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Report

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Gonad Development of male Sole, S. solea (L.), in natural and culture conditions between March and June 2003 in relation to environmental factors

Final report for task 1.2b Upscaling of sole fingerling production: Production of Dover sole eggs

Design and development of commercial scale farming technologies for sole

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Contents

Abstract

The aim of the present study was to detect differences of spermatogenesis between wild, captured and reared (F1) sole (*Solea solea* L.) broodstock. Differences between sampling months March and June 2003 as well as differences between groups were determined.
Wild animals showed a significant decrease in 11-ketotestosterone, spermatogonia, spermatocytes and spermatids from March to June. The captured group did not show this effect, but had significant lower 11kT levels and slightly lower numbers of germ cells in March compared to Wild animals. F1 animals show significantly lower 11-ketotestosterone (11kT) levels and higher conditions than the other groups in June. F1 animals show a pattern of spermatogenesis where spermatids are relatively more abundant than spermatozoa, in contrary to wild and captured animals.
A possible explanation for the low performance of F1 animals is that low levels of 11kT result in partial completion of spermatogenesis and spermiogenesis but are not sufficient to complete spermiogenesis on large scale. It is emphasised that there is no proof for the significant effect of 11kT, though its relevance to spermatogenesis is shown in other species. Incomplete spermatogenesis of F1 males can be caused by too short and less extreme artificial temperature periods for completion of spermatogenesis. This also could explain lower 11kT levels and possible lower performance of captured animals compared to wild groups. A lack of cyclic annual photoperiod and temperature fluctuations during the first years can be a cause for malfunctioning of F1 as well. It is possible that the F1 males do not have sufficient time to complete spermatogenesis or that internal clocks are not imprinted properly.

1. Introduction

1.1 History

Sole farming is a relatively young branch in marine aquaculture. It was a strong need for diversification in production of the sea bass and bream sector that made existent farms seeking new cultivation potentials. Its high economic value made sole one of the interesting opportunities as "new species". A renewed interest, though, since previous studies on sole could not develop economic feasible techniques.
After removing critical problems with reproduction and larval feeding, production of sole seemed to be very easy (Howell, 1997). Still, information on the reproductive biology of sole was very limiting (Zaki, 1989). Reproduction of sole in captivity was routine but growing the larvae and juveniles to market size for instance, proved to be problematic. Devastating, since grow out is the most important part of production with regard to economic viability (Dinis *et al.*, 1999; Howell, 2000; Brown, 2002). The initial lack of larval acceptance of commercial diets was taken away by providing pellet feeds with chemical attractants or invertebrate tissue. Still, vulnerability to restricting diseases like Black Patch Necrosis (BPN) and low growth and survival rates made commercial production impossible. This resulted in decreased interests in sole and a movement towards for example turbot (*Scophthalmus maximus*) by farms in the 1980s. Recent research suggesting that farming of sole does not have blockades different from other cultured marine species showed optimistic prospects for commercial production of sole (Howell, 1997).

1.2 Species

Two species of sole, being *Solea solea* and *Solea senegalensis* both are subject to production by now. It is not the Northern Atlantic *S. solea* that is cultured as main species but its southern relative *S. senegalensis*. The species have a similar biology, though *S. senegalensis* is more adapted to warmer conditions (Dinis *et al.*, 1999). Therefore it is more commonly found in the Mediterranean and more southern regions of the Atlantic. Most of the production of *S. senegalensis* takes place in the Mediterranean.

At the moment, *S. senegalensis* seems to be the species that is most suitable for intensive fish culture systems because of higher growth rates and lower susceptibility to diseases. Still, *S. solea* is produced by a few farms in Europe (Howell *et al.*, 2003)

The Common Sole, *S. solea*, also referred to as *Solea vulgaris* or the "Dover Sole" belongs to the family of the Soleidae, order of flatfishes, Pleuronectiformes. This species is found in the eastern Atlantic from the coast of Senegal to the Mediterranean and up to the North Sea. (Deniel, 1981)

Spawning grounds are found in the complete habitat (Deniel, 1981). Sole is a seasonal spawner. Its reproductive season is strongly related to water temperature which should be 8 to 10 ºC for initialising spawning. The spawning period therefore strongly varies with the geographical area from February in Southern Europe to May in Northern Europe (Deniel, 1981; Ramsay and Witthames, 1996).

1.3 Problem formulation

After removing the main problems concerning reproduction and larval feeding, production of sole was possible (Howell, 1997). Still, the main focus of production of both *S. senegalensis* and *S. solea* lies at the reproduction. According to practice and research it is not egg quality that is deleterious for reproduction (Howell et al., 2003). It seems that female sole is capable of producing good quality eggs. Still larvae production is not reliable and even negligible in several cases. Aspects like the depth of the broodstock tanks as well as density, sex ratio, photoperiod and annual temperature cycles are important features on which research has been focussed. It seems that within these aspects the restricting values have been identified. Failure of fertilization, on the other hand, is quite common still. Both male infertility or a combination of both male and environmental conditions could be causes (Howell et al., 2003).

Failure of predictable reproduction is very restricting concerning fish production. Broodstock of sole only consists of captured broodstock animals nowadays. Captured broodstock consists of animals that are caught from wild populations. However, in view of safe and controlled (re)production and breeding of any organism it is of major importance to be capable of using own produced and reared offspring as broodstock. Independency of natural populations makes safe, predictable and controlled production

possible and therefore can increase commercial opportunities of a species in several ways. Examples are prevention of diseases by import of wild broodstock and possibilities to set up family selection programs, to improve for example growth rates and feed conversion rates.

In case of sole, it turns out that spawning of F1 broodstock does not result in acceptable fertilization rates. F1 broodstock consists animals of the first generation offspring produced and completely reared in artificial circumstances. Again, there seems to be a male problem for F1 females are assumed to reach complete maturation. Environmental circumstances are not being considered as deleterious since captured and F1 groups are kept in the same circumstances.

Therefore it is necessary to investigate the difference in spermatogenesis between wild and F1 sole in order to create a reliable, safe and viable commercial production.

1.4 Aim

This project is aimed to describe the differences in spermatogenesis between wild, captured and F1 sole, *S. solea L.*, before and shortly after spawning, respectively Marc and June, in order to give a possible explanation of the failure of fertilization by F1 broodstock males. Wild and Captured animals are both compared in the months March and June. F1 is only compared with Wild and Captured in June.

1.5 Hypothesis

1) Testes of wild and captured animals differ slightly in between per month and show, within each group, individual development between months.

2) Testes of F1 sole broodstock show different numbers of germ cells, GSI's and steroidical patterns when compared to testes of wild and captured sole in June.

2. Review of Literature

The next chapter is set out in two parts. In the first, elements of environmental conditions, fish characteristics and reproductive determinants are set out and characterised. A description of the reproduction of sole will be given as it is shown by wild animals in natural conditions. Besides, the production of sole as it is generally performed in the artificial circumstances will be explained.

In the second part of this chapter spermatogenesis as a process, steroidical levels during spermatogenesis and further factors of influence are being considered.

2.1 Environmental conditions

Clearly, environment plays a very important regulating role in fish and their internal processes and inevitable on their reproductive performance (Bromage *et al.*, 2001). The fertilization process, often described as very sensitive and uncertain, is a most important determinant for the year-class strength of wild fish populations. Therefore, environmental aspects of fertilization of eggs are very important factors concerning reproduction of many species. (Howell *et al.*, 1991)

Fertilization rates of eggs in wild fish stocks are recorded to show much higher proportions than in artificial circumstances. Research showed a fertilization rate of approximately 99% (Howell *et al.*, 1991), whereas rates in cultured circumstances are reported to be very fluctuating and low on general. Recent data from practice gave average 50% fertilization for *S. solea. S. senegalensis* showed higher proportions of approximately 80% eggs of the buoyant fraction (Howell *et al.*, 2003). Other experiments found fertilization rates of approximately 50% as well (Houghton *et al.*, 1985). These data result from culture conditions in which natural spawning occurs. Whether the low rates are attributable to unfavourable environmental conditions or not remains unclear. Though somewhat lower, produced egg quantities of F1 females do not seem consistently lower than of captured females. (Howell *et al.*, 2003).

The high portions of unfertilized eggs in artificial production under simulated natural conditions even better show the sensitivity and importance of this process (Howell *et al.*, 1991). Ripening of gametes in fish is very seasonal dependent and can take many months, 36 days for the guppy for instance (Schulz and Miura, 2003). As a result it is logical to investigate long term annual fluctuations in the several conditions (Bromage *et al.*, 2001; Howell *et al.*, 2003).

From literature several environmental aspects to be discussed evolve. Examples are: annual photoperiods, temperature, food supplies (Bromage *et al.*, 2001; Howell *et al.*, 2003), light intensity, stocking density (Howell *et al.*, 2003) and spawning substrate (Amezcua and Nash, 2001).

2.1.1 Annual Photoperiod and Temperature

Both photoperiod and water temperature are very important factors for spawning in many fish species. Regarding timing of spawning of fish like seabass (*Dicentrarchus labrax)*, seabream (*Sparus aurata*), several flatfish etc., it is probably a seasonally changing pattern of daylength and temperature that is initiating spawning (Bromage *et al.*, 2001). An indication of annual natural photoperiods and temperature fluctuations in the North sea is given in figures 1 and 2. As can be seen, the yearly change of daylight between sunrise and sunset is huge, ranging from 8.1 to 16.7 hours. It must be noted that temperature records as shown in figure 2 are in shore surface temperatures. Off shore temperature has less extremes (Van der Land, 1991).

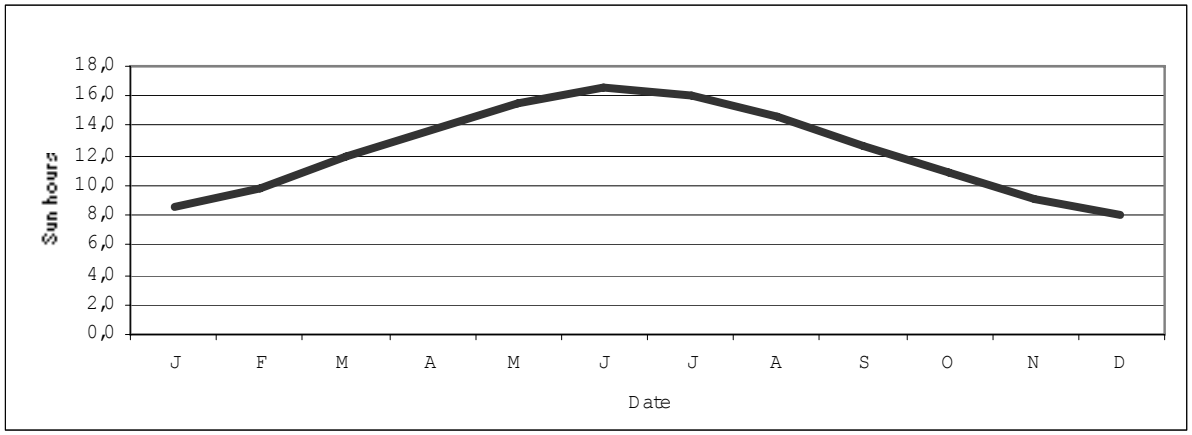


Figure 1. Annual natural photoperiod, Vlissingen (KNMI 2004). Daylength in hours between sunrise and sunset.

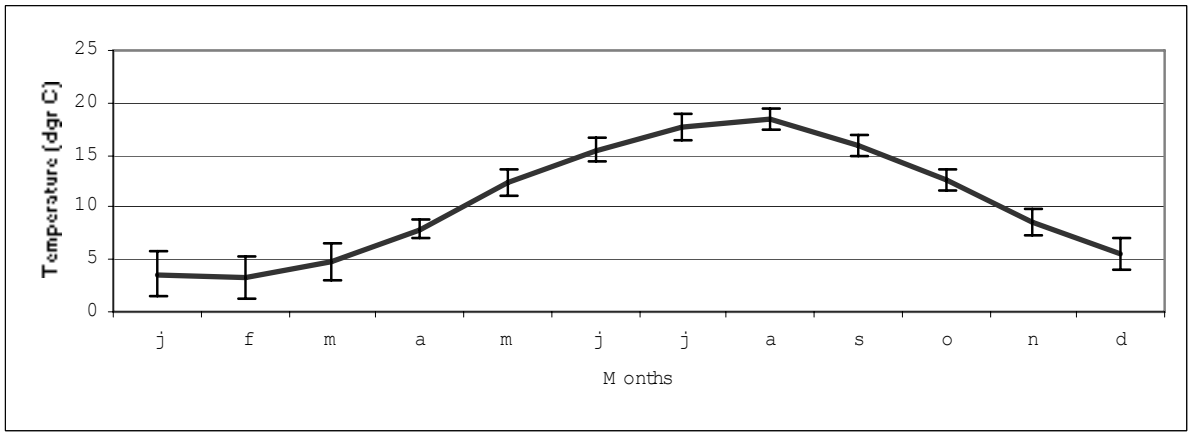


Figure 2. Monthly average North sea water temperature (°Celcius), 't Horntje, Texel 1981-2001 (RIVO 2004).

Natural spawning season

The spawning season of sole shifts across altitude beginning in the Bay of Biscay (France) from January to the end of May. Spawning of sole in the North Sea starts in April and ends in June (Deniel, 1981; Rijnsdorp, *et al.*, 1992). Spawning of *S. solea* observed between the altitudes of 45° to 52° occurs between February and May, though this is quite variable, depending mostly on temperatures before and during the spawning season.

Temperature can delay beginning and ending of spawning periods (Rijnsdorp and Witthames, 2004). In the South-eastern North Sea winters with temperatures of approximately 2 or 3 degrees lower than other winters show delayed spawning. Winters and springs where delayed spawning took place had temperatures of about 4°C in months previous to peak spawning. Advanced spawning was preceded by months with temperatures of 7-8°C. Here, peak spawning occurred at approximately 9 – 10°C (Van der Land, 1991).

In the Bay of Biscay timing of the spawning season of *S. solea* is not affected by temperature differences (Koutsikopoulos and Lacroix, 1992) though in the North Sea it is. In the Bay of Biscay no delay in spawning was found after a longer and colder period of lower temperatures. Differences between the winter temperatures were about 2 or 3 °C. Minimum and maximum temperatures were on average respectively 8 and 11 °C in months previous spawning (Koutsikopoulos and Lacroix, 1992)

The previous mentioned different records suggest that there is a temperature range for approximately 2 to 3 months previous spawning between 4 and 11°C, where lower temperatures within this range cause delay in spawning (Van der Land, 1991; Koutsikopoulos and Lacroix, 1992).

Most eggs in wild conditions are found at approximately 9 – 10 °Celcius. Optimal temperatures for spawning are believed to be the same, since during surveys most eggs were found at these temperatures. Other experiments found some eggs at lower temperatures (Howell, 2000; Koutsikopoulos and Lacroix, 1992).

Migration

Though spawning of sole does not seem to be affected directly by temperature changes, animals do show migration. Younger (1 – 4 years) soles dwell more at estuarine regions (Amara *et al.*, 1998) where temperatures raise from 3.5°C in winter to at maximum 18°C at the end of the summer. When temperatures decrease in winter, fish in estuarine grounds move deeper to warmer regions (Rijnsdorp *et al.*, 1992). In summer most adults are recorded to dwell at southern regions of the North Sea at depths of approximately 40 m. Here temperatures reach up to 17°C (Rijnsdorp and Van Beek, 1991). Apparently, sole will not receive temperatures of less than 4.5°C since off shore water temperature do not reach these extremes (see figure 3). Probably, sole will follow off shore temperature fluctuations which does show less extremes than shore water temperatures. From this we can obtain a curve for assumed optimal temperatures for spawning of sole. This curve is shown in figure 3. Data are derived measurements at off shore research vessels (Van der Land 1992), shore surface water temperatures at Den Helder (Rijnsdorp and van Beek, 1991) and shore surface water temperatures at Texel (Rivo 2004).

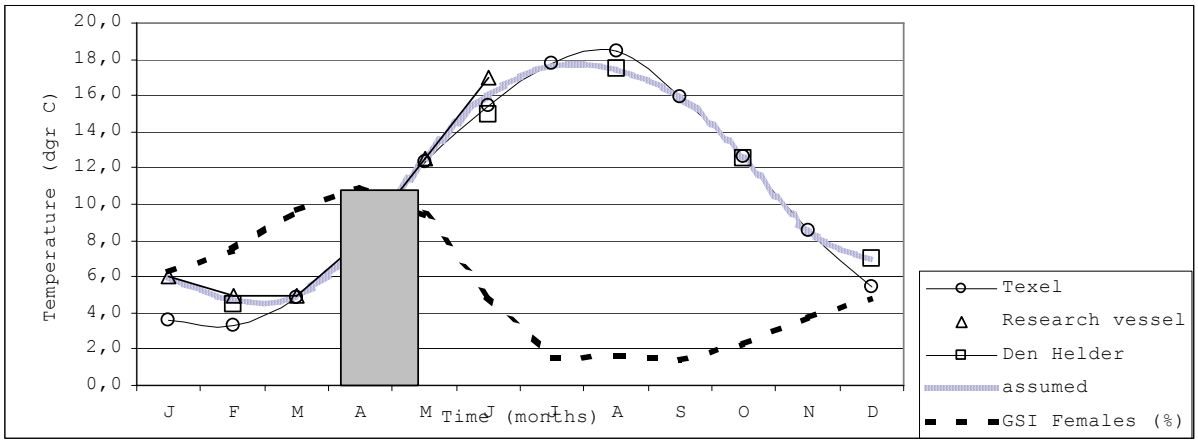


Figure 3. North sea surface water temperatures off shore (Van der Land 1992), shore in Den Helder (Rijnsdorp and van Beek, 1991) and shore at Texel, 't Horntje (Rivo 2004). The assumed approximate optimal temperature cycle for sole is indicated by the grey line. The spawning period (approximately 10°C) is indicated by the grey block.

Cyclic temperature and photoperiod

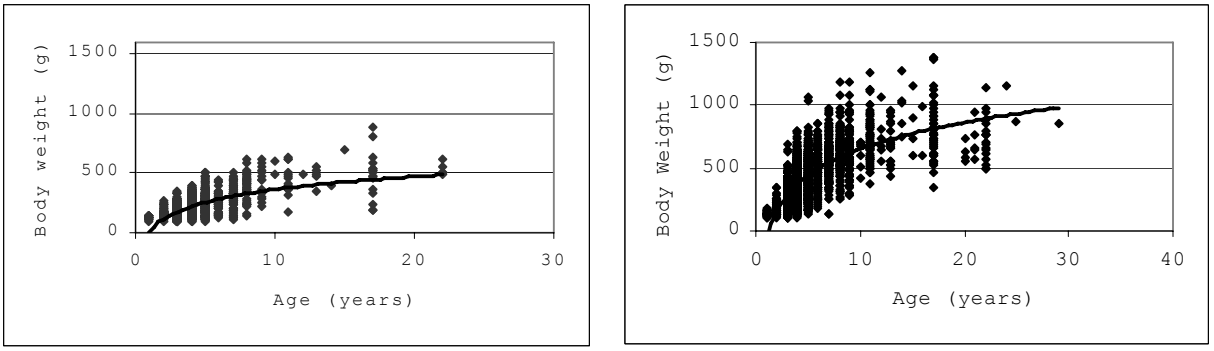
In cultured circumstances, problems with fertilization rates are attributed to disruptions of cyclic temperatures during the year. Emphasis in this case lies on temperatures during winter. In many cases low or zero fertilization rates are attributed to too high temperatures (above 10°C). In occasions where temperatures did decrease below 10°C for some weeks higher rates were found (Howell *et al.*, 2003). In other references temperature indeed seems to be of great importance for spawning. Manipulation of temperature with or without photoperiod manipulation has proved to be capable of shifting spawning periods (Bromage *et al.*, 2001). Besides, there is evidence that cyclic temperature regimes are essential for both quality and quantity of egg production of sole (Howell, 2000).

In case of female catfish, *Clarias Gariepinus*, high temperatures are determining continuous propagation all year round and daylength is not a regulating factor. The internal rhythms of catfish broodstock, however, probably are determined by environmental factors at early stages of development. In experiments fish were showing cyclic gonad growth independently of a new local photoperiod. The fish had a cyclic gonad growth according to the previous photoperiod in which they were reared. Apparently they had an internal clock or endogenous rhythm (Richter *et al.*, 1987). This suggests that the biological clock can be fixed at very early age. When extending this phenomenon to F1 sole which are kept at relative high water temperatures without cyclic patterns during their first life stages, this could explain inability or less performance of reproduction.

In contrast, for Atlantic Cod (*Gadus morhua*) it is possible to shift to different photoperiods and thus shift spawning periods without effect on production (Norberg *et al.*, 2004).

2.1.2 Age and weight: Maturation and spawning

S. solea shows a clear sexual dimorphism in growth. Females grow faster to higher body weights than males. This is shown in figure 4. (Rivo data 1980; Bromley 2003)



Male sole Female sole

Figure 4. Body weight versus age of male and female North Sea sole (RIVO, 1980)

Age of first maturity in wild conditions of female sole is estimated on three years, though only 70% of all animals mature in this year. The fourth year is proved to cover more animals, up to 100%. (Van Beek, 1985; Ramsay and Witthames, 1996; Bromley, 2003). In contrast to females where age is of more influence on first maturity than weight, in males weight is a more important determinant for maturity (Bromley, 2003). Also in Chinook salmon, body weight is described to be of influence on puberty, i.e. first maturation. Maturing fish were significantly heavier than non-mature fish of the same age (Shearer and Swanson, 2000). For Turbot the same pattern is shown. The heaviest animals were mature earlier than less heavy animals, at several temperatures. In relation to this, a correlation between temperature and first maturation was found. Animals kept at higher temperatures were mature earlier (Imsland *et al.*, 1997).

Nevertheless, sole males mature earlier and at smaller size than females. The precise fractions of males that indeed spawn at a certain age are very difficult to determine, though. Stages of maturation in males have to be determined by techniques as histological screening. Macroscopic evaluations of testes are not sufficient and reliable (Bromley, 2003). In case of for example Greenback Flounder, *Rhombosolea tapirina*, macroscopic change is not accompanied by change in equivalent change in germ cell presence (Barnett and Pankhurst, 1999).
In commercial and research culture circumstances, a wide range of sizes for both males and females is used: 0.45 to 1.5 kg for females and 0.2 to 1.4 kg for males (see table 1). This does not seem to be out of natural ranges. No relation between size of the males and fertilization rates could be found (Howell *et al.*, 2003).

Table 1. Weight and minimal age of captured and F1 and wild S.solea broodstock.

Captured and F1		
Weight Female	450-1500 (average)	Howell *et al.*, 2003
Weight Male	200-1400 (average)	Howell *et al.*, 2003
Minimal age at spawning both sexes	3-6	Howell *et al.*, 2003

Wild		
Weight Female	200-300 (minimal)	Bromley, 2003
Weight Male	160-200 (minimal)	Bromley, 2003
Minimal age at spawning both sexes	2-3	Van Beek, 1985 Ramsay and Witthames 1996 Bromley, 2003

2.1.3 Nutritional requirements

In general, different diets are used for broodstock fish in commercial circumstances. An emphasis lies on natural diets. Though dietary aspects are believed to be of direct influence on broodstock performance, work on nutrient requirements for broodstock is limited on a few species. Often, it is believed that for optimal reproduction natural resources should be used as much as possible. However, natural food does have some disadvantages as well. A less stabilised and homogenous, natural, diet can have negative effects (Pavlov *et al.*, 2004). Therefore many times sole broodstock diets are a combination of both formulated and natural diets (Howell *et al.*, 2003).

For sole, a carnivorous fish, natural resources are used. Examples are polychaetes, molluscs and crustacea (Pavlov *et al.*, 2004). Stomach content analyses of wild *S. solea* resulted in ten most abundant prey species being several crustacea and worms. Of these, the crustacea species *Callianassidae* and *Alpheidae* have highest proportions amino acids. *Callianassidae* contain the highest concentration of essential amino acids (EAA's). The three EAA's phenylalanine, histidine and lysine were the most abundant in sole stomachs (Molinero et al., 1994).

There is one case where a diet that only consisted of formulated feed resulted in could be the cause of total failure of fertilization by F1 broodstock. However, there is no real evidence since F1 animals also show disorders when complete natural diets are administered (Howell *et al.*, 2003).

In many species highly unsaturated fatty acids (HUFA's), vitamin E and ascorbic acid (Vitamin C) are important in broodstock dietary requirements. An other influence on steroidogenesis and fertilization processes is shown by eicosapentaenoic acid (EPA) and arachidonic acid (AA) levels. In goldfish, EPA and AA modulate steroidogenesis by acting on testosterone. Further, both EPA and AA are precursors of prostaglandins which can act as pheromones. Pheromones are known to stimulate sexual behaviour and synchronise spawning (Izquierdo et al., 2001).

Dietary lipid levels and resulting whole body lipid have an effect on the moment of maturation in Chinook salmon (*Oncorhynchus tshawytscha*). A significant percentage of Chinook salmon fed high lipid diets matured earlier than fish fed diets containing lower lipid concentrations (Shearer and Swanson, 2000).

The period of administering the broodstock diet depends on the mode of spawning per species. In batch spawners, ovaries are affected by nutrition during vitellogenesis, indicating slightly longer periods of vulnerability for diet. An exception on turbot: spermatogenesis in this species is most affected by diet during the early stages of gonadal development (Izquierdo et al., 2001). This indicates that feed administration for broodstock candidates-to-be can be important. F1 animals which have not been reared under wild conditions with wild "optimal" feed, could for this reason miss certain basic needs for proper spermatogenesis of oögenesis. Nevertheless, it must be noted that many occasions deficiencies in diets are reversible (Izquierdo et al., 2001).

2.1.4 Physical environment.

In the Bay of Biscay sole this is recorded to be a shoreward spawner (Amara *et al.*, 1998) at depths of about 40 to 100 m (Koutsikopoulos and Lacroix, 1992) whereas in the Irish sea fish are spawning at <40 m (Symonds and Rogers, 1995). Tank depth in artificial spawning differs from 0.7 – 1.2 meters. No effect on fertilizations rates could be detected (Howell *et al.*, 2003).

During the wild spawning season in the Irish sea, sole prefers sandy bottoms though when density increases, during spawning seasons, also other sediments like mud and gravel where used (Amezcua and Nash, 2001). In the North Sea and the Bay of Biscay sole spawns on sandy and muddy banks as well (Koutsikopoulos and Lacroix, 1992). It is therefore not likely that sole prefers one certain substrate. In many successful cases, no substrate is used for artificial circumstances (Howell *et al.*, 2003).

In culture, several different broodstock tanks are used. Rectangular and round are both used in concrete, Glass Re-enforced Plastic (GRP) or Polyester. Both recirculation and flow-through systems are used. No deleterious effects on fertilization rates of these systems could be detected (Howell *et al.*, 2003).

Broodstock animals are maintained on densities between approximately 2 and 13 fish per m^2, with an average of 6.1 animals per m^2. A ratio of approximately 0.9 males per female is maintained. No influence of these parameters on amount of egg produced or fertilization rates could be found. For both F1 and captured animals the same substrates are used. See also table 2 (Howell *et al.*, 2003).

Table 2. Sex ratio and density in captured and F1 (a) and wild (b) fish stocks.

(a) Captured and F1		
Sex ratio male/female	0.9/1	Howell *et al.*, 2003
Density (fish/m^2)	2-13	Howell *et al.*, 2003
(b) Wild		
Sex ratio male/female	1/1?	Bromley, 2003
Density (fish/m^2)	-	-

2.1.5 Gonads and spermatogenesis

Sole mates in couples and in groups. This counts for culture conditions and probably for the wild as well (Baynes *et al.*, 1994). Spawning of captured sole was monitored on camera. Fish were seen mating in couples. The males start the ritual by crawling under a female in that way that their genital pores are close to each other. Then the couple swims upwards with synchronised body movements for about 1,5 minutes. Most likely this is the moment of release of eggs and fertilization. Though no eggs were seen being released. After monitoring the above described behaviour eggs were found at the same time (Baynes *et al.*, 1994). Spawning activity in both wild and captured sole was recorded to occur between about 18.00 pm to 21.00, the first hours of darkness (Baynes *et al.*, 1994; Child *et al.* 1991). Possibly in relation to the spawning behaviour with intensive body contact, sole males show relatively small testes (Howell *et al.*, 1991; Baynes *et al.*, 1994; Pavlov *et al.* 2004); there is no need of excessive high amounts of milt. This is also shown in turbot (Pavlov *et al.*, 2004).

Gonadosomatic indices (Gonado Somatic Index (gonad weight/(body weight - gonad weight) x 100)) of the common sole even are the smallest among coldwater marine fish with external fertilization. Average values are 0.2% (Pavlov *et al.*, 2004*)*.

Female GSI value do show seasonal variation, with increasing values towards the spawning season in March, see figure 5.

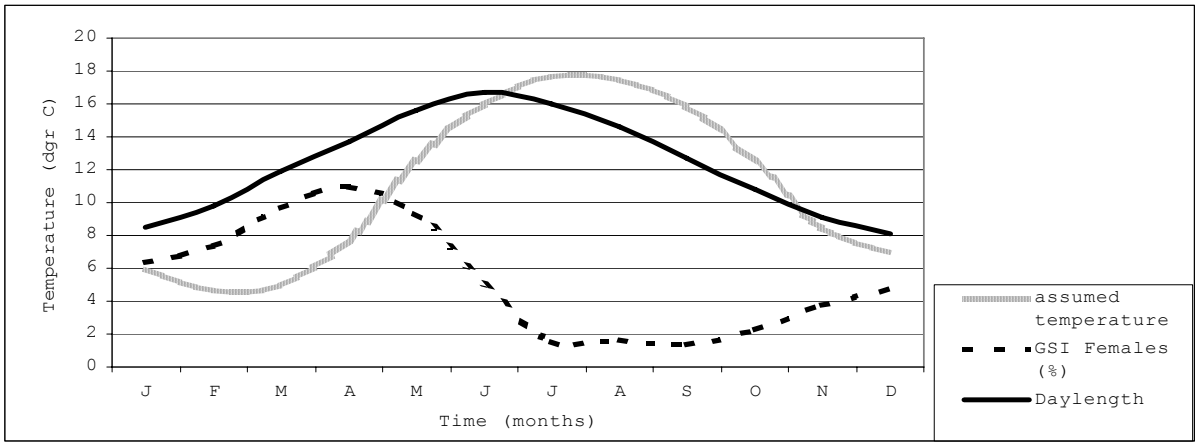
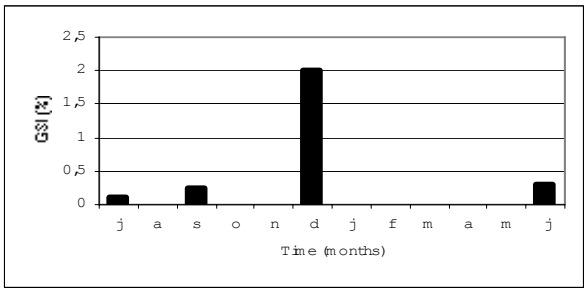


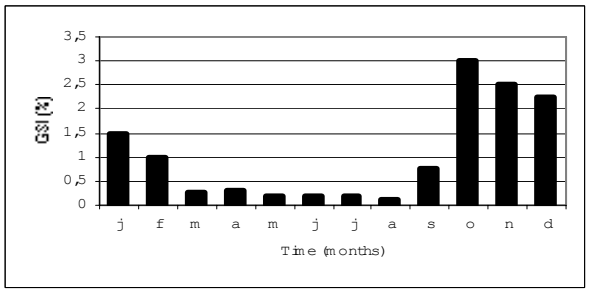
Figure 5. Annual GSI% of female North Sea sole (RIVO 1980) versus annual daylength (hours sun light) (KNMI 2004) and assumed temperature (°C) fluctuations.

It is very likely that sole testis show a similar seasonal variation. Plaice, *Pleuronectes platessa*, Atlantic halibut, *Hippoglossus hippoglossus*, and Flounder, *Platichthys flesus* (L.), for example, enter a phase of testicular regression after the spawning season (see fig 6). In figure *5*, female GSI (Gonadosomatic index) is shown as a function of annual cyclic temperature and day length. Here regression starts when temperature

rises above 10°C in spring (just after spawning). After some time, gonads start to grow. Female sole gonads start growing at approximately 14°C.

Testes showing this variation are called prenuptial testes (Barr, 1963; Norberg et al., 2001; Weltzien *et al.*, 2002). The successive macroscopic developmental stages of sole testis are described in table 3.





Atlantic halibut Flounder

Figure 6. Annual variation in GSI of Atlantic halibut under simulated natural photoperiods (After: Norberg et al., 2001) and Flounder (Platichthys flesus (L.)) under natural photoperiods (Janssen, 1996)

Table 3. Macroscopic stages of sole testis. (After: Bromley 2003)

Stage	Features
Immature	Very small, usually not larger than about 0,2 cm.
Spent, recovering	Thin, redness lost, little sperm in ducts.
Half full	Filling, half full, no sperm in ducts
Full	Fully swollen
Running	Sperm in duct? sperm can be extruded.
Spent	Red colour, little sperm in ducts.

2.2 Histology and morphology of spermatogenesis

In this part, the structure of the male gonadal development is explained. The process of formation of consecutively spermatogonia, spermatocytes and spermatids to spermatozoa is explained as well as the function of supporting and regulating Sertoli and Leydig cells. The complete process is stimulated by steroid hormones and FSH of which the basic functions are described too.

2.2.1 Spermatogenesis in sole

Spermatogenesis is the process of production of male gametes. In this process germ cells undergo three different stages; mitotic proliferation, meiosis and spermiogenesis. Mitotic proliferation results in spermatogonia and later in spermatocytes whereas meiosis of spermatocytes brings forth spermatids. Spermiogenesis results in spermatozoa (photo 1) (Schulz and Miura, 2002). Figure 7 is described in the following paragraphs with emphasis on spermatogenesis typical for sole.

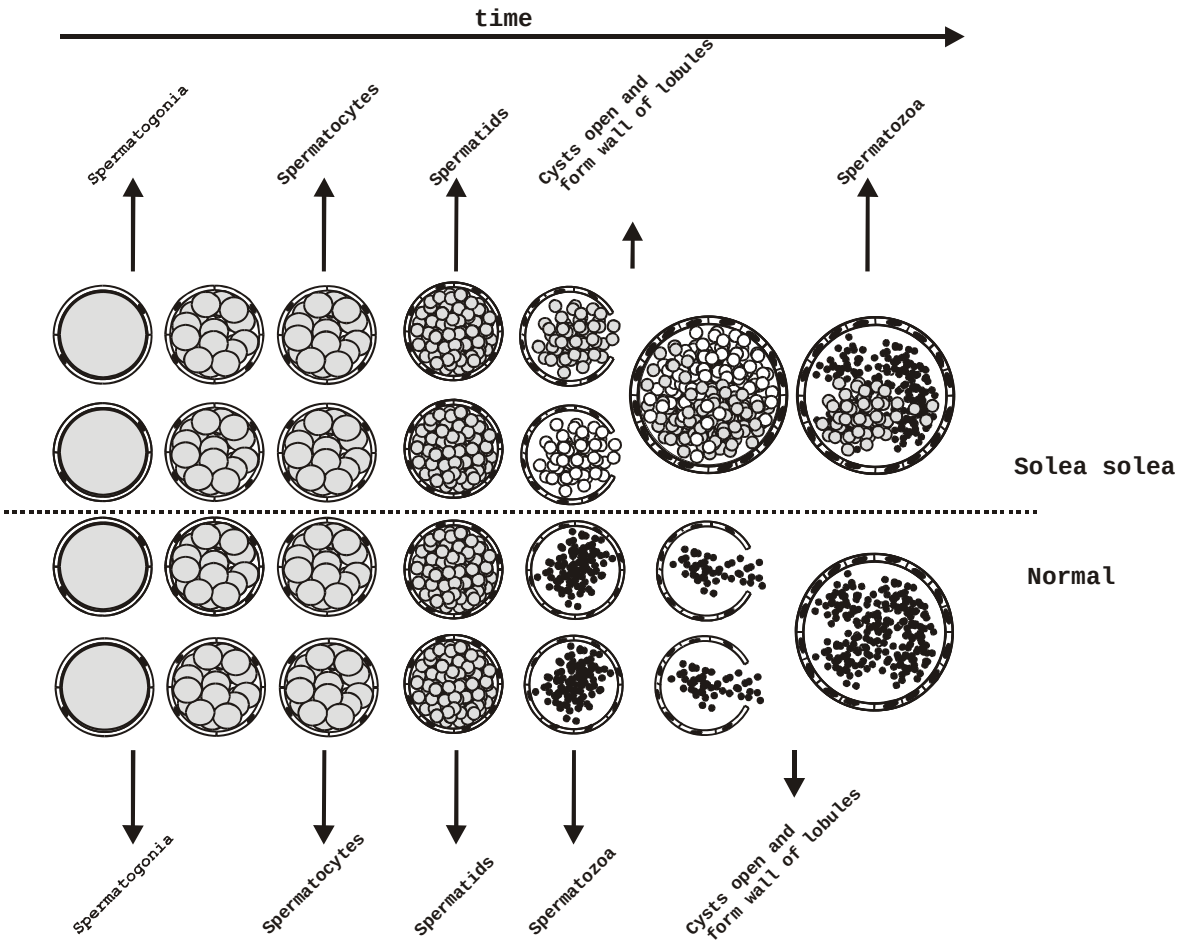


Figure 7. Spermatogenesis in Sole and normal spermatogenesis. Sole: spermatogonia proliferation to meiosis; opening of the cysts with spermatids, Sertoli cells form wall of lobules; spermiogenesis: spermatids and spermatozoa mixed. Normal: spermatogonia proliferation and meiosis; spermiogenesis; opening of the cysts; Sertoli cells form wall of lobules (Blonk, 2005)

Proliferation

Spermatogonia are generated from (spermatogonial) stem cells. The name (spermatogonial) stem cells is used for spermatogonia that have the capacity of cell renewal. Spermatogonial stem cells have two functions: i) renewal of stem cells for long term production of gametes and ii) differentiation into spermatogonia with the destiny of further development to spermatozoa. Once being committed for proliferation, germ cells do have incomplete cytokinesis: daughter cells are connected by cytoplasmic bridges (Schulz and Miura, 2002; Weltzien et al, 2004).

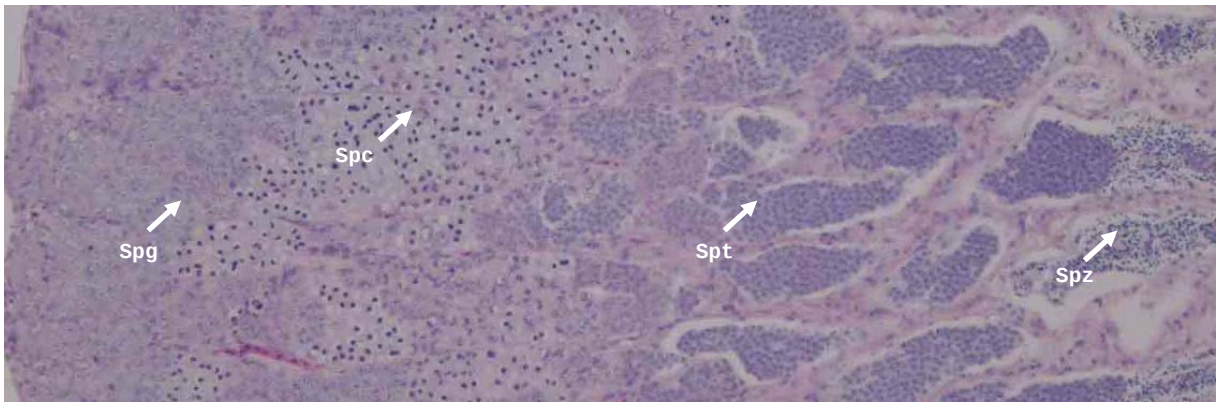


Photo 1. The different successive stages of spermatogenesis in sole. (Spg) Spermatogonia; (Spc) Spermatocytes; (Spt) spermatids; (Spz) spermatozoa.

After a genetically determined number of divisions the spermatogonia enter meiosis, the process to develop haploid gametes. In these divisions there are several different germ cell types. Every process that takes place to eventually end up in several spermatozoa starts with one spermatogonium. After the first mitotic division, two new spermatogonia emerge. A series of mitotic divisions of proliferation results in more spermatogonia. The number of divisions differs per species. (Schulz and Miura, 2002)
At a certain moment the cells differentiate so-called B spermatogonia. (Schulz and Miura, 2002)

Meiosis
In meiosis DNA in cell nuclei is first duplicated after which two cell divisions take place. During the first division (Meiosis I) the duplicated and condensated chromosomes are separated. The second division (Meiosis II), without DNA replication, results in haploid cells. Summarized: after meiosis one cell results in four gametes (Schulz and Miura, 2002).

Meiosis I in fish starts after differentiation of B spermatogonia into primary spermatocytes. Division of primary spermatocytes results in secondary spermatocytes. These cells are divided again by Meiosis II and haploid round-shaped spermatids are formed (Schulz and Miura, 2002).

In most teleosts, the cysts open and release the germ cells into the tubuli at a stage following that of spermatids (the spermatozoa stage). Here the last development takes place (Schulz and Miura 2002). In sole, this is different. Here, the cysts already open at the stage of spermatids (Spt), which are then are released into the lobules. Because the cysts open before completion of spermiogenesis batches of cells of different stages are combined resulting in a mix of spermatids and spermatozoa in one lobule. See figure 7.

Spermiogenesis
Spermatids differentiate in small spermatozoa. The size of the cells, compared to the previous round spermatids, is decreased by chromatin condensation and extrusion of cellular material. Further flagella, a midpiece and a head are formed. In fish sperm there is no acrosome present, since the sperm enters the oöcyte through the micropyle. During the final development of the spermatozoa from spermatids, the milt hydration takes place. It is at this moment that the spermatozoa receive their capacity of fertilization. (Schulz and Miura, 2002; Pavlov *et al.*, 2004; Weltzien *et al.*, 2004).

2.2.2 Testis structure
The reproductive organ of teleost males is build up from double testes and an efferent spermiduct. The spermiduct and the urinary canal joins short before the ventral side of the abdominal cavity ending in the urigenital papilla (Pavlov *et al.*, 2004). In the testis we see two integrated compartments; the interstitial and the germinal compartment. The latter contains the germ cells (Schulz and Miura, 2002).

In fish we can divide the structure and infrastructure of the testis in two ways. One classification is done by recognising differences in the morphology of the germinal compartment. In most teleosts anastomosing tubular testis types are found, whereas in some teleost fish lobular testes are described (Schulz and Miura, 2002; Weltzien *et al.*, 2004). Lobular testes contain lobules terminating at the periphery of the testis and opening at the central efferent duct. Anastomosing testes contain branching canals (tubules). Nevertheless, during time it evolved that this classification was not used properly many times, mostly by not using the proper definition of tubule or lobule (Weltzien *et al.*, 2004).

An other feature is made by using the distribution of germ cells within the germinal compartment. Here two types can be found: unrestricted and restricted types. These classifications respectively refer to the spermatogonia that are respectively found anywhere along the tubule or lobule or only at the periphery of the testis (Weltzien *et al.*, 2004). The last only is found in higher perciform and atheriniform fish (Schulz and Miura, 2002).

In sole a lobular restricted testis can be recognised. The testis completely consists of bundles of many strong sinuous lobules. Within each lobule spermatozoa are produced in a apparently normal way. The process starts with the spermatogonial stem cells (Spg) at the very closed end, then followed by spermatocytes (Spc) and spermatids (Spt) (Blonk, 2005).

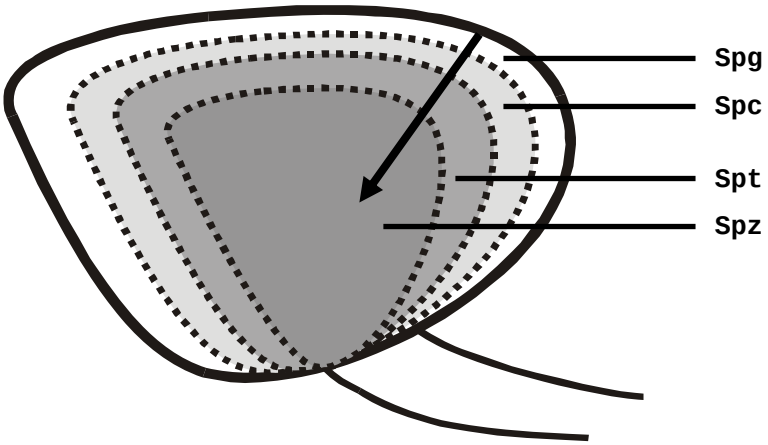


Figure 8. Top view of the sole testis. Regions with continuing development of germ cells from spermatogonia to finally spermatozoa. Cells in cysts move deeper inside the testis according to the arrow.

Along with the continuing spermatogenesis, the germ cells mover deeper into the testis. This can be seen in figure 8. The development of spermatogonia, the mitotic proliferation, takes place within cysts. As development continues the cysts move along the lobule in ventral-lateral direction of the testis (Blonk, 2005).

In sole testes the cells are homogenous distributed between 30 and 70% (Figure *9*). The centre (50%) of the organ is the optimal location to sample and compare. Here the spermiduct can be recognised and all cell stages are found. This is in contrast to the periphery of the testes were more or even only spermatogonia are found (Blonk 2005).

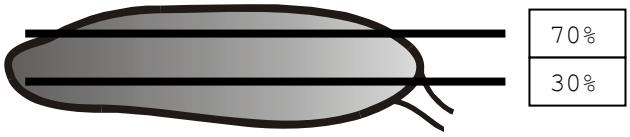


Figure 9. Side view of the testis of sole. Homogeneity of cell distribution is found between 30 and 70% as indicated.

2.2.3 Sertoli and Leydig cells

At the very start of mitotic proliferation, each spermatogonium is enclosed by Sertoli cells that probably provide the sex cells with stimulating factors. This complex of cells is called a cyst or germ cell-Sertoli complex and is forming a micro-environment for the germ cells. The cysts remain intact upon maturation, just before release of spermatozoa (Weltzien *et al.*, 2004). Because the grouped clones of cells are now separated by cysts, each cyst consists of exclusively cells with the same age and stage (Schulz and Miura, 2002; Miura *et al.*, 1996).

Sertoli cells provide the germ cells throughout the process of spermatogenesis with physical support and stimulating factors to complete the process from proliferation to differentiation. Phagocytotic activities to remove dead germ cells also is one of the functions of these somatic cells (Weltzien et al., 2004). In Japanese eel, *Anguilla japonica*, spermatogenesis can not take place without the complex of Sertoli cells (cysts) and germ cells (Miura *et al.*, 1996).
Leydig cells are of importance for supply of steroids for stimulating the process of spermatogenesis as well as for the secondary sexual characters and sexual behaviour (Weltzien 2004).

2.3 Biochemistry during spermatogenesis

Puberty in fish is indicated by the onset of spermatogenesis. The regulating key to the start of puberty, as well as the continuing process of gamete production is controlled by the endocrine pathway of the BPG (Brain Pituitary Gonad) axis (Schulz and Goos, 1999; Weltzien et al., 2004). Androgens and pituitary GTH (gonadotropin hormone, FSH and LH) are the most significant hormones for spermatogenesis. Sertoli cells are most likely activated by FSH, whereas LH is stimulating Leydig cells androgen production (see figure 10). Androgens mainly produce testosterone (T) and 11-ketotestosterone (11kT)(Schulz *et al.*, 2001; Weltzien *et al.*, 2004). It is likely that there also are one or more negative feedback loops on the BPG axis. This as a prevention against excessive production of steroids, see figure 11 (Schulz and Miura, 2002).

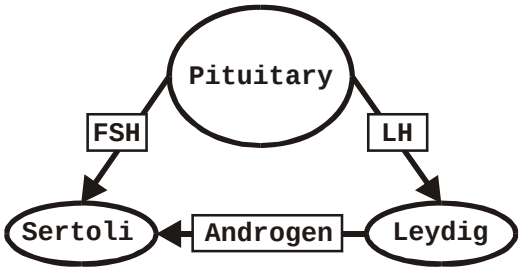


Figure 10. Induction of Sertoli cells by Leydig cells androgen steroid production and by the pituitary FSH production

2.3.1 Steroid hormones

Higher 11-ketotestosterone (11kT) steroid profiles seem to be associated with gonad growth and higher GSI's. GSI, testicular weights and 11-ketotestosterone levels seem to be positively correlated in Atlantic halibut and Plaice *(Pleuronectes* platessa) (Barr, 1963; Weltzien *et al.*, 2002). The same relation with testosterone (T) is observed in Atlantic halibut.
As shown in the previous paragraph 2.1.5., both Plaice and Atlantic halibut show the an annual testicular growth pattern. After spawning the testes first enter a period of regression after which the gonads again increase in mass. This type of cyclic development of male gonad is called prenuptial spermatogenesis (Weltzien *et al.*, 2002). Even so in rainbow trout (*Salmo gairdneri*) a comparable situation and relation is described with 11kT, the major androgen in salmonids (Schulz, 1984). 11kT could not induce spermatogenesis in Rainbow trout in vitro, without the presence of Sertoli or Leydig (Loir, 1999) emphasising the relevance of these cells. In Greenback Flounder, *Rhombosolea tapirina* (Günther 1862) no indication for such a relationship is found (Barnett and Pankhurst, 1999).

With its possible relation to gonadal weights, 11kT can have an effect on gamete development in several species with prenuptial testis (Norberg *et al.*, 2001; Weltzien *et al.*, 2002). In case of the previous described two species Atlantic halibut and Plaice, successive stages of germ cells in testes were accompanied by elevated levels of 11kT. Higher levels were associated with further developed testes i.e. testes with more spermatids and spermatozoa. This relation suggests that the level of 11kT determines the activation of Sertoli cells to induce development of germ cells. (Norberg *et al.*, 2001; Weltzien *et al.*, 2002). No dose-response experiment has been performed on this effect to prove the described relation.

A possible similar effect is found in Japanese eel, *A. japonica*. Experiments did test for effects of different 11kT concentrations on spermatogonia in vitro (0.01, 1, 10 and 100 ng/ml). No effect on cell mitosis of spermatogonia was recorded for levels of 0.01 and 1 ng/ml. Both 10 and 100 ng/ml 11kT did induce mitosis of spermatogonia but equally (Miura *et al.*, 1991a). Possibly different responses could be detected when measuring effects of hormone levels between 1 and 10 ng/ml 11kT.

According to literature, T is capable of blocking 11kT induced spermatogenesis in catfish (Cavaco *et al.* 2001). Probably, 11kT production is indirectly suppressed since T negatively affects GTH production (in particular FSH). The same effect is observed in Atlantic salmon, *Salmo salar* (Borg *et al.*, 1998). It must be noted here, that catfish does not utilise T for spermatogenesis, and T therefore only can block the process via negative feedback (Cavaco *et al.*, 2001). An other relationship is described in Atlantic halibut. Here, increasing T and 11kT levels are described together in combination with continuing spermatogenesis, indicating T does not necessarily block spermatogenesis (Weltzien *et al.*, 2002).

2.3.2 Gonadotropins

11kT and to lesser extend T are not the only factors that can determine spermatogenesis. In Japanese eel, gonadotropin (GTH) separately from 11kT can induce development of all subsequent cell stages of spermatogenesis and spermiogenesis (Schulz and Miura, 2002; Miura *et al.*, 1991a and 1991b). It is found that 11kT production actually is dependent on GTH production by the pituitary which in turn is activated after induction by Gonadotropin-releasing hormone (GnRH). GnRH is released after induction by internal stimuli as biological clocks and external stimuli. External factors are for example nutritional status, temperature cycles, photoperiod or pheromones (Weltzien *et al.*, 2004). See figure 11.

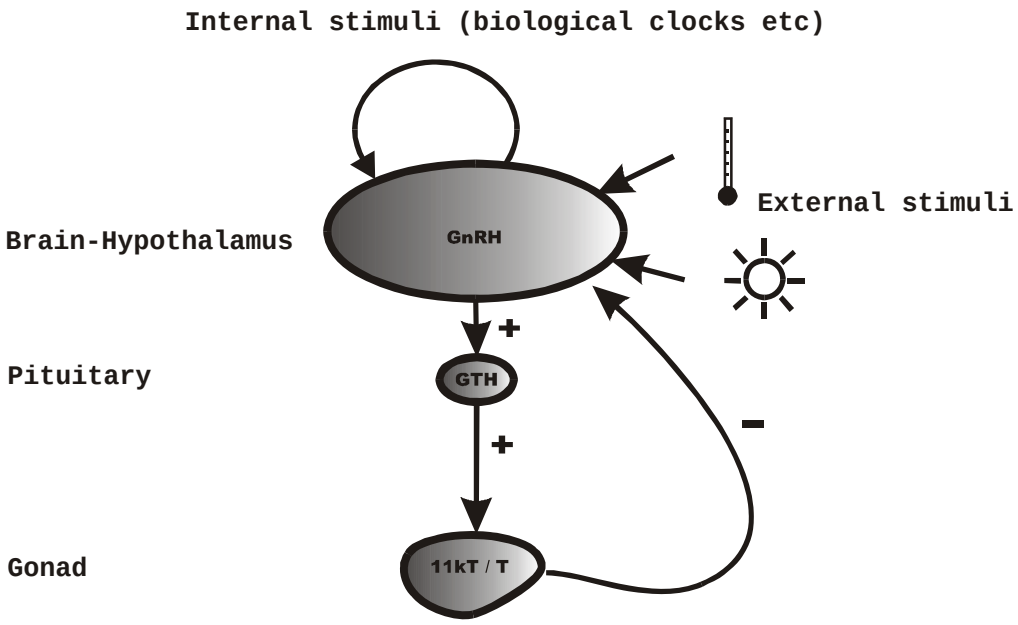


Figure 11. The BPG (Brain-Pituitary-Gonad) axis is induced by internal and external stimuli and shows a negative feedback loop by 11kT and T.

As described before the Leydig cells are responsible for the steroid supply of the germ cells. In experiments on Japanese eel, 11kT producing Leydig cells in vitro remain inactive when only 11kT is added. However, they are activated when HCG (Human Chorionic Gonadotropin) is administered (Miura *et al.*, 1991b). When administering HCG to wild sole females it was possible to induce spawning and improve fertilization rates and amounts of eggs spawned. It is likely therefore, that GTH is inducing spermatogenesis and spawning of sole, though this experiment was not done on males (Ramos, 1986).

Sertoli cells become active through 11kT produced in Leydig cells (Miura *et al.*, 1991b; Miura *et al.*, 1996). In the Japanese Eel, both 11kT and gonadotropin separately induce development of all successive cell stages of spermatogenesis (Miura *et al.*, 1991a and 1991b), but only in coculture with Sertoli cells. Without the presence of either Sertoli cells or 11kT, no development of spermatogonia takes place (Miura *et al.*, 1996). This indicates that GTH or 11kT are restricting induction of spermatogenesis in eel (Miura *et al.*, 1991a; Miura *et al.*, 1996; Schulz and Miura, 2002). Other steroids than 11kT, like 11β-hydroxytestosterone and testosterone, did not initiate cell proliferation (Miura *et al.*, 1991a).

2.3.3 Maturation inducing steroid

During the final development of the spermatozoa from spermatids, the milt hydration takes place. It is at this moment that the spermatozoa receive their capacity of fertilization; the capacitation (Schulz and Miura, 2002; Pavlov *et al.*, 2004).

Capacitation is induced by the maturation-inducing-steroids (MIS's). The exact responsible steroids for this process, have not clearly been identified in (flat)fish. Nevertheless, there are strong indications of existence and function of several hormones. Examples, unique for teleosts, are 17α,20β–dihydroxy-4-pregnen-3-one (17,20β-P) and 17α,20β,21–trihydroxy-4-pregnen-3-one (17,20β,21-P). By administering GnRHa (Gonadotropin Releasing Hormone agonist) to male broodstock of Atlantic halibut, milt hydration was

enhanced and spawning was prolonged. This was accompanied by a significant rise in level of the gonadal steroids, 3β,17,20β-P-5β-S and 17,20α–dihydroxy-4-pregnen-3-one, suggesting a relation of this steroids with spawning. It must be noted that the authors stated that 3β,17,20β-P-5β-S levels reflect 17,20β-P levels but at 10-fold higher concentrations (Vermeirssen *et al.*, 2004). In common Dentex, *Dentex dentex*, GnRHa also induced spawning and 17,20β-P levels. In flatfish as Sole, Plaice and Dab (*Limanda limanda*) 17,20β-P was detected as well and of possible influence (Weltzien *et al.*, 2004).

2.3.4 Other factors

Even though its important determining influence 11kT probably is not the single final factor for normal gametogenesis (Schulz and Goos, 1999). A point of consideration could be the fine-tuning of spermatogenesis by several (paracrine and autocrine) factors induced by 11kT (Miura *et al.*, 1997). Activin B for instance, is produced by Sertoli cells in response to 11kT and induces cell mitosis of spermatogonia but not further stages in gametogenesis (Schulz and Miura, 2002; Weltzien *et al.*, 2004). The existence of Activin B does not make it unlikely that there are comparable factors with influence on later stages in spermatogenesis and spermiogenesis. The exact process of meiosis for example, is still not known (Weltzien *et al.*, 2004).

Regulation and fine-tuning of spermatogenesis occurs in mainly two ways; hormonal pathways i) and genetically fixed programs ii). A considerable part of the spermatogenesis is explained by steroid hormonal effects. However, hormones probably are not regulating every single step in the cascade. There is a certain amount of so-called “hormone-dependent checkpoints”. Reactions between these points are not likely to be dependent on hormonal responses. Few but important points seem to be influenced by endocrine regulation. Stem cell activity, proliferation, start of meiosis, apoptosis (disintegration of cells), Sertoli cell proliferation and differentiation are such points (Schulz and Miura, 2004).

3. Materials and Methods

This thesis research is aimed to describe the differences in spermatogenesis between Captured, Wild and F1 sole, *S. solea*, before and during spawning. This is done in order to *i)* describe the development of the testes in time and *ii)* to give a possible explanation of the failure of fertilization by F1 broodstock males.

Therefore, three groups of respectively Captured, Wild and F1 fish in the months March and June were compared on both histological and steroidical features of spermatogenesis.

3.1 Experimental animals

The origin of the three groups of experimental male sole is described in table 4

Table 4. Origin of experimental Captured (C), F1 (F) and Wild (W) broodstock groups per month.

Group	Month	Definition	N	Origin
Captured	March*	Animals caught at sea and kept in culture conditions.	7	Tank 7 Solea B.V.
Wild	March*	Animals sampled at sea.	14	North Sea
Captured	June	Animals caught at sea and kept in culture conditions.	7	Tank 7 Solea B.V.
F1	June*	Animals reared in culture conditions.	5	Tank 8 Solea B.V.
Wild	June*	Animals sampled at sea.	14	North Sea

* Year: 2003

Males were sampled before (March 2003) and shortly after spawning (June 2003) to obtain an impression of the active male gonad of sole over time. All animals were intended to be of approximately equal physiological age, weight and condition in order to be able to compare data from Captured, Wild and F1 sole.

3.2 Experimental Design

This study is focussed on the process of spermatogenesis in sole, *S. solea*, in relation with the steroid hormone 11kT profile. The aim of the experiment was to determine differences in histology, gonadosomatic index (GSI) and blood plasma steroid 11-ketotestosterone (11kT) levels between the three treatment groups Captured, Wild and F1 animals before (March) and shortly after (June) spawning.

Three points of consideration can be distinguished:

1) Differences in histology, GSI and steroid (11kT) levels between March and June for Captured and Wild animals;
2) Differences in histology, GSI and steroid (11kT) levels between Captured and Wild animals in March and in June;
3) Abnormality of F1 in histology, GSI and steroid (11kT) levels compared to Captured and Wild animals in June.

Differences in spermatogenesis between treatment groups will be characterised by histology (germ cell abundance), GSI and 11kT levels according to the scheme in table *5*. Treatment groups were compared on spermatogenesis in order to detect possible indications for hampered spermatogenesis in F1 animals. To support the histological results, levels of 11kT were measured.

Table 5. Experimental design.

Measurements	Treatment groups				
	March		June		
	Wild	Captured	Captured	Wild	F1
Spermatogenesis • Histology • GSI • Steroid (11kT) patterns	n = 14	N = 7	n = 7	n = 14	n = 5

3.3 Measurements

In the following part the environmental circumstances of the experimental animals, as well as the methods for determination of histology parameters and 11kT levels are set out.

3.3.1 Sampling procedure

Each sampled animal was anaesthetised with an overdose TMS, weighed and sacrificed. From each group left and right testis were sampled on location. After weighing the testes were directly fixated in Bouin's fixative for 4 to 8 hours. The testes of the Wild group were not weighted, since it was not possible to weigh on board of research vessel Tridens. After fixation the testes were successively treated with an ethanol series, Amyl-acetate and finally embedded in paraffin according to standard procedures (See appendix I). Sections of 5µm were made from the embedded samples with a microtome. Every sampled section was fixed on a object glass and stained with Haemaluin-Eosin (See appendix I). Blood samples were taken and processed as described in appendix I. 11kT blood plasma levels were measured using Radio Immuno Assay (Schulz, 1984).

3.3.2 Testicular histology

Of each animal one testis was analysed using a microscope (Olympus BH-2, 100x magnification) and a grid ocular. Each cell covered by a cross of the grid was scored. This was done for four germ cell types; spermatogonia, spermatocytes, spermatids and spermatozoa.
A relative constant surface area per testis, 20% of the width along the complete length of the section, was scored. This is shown in figure 12.

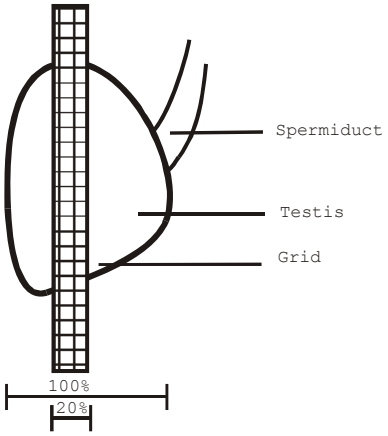


Figure 12. Orientation of testis and position of the grid during scoring of cell types.

3.3.3 Determination of environmental conditions

A description the measured environmental conditions of the used samples is given in table 6.

Table 6. Measured environmental conditions of all experimental groups.

Characteristic	Unit
System design	recirc./flowthrough/open sea
Tank design/spawning ground	Depth, substrate, m^2, m^3
Temperature	°C
Day degrees	°C
Photoperiod	Light hours
Water quality (pH, salinity, hardness)	pH, ‰, °DH,
Feeding ratio	% bodyweight
Composition diet	Type of feed used.
Density	Fish/m^2
Sex ratio	M/F

3.4 Measurements and calculations

On each animal the parameters were measured as shown in table 7:

Table 7. Measurements of parameters per animal.

Measurements	
Steroid level (11kT)	(ng/ml)
Body weight (BW)	(g)
Standard Length (SL)	(mm)
Gonad weight (GW)	(g)
Spermatogonia	(absolute and relative amount)
Spermatocytes	(absolute and relative amount)
Spermatids	(absolute and relative amount)
Spermatozoa	(absolute and relative amount)

GSI was calculated as follows:

$$\frac{\text{Gonad weight}}{\text{Body weight}} * 100\% \text{ (Janssen, 1996)} \qquad \text{(Equation 1)}$$

Condition:

$$\frac{\text{Body weight}}{\text{Body length}^3} * 100\% \text{ (Bromley, 2003)} \qquad \text{(Equation 2)}$$

Day degrees:

$$\sum (\text{Mean daily temperature}) \qquad \text{(Equation 3)}$$

Relative amounts of germ cells type (spermatogonia, spermatocytes, spermatids and spermatozoa):

$$\frac{\text{Scored cells per germ cell type}}{\text{crosses in measured surface}} \qquad \text{(Equation 4)}$$

3.5 Statistical analysis

For tests of significance between quantitative data SAS v.6.12 software was used. First recorded mean data were tested per cell type for normality of variance using a Shapiro-Wilk W test ($\alpha = 0.05$). Data were transformed when necessary with square root or log transformations. Significant differences between groups were determined using an t-test and least square means methods ($\alpha = 0.05$). Pearson correlation tests were performed for relations between data.

4. Results

Of all fish, one testis was analysed on variables. The described results are accompanied by detailed (Tables 8, 9 and 11) tables and graphs. In the graphs significant differences between the columns are reflected by lack of resembling characters.
The complete dataset is shown in appendix II.

4.1 Summarized differences

In this paragraph relevant average data per group and significant differences per group are given.
In table 8 and 9 mean, standard deviations (sd.) and number (n) are found whereas tables 10 and 11 give the significant differences between groups, determined by the least square means method. Table 10 gives the differences between the months March and June between Wild and Captured animals. Table 11 analyses differences between groups in the same months, March and June.

Table 8. Means and Standard Deviations (Sd.) and number (n) for measured variables per Captured and Wild group in March.

	March					
variable	Captured			Wild		
	Mean	Sd.	n	Mean	Sd.	n
11kT (ng/ml)	13,63	12,13	7	33,97	12,54	12
SL (cm)	26,14	3,33	7	26,27	2,14	14
BW (g)	180,14	98,86	7	153,00	35,70	14
GW (g)	0,40	0,24	7	[1)]n.m.	[1)]n.m.	
GSI (%)	0,23	0,14	7	[1)]n.m.	[1)]n.m.	
Condition	0,93	0,19	7	0,83	0,06	14
Spermatogonia	52,50	17,68	2	64,11	44,70	9
Spermatocytes	177,50	16,26	2	109,22	53,38	9
Spermatids	250,00	168,29	2	522,56	451,78	9
Spermatozoa	182,50	94,05	2	339,22	280,81	9
Spermatogonia %	2%	1%	2	3%	2%	9
Spermatocytes %	8%	0%	2	5%	3%	9
Spermatids %	11%	8%	2	25%	17%	9
Spermatozoa %	8%	5%	2	14%	10%	9

[1)]n.m. = not measured

Table 9. Means and Standard Deviations (Sd.) and number (n) for measured variables per Captured, F1 and Wild group in June.

	June								
Origin	Captured			F1			Wild		
	Mean	Sd.	n	Mean	Sd.	n	Mean	Sd.	n
11kT (ng/ml)	6,66	5,83	7	1,01	1,82	5	5,62	5,81	12
SL (cm)	27,13	4,66	7	26,74	0,82	5	25,14	2,79	14
BW (g)	171,43	101,82	7	194,00	19,53	5	125,79	37,44	14
GW (g)	0,30	0,44	7	0,16	0,05	5	[1)]n.m.	[1)]n.m.	
GSI (%)	0,14	0,11	7	0,08	0,02	5	[1)]n.m.	[1)]n.m.	
Condition	0,78	0,06	7	1,01	0,06	5	0,78	0,09	14
Spermatogonia	40,50	18,52	4	34,25	23,61	4	27,20	8,98	5
Spermatocytes	102,50	63,32	4	53,25	62,72	4	25,40	18,22	5
Spermatids	195,25	209,88	4	541,25	216,86	4	177,00	264,94	5
Spermatozoa	398,50	287,08	4	150,75	116,36	4	137,40	102,55	5
Spermatogonia %	3%	2%	4	3%	2%	4	5%	3%	5
Spermatocytes %	6%	3%	4	5%	5%	4	6%	6%	5
Spermatids %	12%	13%	4	44%	4%	4	18%	21%	5
Spermatozoa %	28%	22%	4	12%	10%	4	16%	11%	5

[1)]n.m. = not measured

Table 10. Differences between variables per group over months: Wild and Captured differences between March and June.

	Wild	Captured
variable	March – June	March - June
11kT (ng/ml)	0,0001 ***	0,3980
SL (cm)	0,3109	0,5324
BW (g)	0,1278	0,7206
GW (g)	[1)]n.m.	0,2251
GSI (%)	[1)]n.m.	0,1600
Condition	0,1311	0,0116 ***
Spermatogonia	0,0420 ***	0,5824
Spermatocytes	0,0083 ***	0,2215
Spermatids	0,0312 ***	0,6761
Spermatozoa	0,2043	0,5756
Spermatogonia %	0,2836	0,8805
Spermatocytes %	0,7201	0,6479
Spermatids %	0,2852	0,9646
Spermatozoa %	0,8092	0,2482

[1)]n.m. = not measured

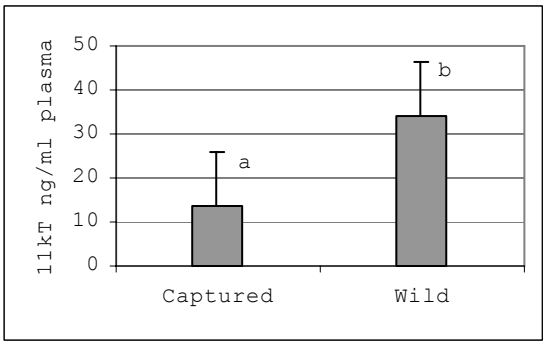
*Table 11. Significant differences (P) with least square means method (α = 0.05) between Captured (C), F1 (F) and Wild (W) group per variable in March and June. Significances are indicated by ***.*

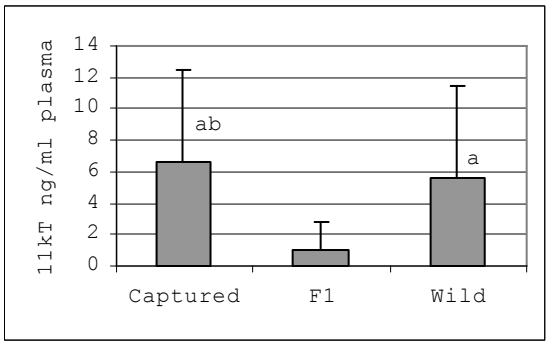
	March		June					
Origin	C	W	C	F	C	W	F	W
11kT (ng/ml)	0,0214 ***		0,0097 ***		0,7137		0,0112 ***	
SL (cm)	0,9249		0,8219		0,1491		0,2992	
BW (g)	0,6283		0,2322		0,1873		0,0144	
GW (g)	[1)]n.m.		0,8128		[1)]n.m.		[1)]n.m.	
GSI (%)	[1)]n.m.		0,3024		[1)]n.m.		[1)]n.m.	
Condition	0,0569		0,0004 ***		0,8691		0,0001 ***	
Spermatogonia	0,7897		0,6730		0,4478		0,7500	
Spermatocytes	0,1870		0,1496		0,0261 ***		0,4171	
Spermatids	0,3714		0,0816		0,7527		0,0359 ***	
Spermatozoa	0,6006		0,3043		0,2418		0,9252	
Spermatogonia %	0,7677		1,0000		0,2980		0,2980	
Spermatocytes %	0,3421		0,4148		0,4119		0,9686	
Spermatids %	0,2818		0,0117 ***		0,6689		0,0215 ***	
Spermatozoa %	0,5921		0,2574		0,4929		0,6011	

[1)]n.m. = not measured

4.2 11-ketotestosterone (11kT)

No normality of variance in the dataset of 11kT was found, possibly due to the large fluctuations of the several 11kT levels. Still, in figure 13 it can be seen that there are differences between the levels between groups and among groups over time. Wild groups showed much higher levels in March than in June. Levels of 11kt were significantly (P = 0.0001) higher levels in March with 33.97 ng/ml blood plasma than in June with 5.62 ng/ml. See table 10. Wild animals also had higher levels 11kT than the Captured group in March. Striking low levels for F1 animals (1.01 ng/ml) versus the other two groups were found. Significant differences with Wild and Captured animals were respectively P = 0.0097 and P = 0.0112, as shown in table 11.



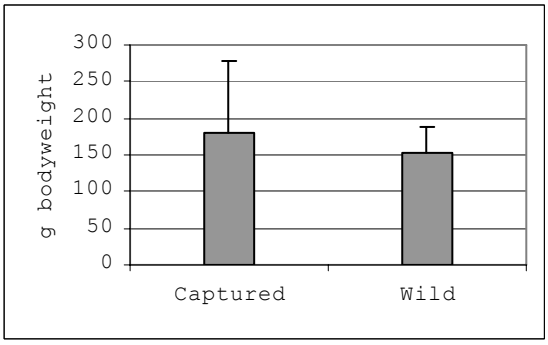


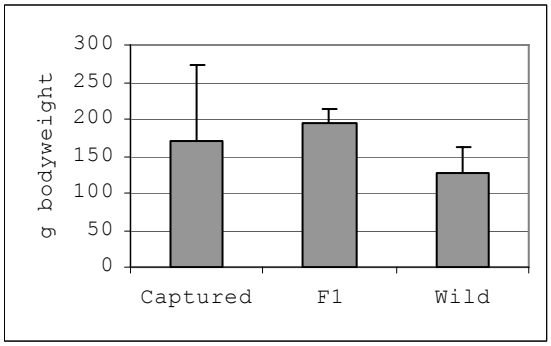
March *June*

Figure 13. Average 11-ketotestosterone (11kT) (ng/ml blood plasma) levels per for Captured (n=7) and Wild (n=12) groups in March and Captured (n=7), F1 (n=5) and Wild (n=12) groups in June.

4.3 Bodyweights, Condition Factors and GSI

As intended, differences in mean bodyweights figure *14* per group were not present. Though somewhat larger and with lower variance by eye, the F1 were not larger than the Wild and Captured animals.

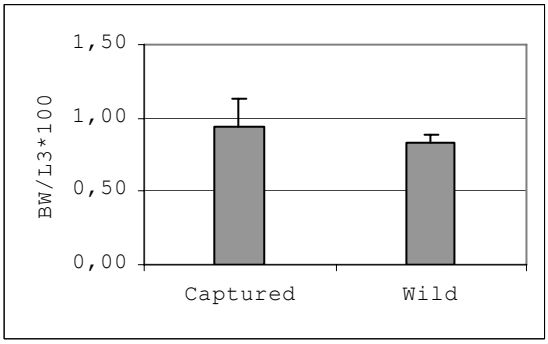


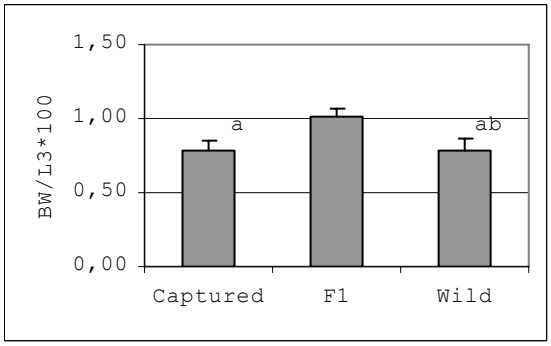


March *June*

Figure 14. Bodyweight (g) per for Captured (n=7) and Wild (n=14) in March and Captured (n=7), F1 (n=5) and Wild (n=14) groups in June.

This is not the case concerning condition factors, see figure 15. F1 animals had (in June) a clearly and significant larger condition factor than Captured and Wild groups (P = 0.0004 and P = 0.0001). For more details see tables 8, 9, 10 and 11. Striking is to see that captured animals have an higher condition factor in March than in June (table *10*).

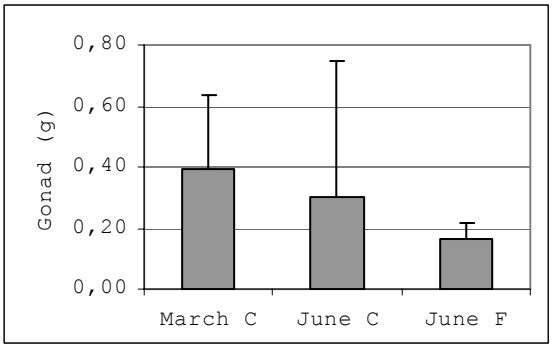




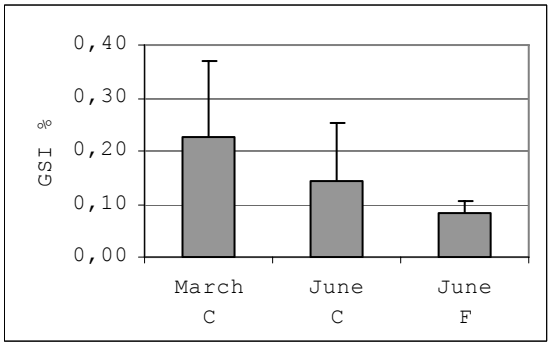
March *June*

Figure 15. Condition ((Bodyweight (g))/(Standard Length (mm))^3) for Captured (n=7) and Wild (n=14) groups in March and Captured (n=7), F1 (n=5) and Wild (n=14) groups in June.

Gonad weight and Gonadosomatic index were not measured in Wild groups since it was not possible to measure gonad weight on board of the research vessels. Therefore only for Captured and F1 gonad weights and GSI's were received, see figure 16. No significant differences were found though F1 animals showed lower GSI's than Captured animals, which in turn had lower GSI in June than in March, see tables 8 and 9.



Gonad weight (g)

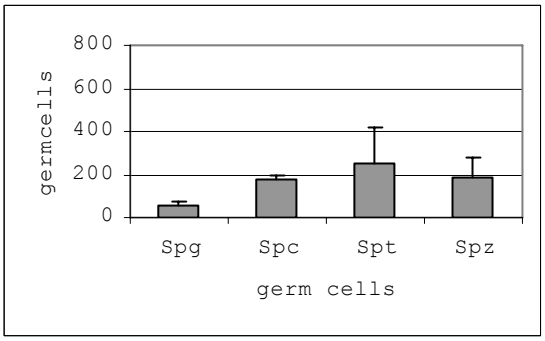


GSI (%)

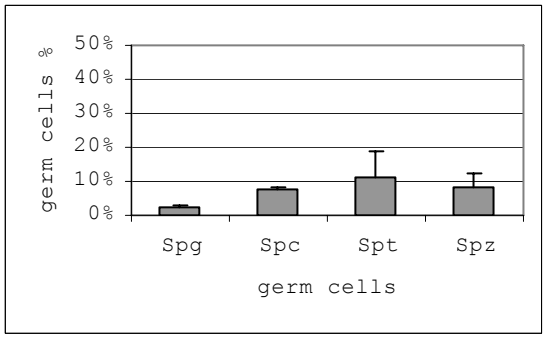
*Figure 16. Average Gonad weight (g) and GSI (g gonad weight/g body weight*100%) per Captured in March (n=7) and Captured (n=7) and F1 (n=5) groups in June.*

4.4 Germ cells

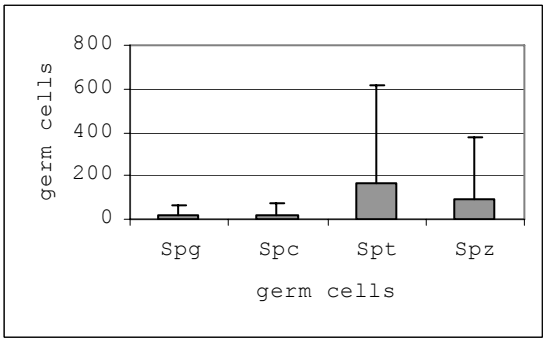
In presence of the different types of germ cells several significant differences were found. Mean absolute amounts of spermatogonia, spermatocytes and spermatids did differ between March and June in Wild groups, compare figs 17 and 18. For all three cell types, amounts decreased as can be seen in tables 8 and 9 with significances of P = 0.0420, 0.0083 and 0.0312. Captured animals did not show a difference in cell number. For both groups no differences was found in percentages of cell abundance over months.



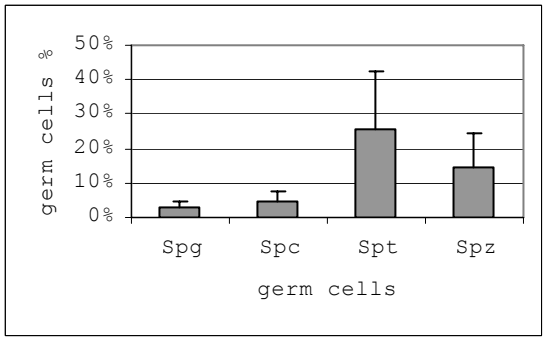
Captured absolute March



Captured relative March

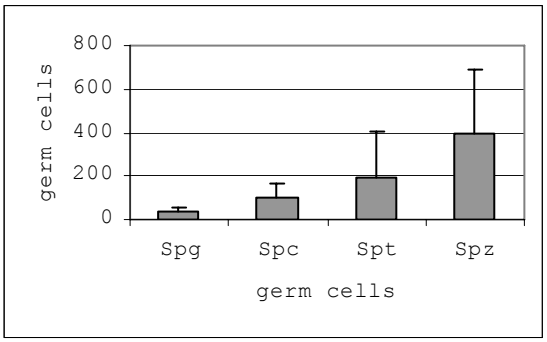


Wild absolute March

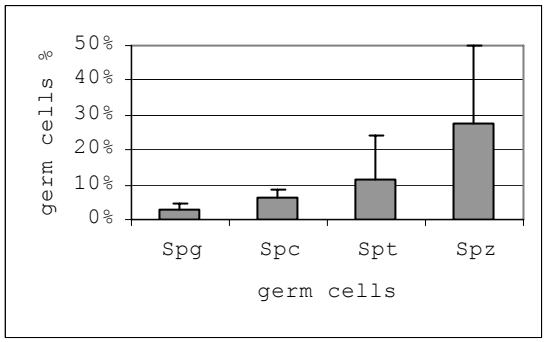


Wild relative March

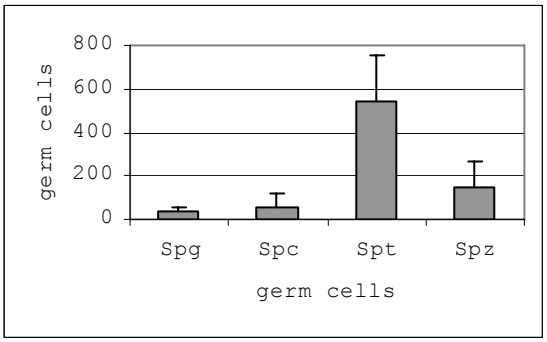
Figure 17. Average absolute quantities and average relative numbers (%) of germ cells in a section from the centre of testes of sampled sole per Captured (n=2) and Wild (n=9) group in March.



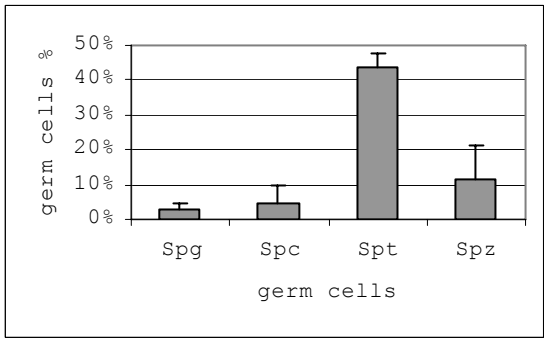
Captured absolute June



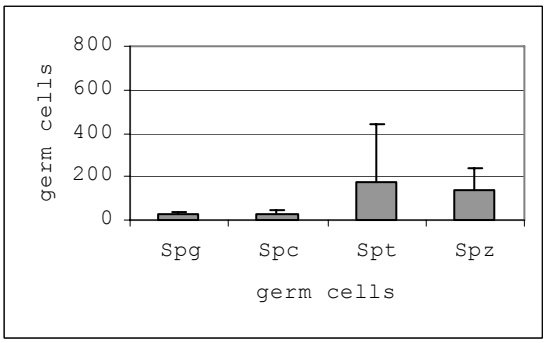
Captured relative June



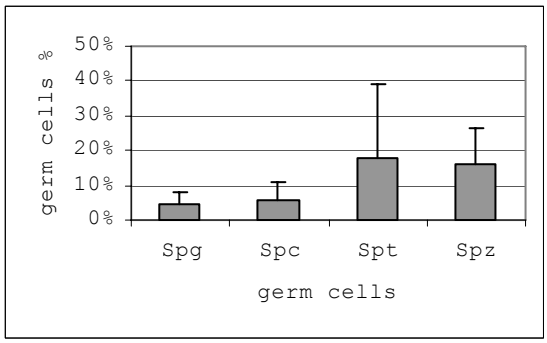
F1 absolute June



F1 relative June



Wild absolute June



Wild relative June

Figure 18. Average absolute quantities and average relative numbers (%) of germ cells in a section from the centre of testes of sampled sole per Captured (n=4), F1 (n=4) and Wild (n=5) group in June.

When comparing groups, mainly differences in quantities of spermatids, both in absolute and relative number, were found in June. This is presented in figure 18. Regarding F1 and Wild groups on absolute numbers of spermatids, a difference with a significance of P = 0.0359 is found. Here F1 groups show a much higher number of spermatids than wild animals. When analysing mean relative number of spermatids per group, this difference becomes clearer. Both Captured and Wild animals show lower proportional abundance of spermatids with significances of P = 0.0117 and P = 0.0215 respectively.
More importantly, differences of abundance of the four difference cell types can be determined. Whereas Captured and, to a less extent, Wild groups show a pattern with increasing percentages and amounts of germ cells along with the consecutive stages, F1 animals show a considerable over expressed abundance of spermatids (see figure 18)

4.5 Environmental conditions

A description of environmental conditions of the used samples is given in table 13 for Captured, F1 and Wild groups. Annual cyclic temperature fluctuations are given in figure 19. The history of temperature fluctuations of the Captured and the F1 group and assumed natural temperatures followed by Wild fish are given in figure 20. Captured animals are assumed to receive (after being caught) the same cyclic pattern as F1 animals, with exception of the first years. As can be seen, in F1 animals this regime is constant (approximately 18°C). For simplicity, the separate curve for Captured animals therefore is not shown.
Day degrees per treatment group until sampling are shown in table 13.

Table 12. Environmental conditions per experimental group : Captured, F1 and Wild sole.

Characteristic		Captured	F1	Wild
System design		Recirculation.	Recirculation	Sand, gravel, mud (Amezcua and Nash, 2001)
Tank design/spawning ground		Circular 1.2m depth, 4m dia[1)], 12.5 m^2 , 15 m^3, sand.	Circular 1.2m depth, 4m dia[1)], 12.5 m^2 , 15 m^3, sand.	Sand and mud (Koutsikopoulos and Lacroix, 1992).
Temperature		8-16°C.	8-16°C.	5-17°C (KNMI, 2004)
Day degrees		See table 13	See table 13	See table 13
Photoperiod		Natural light plus overhead fluorescent lights. Approximate natural cycle (Graph 19).	Natural light plus overhead fluorescent lights. Approximate natural cycle (Graph 19).	Natural cycle
Water quality	- pH	7.5	7.5	+/- 7.8 (Gustafsson and Stigebrandt, 1996)
	- Salinity	25 ‰	25 ‰	35 ‰ (Gustafsson and Stigebrandt, 1996)
Feeding ratio		N.K[2)]	N.K[2)]	N.K. [2)]
Composition diet		Just before and during spawning: Lug worm[3)], Ragworm[4)], Mussel[5)]. All year: Moist pellets (fish oil,fish meal,mussel)	Just before and during spawning: Lug worm[3)], Ragworm[4)], Mussel[5)]. All year: Moist pellets (fish oil,fish meal,mussel)	Several kinds of worms and crustacea. (Molinero et al., 1994)
Density		1.5/m^2	1.5/m^2	N.K. [2)]
Sex ratio		1/2.2-3.8 (m/f)	1/2.2-3.8 (m/f)	1/1 (m/f) (Bromley, 2003)

[1)]Dia = Diameter; [2)]N.K. = Not Known [3)]*Arenicola marina* [4)]*Nereis virens* [5)] *Mytilus edulis*

Figure 19. Annual temperature of Captured and F1 (Average Solea BV) and Wild animals (Wild).

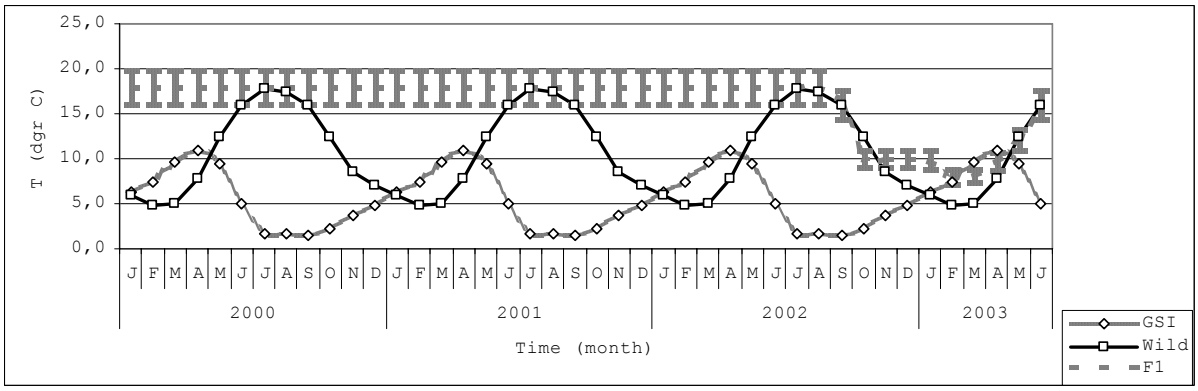


Figure 20. Temperature fluctuations of F1 at Solea BV and assumed natural temperatures followed by Wild fish. Female GSI development in natural conditions is shown as well as indication of the relation between cyclic temperature fluctuations.

Table 13. Day Degrees of sampled Captured, F1 and Wild animals for the whole year, since January 2004, since the last spawning and since the last spawning in 2003.

Period	Captured	F1	Wild
Whole year (dd)	4322	4445	4001
Age per group (years)	4-5[1)]	3?	+/- 2 [2)]

[1)] Caught at 2 to 3 years, from then in culture.
[2)] RIVO 1980 data

5. Discussion

In this part a description of development of spermatogenesis during the spawning of male sole is given together with a comparison of reproductive activity of the three experimental groups.

The final aim is to describe the relation between reproductive features, with special attention to 11kT and presence of germ cells. Furthermore, the relation between the reproductive features and the environmental circumstances will be discussed. This is done in order to find a possible explanation for the reproductive performance of F1 animals.

Captured and Wild groups showed some changes in measured characteristics when being compared between March and June. Both months are mirroring the start or early spawning season and the end of the spawning season (Deniel, 1981; Rijnsdorp, *et al.*, 1992). Captured animals had a significantly lower condition factor in June. Wild animals showed decreased 11kT levels, absolute spermatogonia, spermatocytes and spermatids. Besides, Wild 11kT levels were higher than Captured levels in March though this difference was not detected in June.

5.1 Bodyweight, Gonad weight and GSI

No decreased bodyweights of Wild and Captured animals were found. Apparently male sole bodyweight is not affected by spawning. This could be attributed to the low GSI as found in sole. Spending testes through spawning then would not have great impact on the total bodyweight of the males (Howell *et al.*, 1991; Baynes *et al.*, 1994; Pavlov *et al.* 2004).

Condition on the other hand did decrease in Captured animals. The reason for this is not exactly known. An explanation for the detected difference between the two groups could be low number of sampled Captured fish. The fact that the Wild animals did not show the same pattern makes any other conclusion doubtful, especially when it is realized that captured animals are kept in more favourable circumstances in respect of terms of feeding. This phenomenon is clearly reflected by the F1 group that has a significantly higher condition in June than the other two groups see figure 15.

GSI's only were measured for Captured and F1 groups, see figure 16. No measurements were taken for Wild groups since it was not possible to measure gonad weight on board of the research vessel. Development of GSI in sole was monitored through measuring gonad weight. GSI decreased in Captured animals after the spawning season. The same pattern was recorded in case of Flounder under natural photoperiods and in Atlantic halibut under simulated natural photoperiods (Janssen, 1996; Norberg *et al.*, 2001). Regarding the results, F1 animals tend to have smaller GSI's than Captured animals. Though this statement is not significantly proven, it possibly indicates a less well developed spermatogenesis in F1 which could affect the low performance of these animals.

5.2 11kT and germ cells

Though no normality of variance in the dataset of 11kT was found, still in figure 13 it can be seen that there are differences between groups over time. Regarding the Wild animals, high levels of on average approximately 30 ng/ml blood plasma were detected whereas in June these levels had declined considerably. In March, Captured animals had significantly lower 11kT levels than Wild animals but in the other reproductive aspects no significant differences were found. Looking at the data though, differences can be seen: Wild animals show more germ cells and especially higher proportions of spermatids (table 8).
Analysis of differences between groups in June showed that F1 had significantly lower 11kT levels than the other two groups, though believed to be in the same physiological and reproductive state. This could be an explanation for differences in performance.

In case of germ cell types, differences in number (absolute and relative) were found during the spawning season, though only for wild animals. Abundance of the three types spermatogonia, spermatocytes and spermatids decreased significantly along with the continuing spawning season. Compare figures 17 and 18 and see table 10. In Flounder and Plaice testes show a similar pattern, with increasing amounts of germ cells. At the start of the spawning season spermatozoa are first present whereas at the end they are spent (Barr, 1963; Janssen, 1996). However, spermatozoa did not show a decrease in number. Possibly the sole

males in this experiment were sampled in June when they still were spawning. Therefore the final stage in spermatogenesis, the spermatozoa, was still present in high numbers. Another possibility is that the animals in March were still increasing their production of spermatozoa. These cells were spent during spawning and by June levels had decreased again to previous in March measured quantities.

When comparing the Captured, F1 and Wild groups, it is determined that differences are present in March and June (see table 11). Main deviations are found in number of spermatids in June though, the phase before final maturation of male gametes. In this case, F1 show substantially higher numbers of cells than Wild and Captured groups. Though not significantly, testes of F1 males seem to contain relatively lower amounts of spermatozoa than Captured and to a less extent Wild groups. Patterns of abundance of germ cells in testis point in F1 to extreme accumulation of spermatids, suggesting a non-completion of spermatogenesis.

Regarding partly significant decreasing levels of 11kT with the decrease of germ cells in Wild and Captured animals towards end of the spawning season, suggests the presence of a relation. This pattern is confirmed by research on Atlantic halibut. Here progressing development of germ cells was recorded along with increasing levels of 11kT (Weltzien *et al.*, 2004). This will be discussed in the following.

5.3 11-ketotestosterone; gametogenesis and gonad growth

In several species an indication for an effect of 11kT on spermatogenesis and gonad growth was found (Barr, 1963; Weltzien et al., 2002). In the current experiment, higher 11kT profiles seem to relate to gametogenesis, gonad weight and higher GSI values as well.
An indication for a relationship between 11kT and testicular weight or GSI was found though not causally proven. In other species, for example Atlantic halibut, 11kT and T are positively correlated with testicular weights. Atlantic halibut shows prenuptial spermatogenesis, i.e. the testes successively regress after a spawning season and increase in mass as the next season approaches. Parallel with this development, gradually rising levels of 11kT are demonstrated (Weltzien *et al.*, 2002). The same pattern is also seen in plaice (Barr, 1963). On macroscopic basis the same gonadal cycle is seen in male sole (Bromley, 2003). Nevertheless, other experiments with a dose-response character should be conducted to prove the assumed association of 11kT and gonad growth.

On the other hand, 11kT does not necessarily determine stage of testes. Greenback Flounder, *Rhombosolea tapirina* (Günther 1862), for instance does not show significant relations (Barnett and Pankhurst, 1999).

With its relation to gonadal mass in prenuptial testis, 11kT could have direct effect on the different stages of testis development. Several threshold levels of 11kT determine to which stage testis, and thus germ cell types, develop (Norberg *et al.*, 2001; Weltzien *et al.*, 2002). In this experiment relatively low 11kT levels were found to be accompanied by high amounts of spermatids both in absolute and relative quantities, i.e. cell presence.

A conclusion resulting from above mentioned outcome could be that F1 animals do not complete the process of spermiogenesis due to proved low blood plasma (threshold)levels of 11kT. The differentiation of spermatids to spermatozoa is halted or delayed since the required threshold level is not reached. On the other hand, threshold levels for spermatid production are present. Therefore spermatids accumulate in the testes. This suggests that something in 11kT production in F1 animals is hampered.

This indeed could be an explanation when we regard some experiments on rainbow trout (*Oncorhynchus mykiss*). Different measured levels of 11kT, the most potent steroid in salmonids, directly correlated with different stages of the testis, i.e. presence of different cell types (Schulz, 1983). In Atlantic halibut a comparable situation is described. Here, presence of stages of germ cells in testes were reflected by different levels of 11kT. Higher levels 11kT were found in animals with testes with more haploid cells. This suggests that a threshold level of 11kT determines to which stage the testicular development will proceed (Norberg *et al.*, 2001; Weltzien *et al.*, 2002).

Possibly, a different effect of 11kT is found in Japanese eel. Experiments did test different 11kT doses (0.01, 1, 10 and 100 ng/ml) but no effect on cell mitosis of spermatogonia was recorded for levels of 0.01 and 1 ng/ml. Both 10 and 100 ng/ml 11kT did induce mitosis of spermatogonia but equally (Miura *et al.*, 1991a). This implies that quantities of gametes are not affected by different, relatively high, levels of 11kT. However, effects of concentrations between 1 and 10 ng/ml are not recorded and possibly effects of intermediate doses can be demonstrated. Administering 11kT doses to fish with known low 11kT production might point to the expected response.

It is not known if 11kT in sole indeed does determine to which stage of spermatogenesis testes develop. To prove its assumed relation with spermatogenesis in sole a dose-response test could be performed. Here, animals with known low levels or no 11kT should be treated with several different doses of the steroid. In this way it is possible to detect gonad growth as a result of elevated 11kT levels.

Besides its effect on spermatogenesis, 11kt has an other function in stickleback, *Gasterosteus aculeatus*. During the courtship phase in this species higher levels of 11kT are found. This indicates a possible relationship of the steroid with mating behaviour. In animals that do not show courtship behaviour exhibit low levels (Páll *et al.*, 2002). Courtship behaviour is described to be important in sole reproduction (Baynes *et al.*, 1994). This can support the proposed effect of 11kT levels on fertilization rates. It appears that animals that do not show required 11kT levels, also lack courtship behaviour.

5.4 Gonadotropins

In the Japanese Eel, both 11kT and gonadotropin separately induce development of all subsequent cell stages of spermatogenesis (Miura *et al.*, 1991a and 1991b). Besides, 11kT production is dependent on gonadotropin: 11kT producing Leydig cells in vitro remain inactive when only 11kT is added but become active when gonadotropin is administered (Miura *et al.*, 1991b).
A lower production of 11kT in this way could be subscribed to a lower production of gonadotropin by the pituitary gland (Cavaco *et al.* 2001). Gonadotropin was not measured in the current experiment.

In case of F1 animals a block in spermiogenesis seems to occur, due to which spermatid amounts increase for they do not receive (sufficient) stimuli to complete the transformation to spermatozoa. With regard to the previously described endocrine pathway of trout and halibut it could be that the block is caused by a disorder in the production of the steroid 11kT.

Nevertheless, the indicated mitosis and presence of some spermatozoa is at least a proof that the spermatogenesis is completed partly. In Japanese eel it is shown that when the first step (of 11kt administration) is made, the rest of the cascade in spermatogenesis to spermiogenesis should follow (Miura *et al.*, 1991a; Miura *et al.*, 1996). This seems not the case in sole. It must be noted that eel has a very specific mode of reproduction; this model therefore should not just be generalised.

Though emphasising the importance of 11kT in many species, it must be reminded that this steroid does not seem to be the single final factor for proper gametogenesis. A point of consideration could be the fine-tuning of spermatogenesis by paracrine and autocrine factors induced by 11kT (Miura *et al.*, 1997). Activin B for instance, is produced by Sertoli cells in response to 11kT. It induces cell mitosis of spermatogonia but not further stages in gametogenesis (Schulz and Miura, 2002; Weltzien *et al.*, 2004). The existence of Activin B does not make it unlikely that there are similar factors affecting later stages in spermatogenesis and spermiogenesis. Still it should be a partly effect because in the F1 animals there are spermatozoa.

5.5 Photoperiods and temperature

Besides histology, aspects of culture circumstances were analysed as well. When comparing the several aspects between Captured and F1 broodstock no direct possible causes for malfunctioning spermatogenesis or reproduction could be pointed at.

It is striking that F1 fish seem to be delayed; in the sections very few spermatozoa and low 11kT levels were observed, though in Wild conditions winters with higher temperatures do advance spawning (Van der Land, 1991; Koutsikopoulos and Lacroix, 1992). Under normal circumstances F1 animals should have had advanced testes development as well, see table 13. This emphasises the suggestion that F1 animals lack

normal production of factors supporting spermatogenesis. According to the results 11kT could be one of these factors.

A difference between groups in the amount of day degrees since last spawning (spawning in 2004 before sampling) could suggest that the F1 animals already were too far developed or past spawning for proper and equal comparison (Weltzien *et al.*, 2002). Nevertheless, none of the testis did look regressed, as normal proportions of cells were observed. No phagocytotic actions were seen and regular development still seemed to continue, though mainly until spermatid stage.

When regarding the differences in temperature fluctuations in the three groups (see fig 19) it is determined that the groups did not receive the same treatment. Wild stocks of sole clearly had lower temperatures than Captured and F1 animals. Especially concerning the period prior to spawning differences with Wild conditions are found. During these months Captured and F1 animals received higher temperatures than Wild animals (δ°C approximately 4 - 6 °C, see fig 19). Annual day degrees values of F1 animals are higher than those of Captured and Wild groups (see table 13) as well. This implies that since the groups were sampled on approximately the same time, they probably do not represent the same reproductive state. Modifications for fine tuning of broodstock temperature fluctuations could be lowering temperatures in months prior to spawning.

Still, Captured animals did not seem to be affected by temperature differences compared to Wild conditions for they did produce viable offspring. This is probably due to the fact that those animals still were kept at temperatures within the required range of temperatures (Van der Land, 1991; Koutsikopoulos and Lacroix, 1992). Therefore the temperature profiles in cultured conditions seem correct and in this case not of influence on spermatogenesis. On the other hand, in practice fertilization rates of Captured animals are low compared to Wild animals. When regarding figure *20* it is determined that Wild groups start gonad growth when temperature after summer passes approximately 14°C. Gonad growth is finalized at spawning with an increase of temperature in spring to approximately 10°C. Above approximately 10°C gonad enter regression and a period of rest. This process takes about 7-8 months in the wild. In the Cultured and the F1 broodstock group, which follow the same annual cycle, the temperature cycle only took approximately 6 months to reach 10°C. This is possibly to short for full completion of spermatogenesis. It could be that in the current experiment, this is reflected by the lower 11kT levels en the somewhat lower number of germ cells in the Captured animals: these animals seem to be slightly delayed.

The cyclic photoperiods were not different between the groups as derived from data in table 12. It is assumed that the used artificial cyclic photoperiods, that follow natural photoperiods, do not negatively affect spermatogenesis. However, a difference was found for the F1 group that during the first year received 24 hours light (LL) photoperiods. The lack of a cyclic pattern in the first year of F1 could cause the disorder in spermatogenesis. This will be explained later.

The fact that Cultured animals do fertilize eggs en F1 do not, could be explained by the fact that F1 animals were only recently kept in a cyclic temperature regime. When analysing cyclic photo and temperature fluctuations, there could be an effect caused during the rearing of the broodstock sole. Regarding the temperature regimes of the F1 broodstock during the years previous to spawning (Figure 20), it is seen that annual temperature regimes were quite high and constant. There was almost no natural fluctuation in temperature or day length during the first years. In catfish, *C. Gariepinus*, it is shown that internal rhythms of broodstock fish are determined by environmental factors at early stages of development. However, this was the case for photoperiods (Richter *et al.*, 1987). Still, it is not unlikely that a similar effect of imprinting can be determined by temperature fluctuations. This could affect the setting of the broodstock biological clock. Fish that are not imprinted similarly could mature and thus spawn non-synchronously for (steroid) hormonal profiles follow cyclic variations and internal clocks according or the BPG axis (Weltzien *et al.*, 2004).

6. Conclusions and recommendations

Possibilities that explain the inability of reproduction of F1 males of *S. solea* are:

a) The higher temperature regime for F1 animals compared to Wild animals should result in advanced testes development. Nevertheless, this is not observed which is emphasizing the possibility of a disorder on other factors involved with spermatogenesis.

b) A possible explanation of the low performance of F1 animals could be that too low (threshold)levels of 11kT possibly give partial spermatogenesis to spermiogenesis but are not sufficient to execute spermiogenesis on large scale.

c) A disorder in production of factors preceding 11kT (such as gonadotropin hormones) that indirectly support spermiogenesis could (in)directly be affecting the 11kT levels for the completion of spermiogenesis.

d) Due to lack of cyclic annual temperature fluctuations and light regimes during rearing of animals it is suggested that the F1 animals' internal clocks/endogenous rhythm are not imprinted properly and therefore do not synchronously mature and spawn.

e) A possible cause for lack of completion of spermatogenesis in F1 sole could also be the cyclic annual temperature in artificial circumstances. The period for gonad growth in artificial circumstances is shorter than in natural conditions. Besides it is less extreme in months prior to spawning. It is suggested that the animals do not have time enough for completion of spermatogenesis.

f) Though some small differences with Wild animals, Captured animals mainly show the same reproductive patterns. Lower reproductive capabilities, possibly resulting from Lower 11kT levels and a delay in development in March, could be due to system temperature regimes as described under e).

Recommendations for furthers research are:

a) Testing whether 11kT levels determine the state of development of spermatogenesis or not by dose response tests.

b) Testing if extending the period between start of regression (approximately 14°C) and next spawning (approximately 10°C) to approximately 7-8 months has an positive impact on fertilization rates.

c) Testing if rearing conditions have an effect on spawning and fertilization where cyclic photoperiods and temperature regimes are followed from rearing. This in order to completely simulate natural conditions and thus set endogenous rhythms.

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Appendix I - Protocols sampling blood and tissue

Protocol Blood sampling.

1. Anaesthetize the fish with an overdose TMS.
2. Fill syringes with Sodium Citrate (3 x H_2O) 6% 50 µl/ml blood
3. Take a blood sample of 1ml.
4. Transfer the blood to the micro tubes and put them on ice
5. Centrifuge the blood 6000rpm 10 minutes
6. Take of the plasma
7. Transfer plasma to Eppendorf tubes on ice/fridge

Protocol Testis sampling.

1. Take out both testis of the sole
2. Each testis in one container,

Histological labelling: F/W/C, fish no., left/right testis

3. Put sample in Bouin's fixative 4-8 hrs – Stirring
4. Put sample in alcohol 50% 3 x 30 min
5. Put sample in alcohol 70% transport

Bouin's fixative: Formaldehyde (neutral 40%) 5ml
Picric acid (sat.) 15ml
Acetic acid (100%) 1ml
Filtrate before use.

Alcohol series

1. (Ethanol 50% (3x) 30 min)
2. (Ethanol 70% several hours (transport))
3. Ethanol 80% (2x) 1 hr
4. Ethanol 90% 1 hr
5. Ethanol 96% (2x) 30 min
6. Ethanol 100% (3x) 15 min
7. Amyl acetate (2x) 1 hr
8. Amyl acetate (2x) 1 night
9. Paraplast (3x) 30 min (58°C stove)
10. Paraplast (3x) 20 min (58°C in vacuum stove)
11. Embedding in Paraplast in mould.
12. Cool down at 10°C and stored in fridge.
13. Cutting with microtome, sections of 5µm.
14. Desired sections put in water with a little gelatin.
15. Put on object glass and in stove at 37°C for 24 hours.

Protocol colouring of sections.

Object glasses with sections put through following series.

1. Xyleen 3 min
2. Ethanol 100% 2 min
3. Ethanol 96% 2 min
4. Ethanol 70% 2 min
5. Aquadest (2x) 1 min
6. Haemaluin 5 min
7. Aquadest (2x) 2 min
8. Tap water 10 min
9. Aquadest 2 min
10. Eosine 4 min
11. Ethanol 70% 2 sec
12. Ethanol 96% 2 sec
13. Ethanol 100% (2x) 2 min
14. Xyleen (2x) 2 min

Enclose in with Pertex and dry.

Appendix II - All measurements

Explanation of codes for animals: Origin (C = Captured; F = F1; W = Wild)

	Tag	Month	Origin	11kT	SL	BW	Wgon	GSI	Condition	Spgon	Spcyts	Stids	Spzoa	Spgon rel	Spcyts rel	Stids rel	Spzoa rel
1	T10	M	W	.	28,60	192,00	.	.	0,82	23,00	2,00	189,00	10,00	0,06	0,01	0,48	0,03
2	T17	M	W	41,92	26,90	166,00	.	.	0,85	.	.	.	.	.	.	.	.
3	T18	M	W	38,87	28,60	196,00	.	.	0,84	148,00	102,00	1476,00	87,00	0,04	0,03	0,44	0,03
4	T19	M	W	21,68	28,20	166,00	.	.	0,74	69,00	111,00	211,00	573,00	0,02	0,04	0,07	0,19
5	T20	M	W	32,74	25,40	150,00	.	.	0,92	.	.	.	.	.	.	.	.
6	T21	M	W	64,81	26,90	172,00	.	.	0,88	23,00	147,00	388,00	642,00	0,01	0,05	0,13	0,22
7	T23	M	W	29,66	27,40	180,00	.	.	0,88	51,00	174,00	738,00	272,00	0,02	0,08	0,35	0,13
8	T24	M	W	20,06	28,60	194,00	.	.	0,83	41,00	135,00	138,00	427,00	0,03	0,08	0,09	0,27
9	T33	M	W	32,27	24,50	108,00	.	.	0,73	.	.	.	.	.	.	.	.
10	T36	M	W	43,01	27,80	166,00	.	.	0,77	129,00	76,00	948,00	104,00	0,03	0,02	0,24	0,03
11	T48	M	W	30,59	24,30	134,00	.	.	0,93	45,00	75,00	429,00	144,00	0,05	0,08	0,43	0,14
12	T49	M	W	18,82	24,60	114,00	.	.	0,77	.	.	.	.	.	.	.	.
13	T51	M	W	.	21,70	82,00	.	.	0,80	48,00	161,00	186,00	794,00	0,02	0,05	0,06	0,26
14	T74	J	W	4,11	26,00	110,00	.	.	0,63	.	.	.	.	.	.	.	.
15	T75	J	W	11,63	29,00	156,00	.	.	0,64	.	.	.	.	.	.	.	.
16	T76	J	W	2,37	26,00	116,00	.	.	0,66	15,00	2,00	42,00	108,00	0,01	0,00	0,03	0,07
17	T77	J	W	10,18	28,00	170,00	.	.	0,77	32,00	47,00	55,00	120,00	0,06	0,09	0,10	0,23
18	T78	J	W	2,14	27,00	138,00	.	.	0,70	26,00	36,00	3,00	7,00	0,10	0,13	0,01	0,03
19	T79	J	W	4,22	25,00	134,00	.	.	0,86	.	.	.	.	.	.	.	.
20	T8	M	W	33,26	24,30	122,00	.	.	0,85	.	.	.	.	.	.	.	.
21	T80	J	W	4,60	27,50	169,00	.	.	0,81	.	.	.	.	.	.	.	.
22	T81	J	W	7,83	23,00	94,00	.	.	0,77	.	.	.	.	.	.	.	.
23	T82	J	W	.	21,00	78,00	.	.	0,84	.	.	.	.	.	.	.	.
24	T83	J	W	.	21,50	82,00	.	.	0,83	39,00	12,00	642,00	290,00	0,03	0,01	0,53	0,24
25	T84	J	W	0,15	22,30	88,00	.	.	0,79	.	.	.	.	.	.	.	.
26	T85	J	W	0,15	22,10	98,00	.	.	0,91	24,00	30,00	143,00	162,00	0,04	0,04	0,21	0,24
27	T86	J	W	0,52	29,00	200,00	.	.	0,82	.	.	.	.	.	.	.	.

28	T87	J	W	19,59	24,50	128,00	.	.	0,87	.	.	.	.	.	.	.	.
29	W1	M	C	10,60	24,80	159,00	0,30	0,19	1,04	.	.	.	.	.	.	.	.
30	W10	M	C	20,01	32,60	340,00	0,58	0,17	0,98	.	.	.	.	.	.	.	.
31	W11	M	C	0,15	23,00	106,00	0,08	0,08	0,87	.	.	.	.	.	.	.	.
32	W14	M	C	2,06	24,10	107,00	0,10	0,09	0,76	.	.	.	.	.	.	.	.
33	W2	M	C	36,33	25,10	114,00	0,48	0,42	0,72	.	.	.	.	.	.	.	.
34	W22	J	C	0,15	27,00	155,20	0,09	0,06	0,79	.	.	.	.	.	.	.	.
35	W23	J	C	4,96	23,60	108,60	0,10	0,09	0,83	.	.	.	.	.	.	.	.
36	W24	J	C	5,70	22,70	79,50	0,14	0,18	0,68	16,00	65,00	484,00	0,00	0,01	0,04	0,30	0,00
37	W25	J	C	17,12	35,60	359,30	1,30	0,36	0,80	37,00	62,00	33,00	685,00	0,03	0,04	0,02	0,47
38	W26	J	C	10,17	25,00	111,40	0,20	0,18	0,71	51,00	196,00	217,00	448,00	0,02	0,09	0,10	0,20
39	W27	J	C	0,76	24,80	123,30	0,07	0,06	0,81	.	.	.	.	.	.	.	.
40	W28	J	C	7,74	31,20	262,70	0,22	0,08	0,86	58,00	87,00	47,00	461,00	0,06	0,08	0,05	0,44
41	W29	J	F	0,33	25,50	166,00	0,09	0,05	1,00	24,00	10,00	844,00	157,00	0,01	0,01	0,44	0,08
42	W30	J	F	4,27	27,60	215,00	0,21	0,10	1,02	67,00	145,00	513,00	297,00	0,06	0,12	0,43	0,25
43	W31	J	F	0,15	27,00	183,00	0,13	0,07	0,93	.	.	.	.	.	.	.	.
44	W32	J	F	0,15	27,20	206,00	0,17	0,08	1,02	34,00	42,00	478,00	136,00	0,03	0,03	0,40	0,11
45	W33	J	F	0,15	26,40	200,00	0,22	0,11	1,09	12,00	16,00	330,00	13,00	0,02	0,02	0,49	0,02
46	W7	M	C	12,12	28,60	303,00	0,68	0,22	1,30	65,00	166,00	369,00	249,00	0,03	0,08	0,17	0,11
47	W9	M	C	14,17	24,80	132,00	0,55	0,42	0,87	40,00	189,00	131,00	116,00	0,02	0,08	0,05	0,05