. Similarly, several authors (Engen & Folstad, 1999; Rowe & Hutchings 2004, 2006) have established that in cod the DM mass in spawning males was positively correlated with body size, and also to the condition and fertilization potential. Rowe & Hutchings (2008) concluded that there is a positive link between sound producing musculature and mating success in cod. Such hypothesis could not be investigated because no broodstock of hake was available. On the other hand, no significant correlation was found between hake DM and the sperm production indices studied. Cod DM mass has been negatively correlated with spermatocrit levels (Engen & Folstad, 1999) and positively correlated with gonad mass (Rowe & Hutchings, 2004). There were too few hake males from which both sperm quality data and DM mass estimation were made. Therefore, it is necessary to collect more data to test if DM mass could be an indicator used to predict the hake gamete quality in mature individuals.

In hake, the DM clearly increases in mass as the male becomes sexually mature, suggesting that this morphological trait is a male secondary sexual character under the control of androgen hormones. This is supported by the observation that in female hake, on the other hand, there is no increase in the size of the DM with sexual maturity and no significant increase with increase in the size of the fish. The amount of material was insufficient to precisely delineate at what size DM dry weight start to be significantly different between males and females. However, our results on mature Nw hake imply that DM mass was similar in both sexes prior to spawning and that the DM mass becomes sexually dimorphic at the onset of spawning. According to Lucio

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et al. (2000), first maturity is reached in European hake around 42 cm (both sexes combined). It can therefore be expected that male and female hake do not develop significant differences in DM until their first maturity. Further study on hake DM completing the present data set might help in confirming the size at first maturity for this species, respectively for males and females and also depending on the location, essential data for fishery purposes.

In the context of halieutic studies it can be very difficult to determine the sex of hake landed in the gutted condition by commercial trawlers, because the ovaries (at least of mature females) and a large part or all of the testes of mature males are generally removed with other viscera at sea. Therefore the size of DM could be the primary factor in distinguishing sexes, however, that could only be presently applied accurately to mature male and female hake, female hake. Templeman & Hodder (1958) found the same results for haddock.

The general findings of this thesis represent a first step towards a better comprehension of the male reproductive biology of European hake, for which no literature was found so far. Hake is of high economical value, and as a consequence of the continued decline in most wild stocks there is growing interest in the aquaculture of this species. Knowledge acquired through this work will be therefore of importance not only for purposes of broodstock management, but also for the development of sperm preservation techniques.

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## **5. CONCLUSION AND SOME RECOMMENDATIONS FOR FUTURE INVESTIGATIONS**

In this thesis, consisting of six papers, the sperm biology of European hake and the presence of drumming muscles have been addressed for the first time. The findings of these papers has contributed significantly to a better understanding of the reproductive biology of the hake, which will be beneficial to both aquaculture and future stock management policies. However, further studies should be undertaken to supplement the present data sets.

### **5.1. Main findings**

This thesis provides original data on European hake sperm quality: spermatozoa concentration and volume, spermatocrit, osmolality and pH.

Sperm production characteristics, ATP measurements, and sperm motility parameters showed lower values in hake compared to values recorded in other marine species such as cod, halibut and sea bass.

The total average distance travelled by hake sperm was lower than that observed in cod while this distance was higher for hake sperm compared to sea bass sperm.

Hake sperm motility activation occurred through a signal generated by an osmotic pressure gradient between the inside and outside of the sperm cell.

An activating medium of reduced salinity negatively affected several hake sperm motility parameters, as measured using CASA, but the spermatozoa's swimming duration increased.

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All motility parameters declined during the motility period which reflects consequently a parallel decline of the flagellar movement parameters: the amplitude and length of each flagellar wave, the number of waves (or curvatures) along the flagellum and the flagellar beat frequency.

During the motility period, the distal part of the flagellum became devoid of waves.

The efficiency of sperm forward movement decreased dramatically after 100 s post activation in full seawater: after 100 s, we observed sharp decreases in the percentage of motile sperm, velocities, straightness of sperm tracks and amplitude of lateral head displacement. The diameter of the sperm trajectories also abruptly decreased and led to circling of spermatozoa becoming tighter restricting the effective progression of sperm cells.

Hake undiluted sperm storage potentiality at 4°C (ca. 10 days) was comparable to observations carried out in cod and five times higher than observations reported in sea bass.

During the period of storage at 4°C, the percentages of motile sperm as well as the motility parameters declines were probably due to a corresponding observed decrease in the sperm energetic content.

Cryopreservation of hake sperm was successfully achieved using dimethyl sulfoxide at 10 % as a cryoprotectant.

In this thesis the presence of drumming muscles in hake has been shown for the first time, and suggests that the observed differences in drumming muscle size between male and female hake reflects differences/changes in sound production with sex, sexual maturity, and season, i.e. sound production by adult males being more frequent during the spawning season than during the rest of the year.



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## 5.2. Recommendations

These are split into three: the use of experimental data, the investigations on potential stock differences and the methodology improvements.

### *Use of experimental data*

For the European hake, the availability of a viable broodstock would help to overcome the inherent difficulties of obtaining adult fish from the wild over the complete reproductive cycle. The availability of these fish would allow:

To follow individual changes in sperm indices over the complete spawning season. Furthermore, collecting sperm samples from captive individuals would allow having more replicates, decreasing the within-male variance component of statistical analyses and providing greater ability to detect inter-male differences.

To determine the optimal time of sperm collection over the prolonged spawning season, in terms of maximal sperm storage capacity and cryopreservation success.

To define an optimal storage medium to be used for short or long term sperm storage.

To facilitate easy access to fresh hake eggs for future artificial fertilization studies. Fertilization success is the ultimate measure of sperm quality, including after cryopreservation procedures. Conducting artificial fertilizations with sperm stored in different conservation media would also be the ultimate measure for defining the optimal storage medium used for short or long term sperm storage. Facilitating the access to hake gametes would clarify which fish characteristics or which sperm characteristics could lead to different fertilization success between different males.

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Artificial fertilization studies would help in the determination of the optimal sperm to egg ratio.

To control the time of spawning using, for example environmental manipulations such as photoperiod. Advancing or delaying the spawning period through photoperiod manipulation would allow the production of out-of-season gametes.

To describe the hake reproductive behaviour.

To investigate the effects of different environmental changes on the sperm responses (using CASA) and ultimately on hake fertilization success as for example, changes in water temperature (potential impact of global warming), as well as the potential impact of anthropogenic factors such as environmental contaminants (pollutants) including heavy metals.

### *Potential stock differences investigations*

The availability of hake broodstocks from different geographical areas, held under similar conditions would assist in determining whether these different groups display differences in reproductive characteristics. Such knowledge would be highly beneficial for future management and conservation policies.

Furthermore, in order to fully evaluate hake sperm quality, further studies on evaluating sperm motility traits such as sperm velocity, flagellar beat frequency and trajectories of spermatozoa but also on evaluating hake sperm ultrastructure has to be conducted. Energetics studies investigating the cell energy level and its distribution along the flagellum during the motility period would also be of great interest in that matter.

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*Methodology improvements*

Finally, to be able to establish relevant comparisons of hake sperm quality parameters with other cultured fish there is a crucial need of a harmonization of the methods. A ‘wish-list’ here would be: 1) a common method comprised in a single software should be used; in this respect a software freely accessible as a plug-in of Image J (Wilson-Leedy & Ingermann, 2007) fulfils this need; 2) only a limited number of variables should be used among the whole set offered by many such softwares; e.g. percentage of motile sperm, velocity and linearity allow quite a wide description of sperm motility characteristics and 3) the experimental or laboratory protocols should as far as possible, be standardised.

These recommendations would improve the knowledge presently acquired on hake sperm biology. A better understanding of the sperm biology of the hake would be highly beneficial for the development of gamete management practices, similar to what has been achieved for salmonids.

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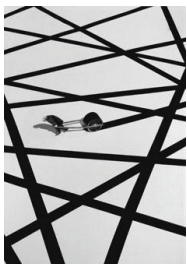
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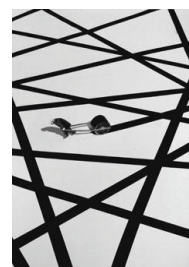
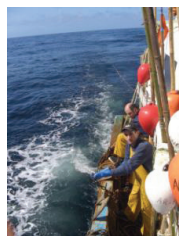
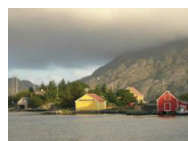
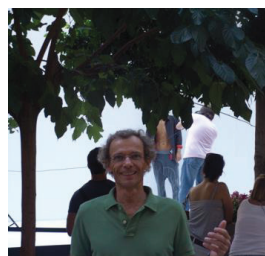
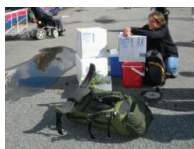
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Merci.



la dernière ligne droite, from gilbert garcin



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