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Spermatozoon ultrastructure and sperm production in wolffish (*Anarhichas lupus*), a species with internal fertilization

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Abstract

The ultrastructure of spermatozoon and sperm production were observed in wolffish (*Anarhichas lupus*), a species with internal fertilization, from a broodstock. The mature spermatozoon has an elongated head, eccentric flagellum, and comparatively well developed middle piece. Based on the observations of sperm cells with the optical microscope, the mean lengths of spermatozoon head and flagellum were 3.3 and 21.2 μm respectively. A high variability of sperm cell size was observed due to the presence of spermatids and degenerated spermatozoa in the ejaculate. In most males, sperm production was registered throughout the whole year with a peak in December and January. Sperm production decreased to the end of the breeding season. The difference in volumes of ejaculate and sperm concentrations between males kept at two light cycles (18D: 6L and 6D: 18L) was not significant. Features of wolffish sperm are compared with data reported in the literature on other internally and externally fertilizing fish.

Keywords: *Anarhichas lupus*, wolffish, sperm morphology, sperm production.

L'ultrastructure du spermatozoïde et la production de sperme chez le poisson-loup (Anarhichas lupus), *une espèce à fécondation interne.*

Résumé

L'ultrastructure du spermatozoïde et la production de sperme ont été observées chez le poisson-loup (*Anarhichas lupus*), une espèce à fécondation interne, à partir d'un élevage. Le spermatozoïde, arrivé à maturité, a une tête allongée et un flagelle excentré, et un corps intermédaire comparativement bien développé. La longueur moyenne de la tête du spermatozoïde et du flagelle sont 3,3 et 21,2 μm respectivement. La taille des cellules du sperme est très variable, due à la présence de spermatides et de spermatozoïdes dégénérés. Chez la plupart des mâles, la production de sperme a été observée tout au long de l'année avec un pic d'abondance en décembre et en janvier. La production de sperme diminue à la fin de la saison de reproduction. La différence de volumes de l'éjaculat et du sperme entre mâles gardés sous deux cycles différents d'éclairement (18 h nuit: 6 h jour et inversement) n'était pas significative. Les caractéristiques du sperme du poisson-loup sont comparées aux données bibliographiques d'autres poissons à fécondation interne et externe.

Mots-clés : *Anarhichas lupus*, poisson-loup, morphologie du spermatozoïde, biologie de la reproduction.

INTRODUCTION

Species of the family Anarhichadidae are regarded as potential subjects for cold-water marine aquaculture. Among the species, the biology of common wolffish (*Anarhichas lupus*) has been investigated more completely. Recently the whole life cycle of this fish was reproduced in captivity (Pavlov and Moksness, 1994*a*; Moksness, Pavlov, 1996).

The eggs of common wolffish are fertilized internally in the female's ovary and released into water several hours after fertilization (Johannessen *et al.*, 1993; Pavlov, 1994*a*). The egg mass is protected by the male during the embryonic period (Pavlov, Novikov, 1993). Such a mode of reproduction is connected with some peculiarities of the sperm. In most viviparous fishes with internal fertilization spermatozoa are grouped into spermatozeugmas or spermatophores. They are transferred through the anal fin transformed into a gonopod. In some families, including Anarhichadidae and Zoarcidae, spermatozoa remain free and they are transferred through a primary (urogenital papilla) copulative organ (Billard, 1986; Makeyeva, 1992). The spermatozoa of wolffish are activated in the seminal plasma, and can retain motility during several days at temperature close to 0°C. At the same time, the volume of ejaculate and sperm concentration are much lower than those in fish with external fertilization (Pavlov and Moksness, 1994b).

A special method was applied for artificial insemination of wolffish eggs *in vitro* (Pavlov, 1994*a*, *b*). In spite of the low volume of ejaculate, for successful fertilization with 90-100% fertilization rate, the minimal sperm/egg ratio should be similar to that in salmonids (Pavlov and Moksness, 1994*b*). To obtain sufficient amounts of good quality sperm for artificial fertilization, and for assessment of the optimal number of males in the broodstock, the features of wolffish sperm production should be studied.

Sperm morphology and sperm production in captive wolffish have not been described before. Such data would be very important for wolffish broodstock management, as well as for understanding the evolutionary trends in modes of reproduction and spermatozoon structure of fishes. The aims of the present work were to describe spermatozoon ultrastructure, using scanning electron microscopy (SEM) and transmission electron microscopy (TEM) methods, and to study peculiarities of sperm production of males from the broodstock.

MATERIAL AND METHODS

Wolffish broodstock kept at the Institute of Marine Research, Flødevigen Marine Research Station originated from larvae hatched from an egg-mass and caught in the sea in 1986-1988. The first maturation of most fish of both sexes was observed in 1991-1992. The males were separated from females and kept in green-walled tanks of 2-3 m^3 capacity at stocking density averaging 14 fishes per m^3. The fish were fed commercial dry pellets (Elite Plus, produced by T. Skretting A/S, Norway). The temperature of sea water was lowest in February-April and highest in August-September with fluctuation from 4.7 to 13.7°C. The salinity was 32.0-34.7 and the photoperiod was constant (18L : 6D). From the beginning of May 1994, a group of males was kept at a constant photoperiod of 6L: 18D.

In 1992-1993 the males were stripped without anaesthetic and in 1994-1995 they were anaesthetized by MS-222 at a concentration of 130-200 $mg.l^{-1}$. Just after stripping, the sperm from each male were placed in the refrigerator in a plastic tube. The volume of ejaculate was measured and sperm concentration was counted in a haemocytometer. To count total number of spermatozoa in the ejaculate, sperm concentration ($spz.ml^{-1}$) was multiplied to volume of ejaculate (ml).

For SEM, drops of 2.5% buffered glutaraldehyde with sperm were placed on a glass slide. After drying of the drops, the sperm were dehydrated in ethanol, acetone, and dried in a HCP2 Hitachi critical-point drier. After being sputter coated with fusion of gold and palladium, the samples were examined with a S405 Hitachi scanning electron microscope.

For TEM, samples of sperm from ejaculates of nine males were fixed in 2.5% glutaraldehyde made on phosphate buffer, pH 7.2-7.4. The samples were centrifuged and the sediment was post-fixed in 1% OsO_4. Subsequent mounting of samples was done according to current methods (Weakley, 1972). Thin sections of sperm cells were examined and photographed with Jem-100B Jeol transmission electron microscope.

To determine the variability of size of sperm cells in the ejaculate, sperm stripped from each male were diluted in 4% formaldehyde to prevent motility of cells, and cells were measured under an optical microscope (×1 000). A video analysis system described by Andersen and Moksness (1988) was used for the measurements. To determine size of mature spermatozoa, the length and width of spermatozoon head, and tail length were measured. As was described earlier (Pavlov and Radzikhovskaya, 1991), recovery of sperm production was observed when the frequency of sperm stripping was at least 12-14 days. Therefore, to observe changes in sperm size composition during the breeding season 1992-1993, size of sperm cells (the largest diameters of cells without tails) obtained from several tagged males was measured monthly, and 200-300 cells were used for each measurement.

The broodstock used in this study originated from the regions of Faroe Islands and the Barents Sea off northern Norway, and the light cycles of these regions can be referred to the natural ones. To determine influence of photoperiod on sperm production, 33 and 12 males kept at light cycles of 18L: 6D and 6L: 18D

respectively were stripped monthly from December 1994 to March 1995.

To observe sperm production of males throughout the year, 20 males kept at the 18L : 6D light cycle were stripped with mean periodicity of one month from April 1992 to March 1993. To determine individual variability in sperm production, five males were tagged by plastic tags and volume and concentration of spermatozoa in the ejaculate were determined several times during the breeding season.

Statistical comparison between characteristics of sperm production was made using Student's *t*-test.

RESULTS

Spermatozoon ultrastructure

Spermatozoon of wolffish has an oval head with dense packed chromatin material (Fig. 1*a*). Fibrillar structures and granules are determined in the nucleus. The head is surrounded by nuclear membrane without pores, and by external envelope, a plasmalemma. The nuclear invagination located inside of the head. Its length is approximately 2/3 from the head length (Fig. 1 *b*). A centriolar complex is found in the upper part of the canal. An electron dense homogeneous

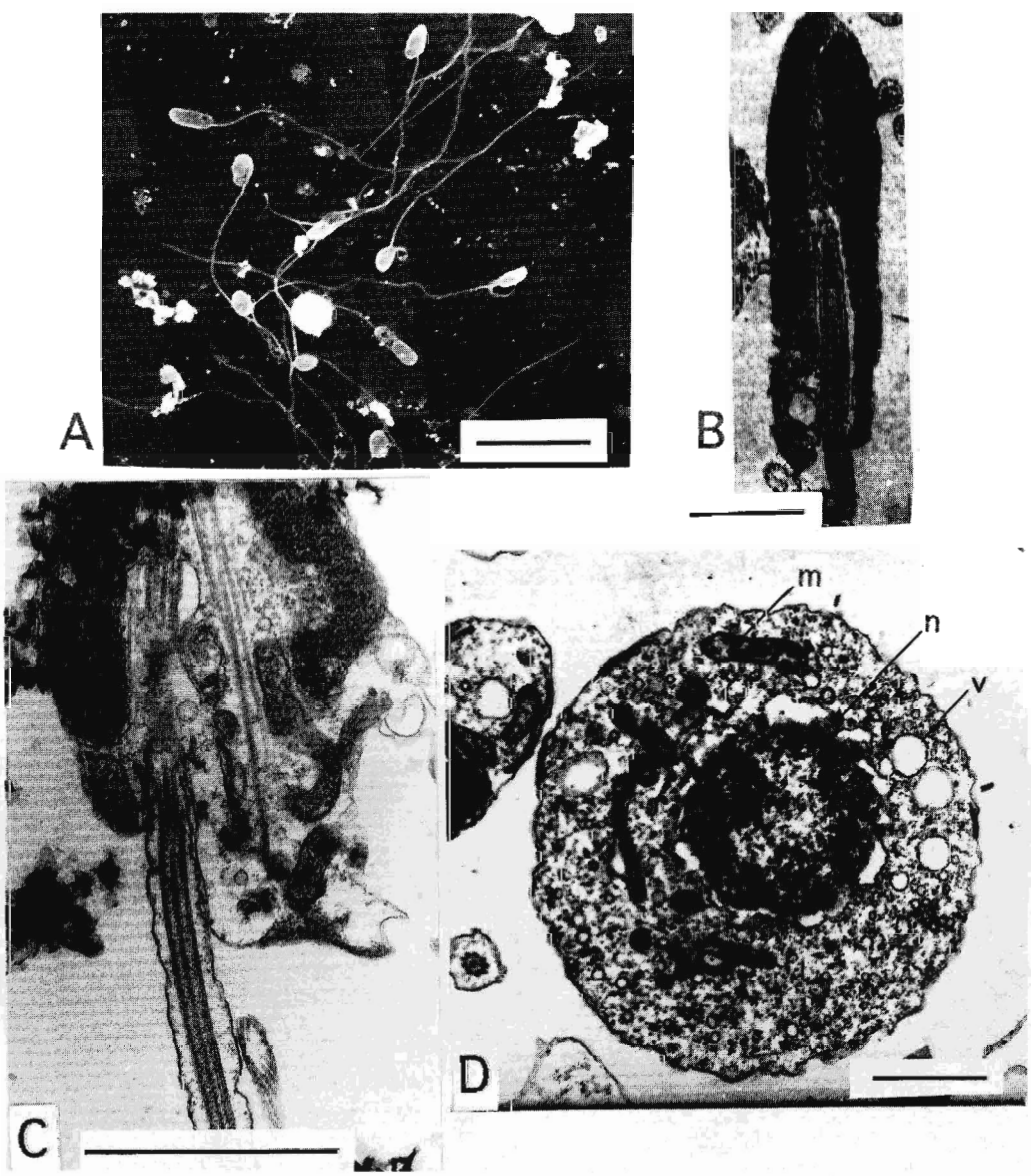

Figure 1. – Electron micrographs of sperm cells in the ejaculate of wolffish. (A) Image of scanning electron microscope of spermatozoa ; ×2 000; bar : 10 μm. (B) Longitudinal section of spermatozoon head and middle piece; ×10 000; bar : 1 μm. (C) Fragment of spermatozoon with two axonemes; ×20 000; bar : 1 μm. (D) Spermatid; ×20 000, bar : 1 μm; m, mitochondria; n, nucleus ; v, vesicle.

spindle-shaped structure is situated above the basal plate of the centriole. A long axis of this structure is perpendicular to the axoneme. A cross-section of the centriole shows the classical composition: 9 triplets of microtubules with particles of electron dense material between them.

Location of centrioles in the centriolar complex is not quite clear. Sagittal sections usually show one basal plate and a spindle-shaped structure. In several cases, an additional centriole is situated above the first one, with a small angle between two basal plates. Several spermatozoa with microtubules originating from the second centriole were observed, and two axonemes were distinguished (Fig. 1*c*). One axoneme with cytoplasmic cover forms a flagellum. The other axoneme probably never reaches the external part of the middle piece. The fate of the additional axoneme is not clear.

The middle piece of the spermatozoon has a form of half-oval on SEM samples. This part is situated eccentric to the nuclear invagination and flagellum, mainly at one side from them (Fig. 2). Many oval mitochondria (usually ten, up to fifteen) and vesicles of different size are distributed in the middle piece. The axoneme consists of nine peripheral and two central duplets of microtubules. They are electron transparent on cross-sections. A cytoplasmic cover, sometimes with vesicles inside, surrounds the axoneme. The width of the cover varies along the axoneme. Therefore,

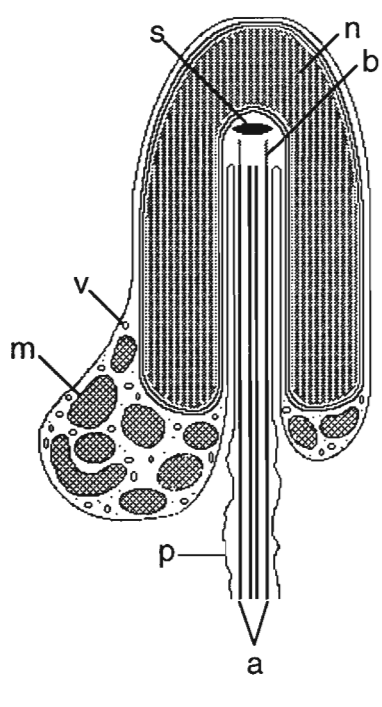

Figure 2. – Scheme of internal structure of wolffish spermatozoon. a, axoneme; b, basal plate; m, mitochondria; n, nucleus; p, plasmalemma; s, electron dense structure; v, vesicle. Bar: 1 μm (based on the measurements of spermatozoa with optic microscope).

transverse sections of flagellum show 1-2 lateral ridges, or can be without ridges.

Spermatids are usually found in the ejaculate together with mature spermatozoa. The ratio between spermatozoa and spermatids varies in individuals. Spermatids at different developmental stages, of different size and form are seen on electron micrographs. Their form varies from round at earlier stages to elongated at stages close to the transition into spermatozoa. A friable chromatin with electron dense granules and fibrils is found in the round nuclei of round spermatids. Mitochondria and elements of endoplasmatic reticulum and Golgi complex represented by vesicles of different size are seen in the cytoplasm (Fig. 1*d*). Microtubules, basal plate and flagellum are found at the latest stages of transition from spermatids to spermatozoa.

Size of sperm cells

Electron microscopy shows that the spermatozoon of wolffish has an oval head with an average size of approximately 0.7-1.5×2.0-3.0 μm. The average size of the middle piece is 1.5×1.5 μm. The diameter of spermatids at earlier stages of their development is 4.0-7.0 μm.

Observation of sperm cells with optical microscope shows a large variation in size composition of cells in the ejaculate. The size distributions of the cells are usually positively skewed. In some males, cell size range is narrow, while in others it is very wide, with the presence of many small and big cells (Figs. 3*a*, *b*). The smallest cells are without flagellum, but show agitated movements in the ejaculate. Other cells have forward movements. Mean length of spermatozoon head was 3.3 μm (SD 1.4, N=465) in two males. The width of spermatozoon head was about 1.47-fold lower than its length. The length of spermatozoon flagellum from a male ranged from 11.2 to 31.7 μm, with a mean of 21.2 μm (SD 5.5, N=40). Comparison of sperm cell size on SEM micrographs and in sperm samples observed with optical microscope shows possible shrinkage of cells caused by their treatment during fixing.

In most males, the majority of sperm cells had the highest size at first and second stripping, and the proportion of these cells declined after subsequent stripping. In male 1, average sperm cell size decreased from the first to the ninth stripping with values 5.4 μm (SD 1.8) and 2.7 μm (SD 0.8) respectively. The difference was significant ($p<0.0001$). (Fig. 4*a*). In male 2, average sperm cell size decreased from the first to the sixth stripping with values 4.8 μm (SD 1.1) and 3.3 μm (SD 0.8) respectively. The difference was significant ($p<0.0001$). To the end of the breeding season, a large portion of males had a greater proportion of cells of lower size without flagellum.

Sperm production

Observation of sperm production in 20 males during the year showed that ejaculate volumes were maximal from August to January (Fig. 5*a*), and a peak of sperm concentration in the ejaculate was found in December (Fig. 5*b*). In most of the males, there was a general trend for decreasing sperm production from the beginning of December for several subsequent months (Fig. 6). In some males, sperm production remained at a very low level during the whole year. The difference in sperm volume and sperm concentration between the males kept at different photoperiods (I8L:6D and 6L:18D) was not significant (Table 1). No significant correlation was observed between ejaculate volume or sperm density and the weight of milters in the range of 0.4–4.2 kg (N=170).

DISCUSSION

As it is known, fish spermatozoon morphology is very different, and depends on taxonomy, as well as on reproductive biology of the species (Ginsburg, 1968; Turdakov, 1972; Baccetti, 1984; Emel'yanova and Makeyeva, 1985; Jamieson, 1991; Mattei, 1991). In fishes with internal fertilization, the middle piece of the spermatozoon is well developed and contains many mitochondria (Ginsburg, 1968; Fribourg *et al.*, 1970; Gardiner, 1978). Wolffish spermatozoon belongs to the perciform type described by Mattei (1991). The middle piece of wolffish spermatozoon is not large, compared to the head, but contains many mitochondria, suggesting a long period of spermatozoon activity. As was shown before, this period could reach several days in undiluted ejaculate, but maximal sperm fertility was observed within approximately 10 h from stripping at 4°C (Pavlov, 1994*a*, *b*).

It is interesting to compare spermatozoon ultrastructure in wolffish and in fishes from the family Zoarcidae. This family is close to Anarhichadidae, and both are included in the suborder Zoarcoidei (Anderson, 1994). Spermatozoon ultrastructure has been described in two species from the Zoarcidae family: viviparous *Zoarces elongatus* (Koya *et al.*, 1993), and oviparous, but internally fertilizing fish, ocean pout (*Macrozoarces americanus*) (Yao *et al.*, 1995). Reproductive biology of the latter species is very similar to that in wolffish: the eggs develop in external medium after their internal fertilization (Yao and Crim, 1995).

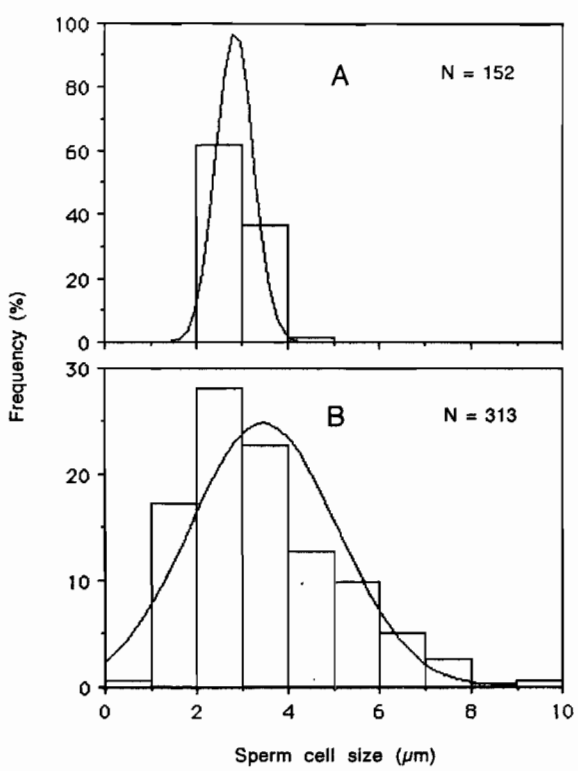

Figure 3. – Size distributions of wolffish sperm cells in the ejaculates obtained from two males (A and B).

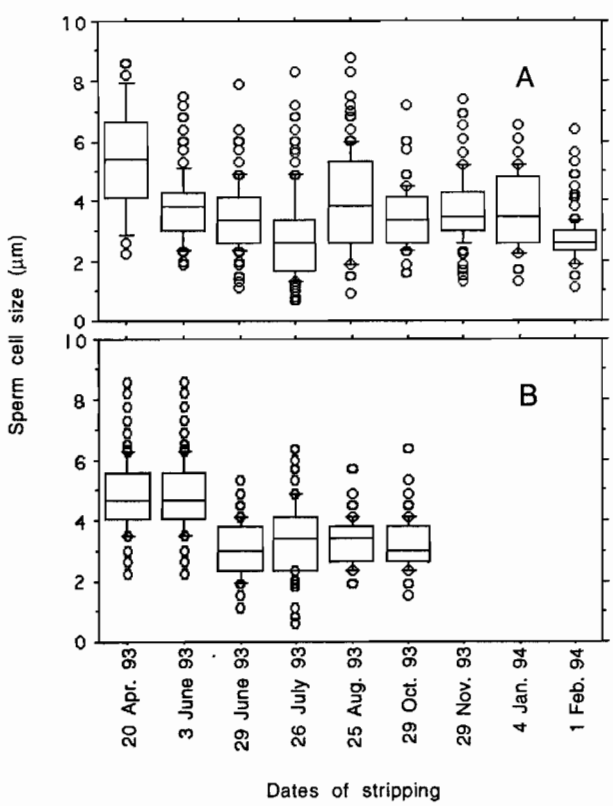

Figure 4. – Box plots showing size distributions of sperm cells in the ejaculates obtained from two males (A and B) during breeding season in wolffish. Each box plot is composed of five vertical lines that display the 10-th, 25-th, 50-th (median), 75-th and 90-th percentiles of sperm cell size. All values for the variable above the 90-th percentile and below the 10-th percentile are plotted as points.

The shape of the wolffish spermatozoon head is similar to that described in *Zoarces elongatus* and ocean pout. A long nuclear invagination with a centriolar complex inside is a unique structure of wolffish spermatozoon. Such a canal has various lengths, or can be absent in different fish species (Emel'yanova and Makeyeva, 1985, 1991; Mattei, 1991). In *Zoarces elongatus* and ocean pout, this canal has not been observed. Well developed middle piece has oval shape in the former species and elongated in the latter one. Two of these species possess spermatozoa with biflagellar tails (Koya *et al.*, 1993; Yao and Crim, 1995). Thus, the variation in the structure of spermatozoon tails within the suborder Zoarcoidei (one or two flagell) is similar to that observed in several families of the order Siluriformes (Emel'yanova and Makeyeva, 1991).

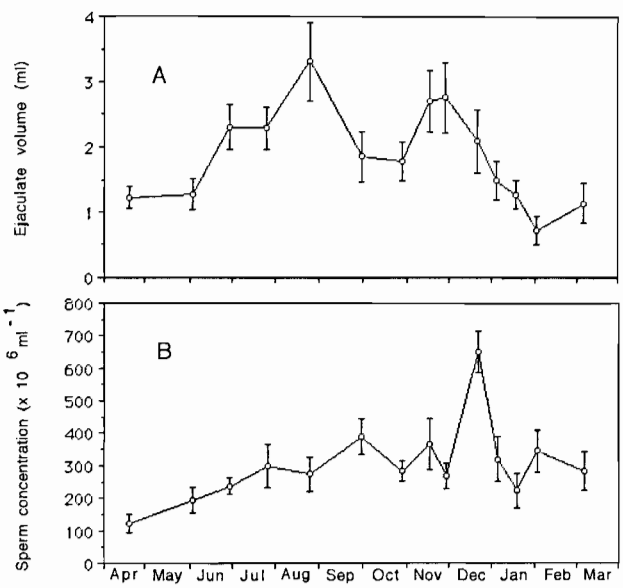

Figure 5. – Volume of ejaculate (A) and concentration of sperm (B) repeatedly stripped in 20 males during the breeding season in wolffish kept at 18L: 6D photoperiod. Error bars are the standard errors.

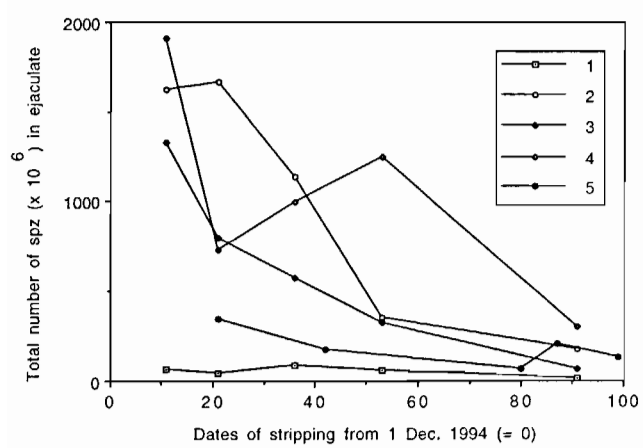

Figure 6. – Sperm production (total number of spermatozoa in the ejaculate) in five males during the breeding season in wolffish kept at 18L:6D photoperiod.

As has been described above, in several cases we have found two basal plates with microtubules. At the same time, longitudinal and cross-sections of the spermatozoon always showed only one flagellum. The presence of some spermatozoa with biflagellar tails has not been observed, but could not be totally excluded in wolffish. Appearance of abnormal spermatozoa was reported in different vertebrate animals. It could be caused by abnormalities in mitosis or meiosis, resulting in duplication of flagellum and chromosome number in sperm cells (Danilova, 1978). A polymorphism in the spermatozoa ultrastructure and size was described in different types of animals (Roosen-Runge, 1980), and apparently takes place in wolffish. It is interesting to note that a polymorphism was found in wolffish eggs too: small proportions of eggs from several females had from 2 to 5 micropyles (Dzerzhinskiy *et al.*, 1992). Similar variability in the number of micropyles was described in ocean pout (Yao *et al.*, 1995).

A large variability in the sperm cells size was observed in the majority of males from the broodstock and apparently was connected with the presence of mature spermatozoa, spermatids of larger size, and smaller cells without flagellum in the ejaculate. Cells of larger size found in the beginning of the breeding season, were probably represented by spermatids. During this season, the proportion of spermatids apparently declined, but of small cells without flagellum increased.

An observation of spermatids in the ejaculate is rather unusual for fishes. In some species with external and internal fertilization, cells at earlier developmental stages were found together with mature spermatozoa in seminal lobule lumen. They were observed in *Neoceratius spinifer* (Neoceratidae), *Lepadogaster lepadogaster* (Gobiesocidae), *Ophidion* sp. (Ophidiidae), several species from Blenniidae (Mattei, 1993), and in *Zoarces elongatus* (Zoarcidae) (Koya *et al.*, 1993). In the latter species, spermatids and spermatozoa were motile in isotonic medium. Spermatids were often observed in turbot (*Scophthalmus maximus*) sperm samples at the beginning of the milting period (Suquet *et al.*, 1994). According to Mattei *et al.* (1993), in most teleost fishes with "cystic" synchronous spermatogenesis, degradation of cysts occurs and spermatozoa release into the sperm ducts. In several other fish species with "semi-cystic" asynchronous spermatogenesis, development of spermatids continues in the seminal lobules due to earlier degradation of cysts. In the latter case, a mixing of spermatids and mature spermatozoa in the sperm ducts is possible. At the same time, the presence of spermatids in wolffish ejaculate is apparently not normal and can be caused by the stripping procedure.

The smaller cells without flagellum observed in wolffish ejaculate were probably represented by degraded spermatozoa, or by abnormal sperm cells. We observed these cells in the ejaculate under the light microscope, but they were not identified on SEM or TEM micrographs. Similar cells, sometimes without heads, or flagellum were observed in several groups of invertebrates (Danilova, 1978). These cells could have agitated movements caused by contractions of microtubules presented in their cytoplasm. The results of this study showed that to words the end of the breeding season, sperm production decreased and the proportion of small degenerated cells increased, probably due to resorption of spermatozoa. Lower sperm quality at the end of the breeding season was described in other fish species (Belova, 1981; Billard and Takashima, 1983).

In captive wolffish, sperm can be produced all-year-round, with a peak in December - January. This time of the year coincides with mass maturation of females in the tanks and apparently with the peak of natural spawning in southern Norway (Pavlov and Moksness, 1994*a*). Similar results were obtained during field studies of wolffish from North-Norwegian waters: spermatozoa were presented in testes during all seasons of the year, with the highest concentrations at the time close to reproduction (Falk-Petersen and Hansen, 1991). Therefore, wolffish possess continuous spermatogenesis throughout the year in nature and in captivity. Very low level of sperm production in several males from the broodstock over the entire year shows a possibility for selection and elimination of such fishes. Another practical conclusion is insignificance of light cycle on sperm maturation. An opposite feature has been found in females: they did not reach final maturation at 6L: 18D photoperiod over the entire year.

The gonadosomatic index, GSI [(gonad weight/body weight)x100] of mature wolffish male is probably one of the lowest in fishes (see a review of Suquet *et al.*, 1994), reaching mean values of 0.11-0.13% (Pavlov and Radzikhovskaya, 1991; Johannessen *et al.*, 1993; Pavlov and Moksness, 1994*b*). According to the latter paper, volume of ejaculate and sperm concentration were approximately 12 and 66 times lower in wolffish than in Atlantic salmon (*Salmo salar*). Apparently, it can be explained by difference in modes of fertilization in these two species. At the same time, in ocean pout, a fish with internal fertilization, the values of GSI and volume of ejaculate were higher than those in wolffish, but sperm concentration was still low (Yao and Crim, 1995).

In spite of internal fertilization, general morphology of wolffish spermatozoon is close to that in oviparous fishes. The ultrastructure of spermatozoa in the species from family Zoarcidae is more specialized, having better developed middle part and biflagellar tail. It corresponds with more derived type of reproduction in some species of the family, which are viviparous. The mode of reproduction of wolffish with internal insemination and releasing of eggs into external medium and corresponding spermatozoon morphology could be regarded as the first step toward the viviparity within suborder Zoarcoidei.

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