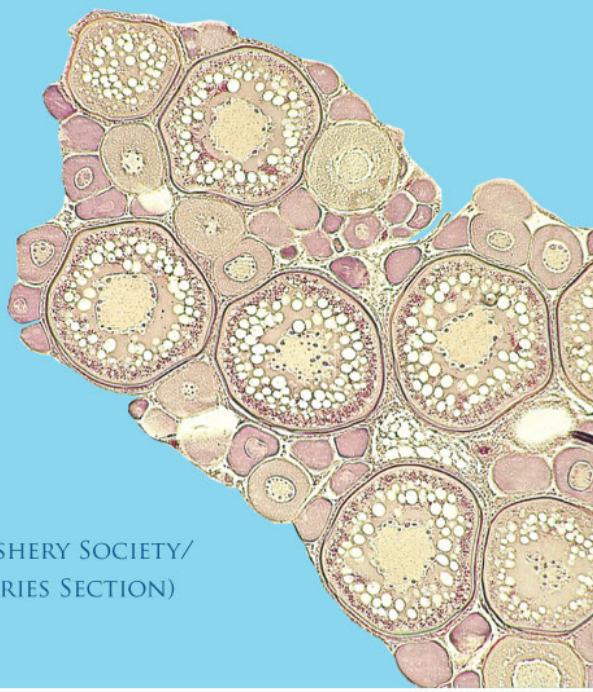
4TH WORKSHOP ON GONADAL HISTOLOGY OF FISHES

EL PUERTO DE SANTA MARÍA, CADIZ, SPAIN
16-19 JUNE 2009



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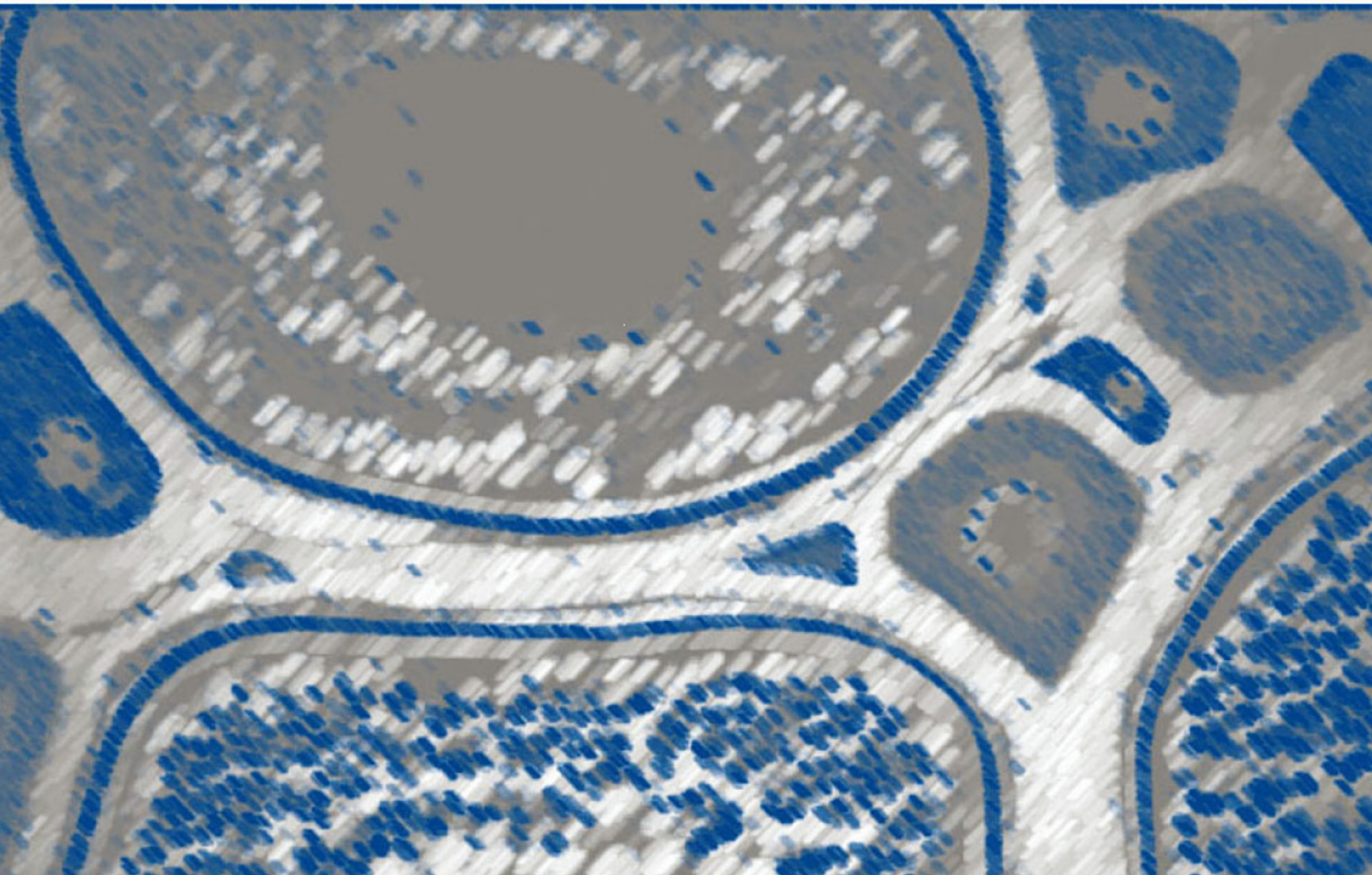

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David M. Wyanski and Nancy J. Brown-Peterson, Co-Editors
Sonia Rábade Uberos, Editorial Assistant

PROCEEDINGS OF THE 4TH WORKSHOP ON GONADAL HISTOLOGY OF FISHES

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The 4th Workshop on Gonadal Histology of Fishes continued a series of successful workshops exploring aspects of fish reproduction that began in 2001. The first workshop occurred in February 2001 at a meeting of the Southern Division of the American Fisheries Society in Jacksonville, Florida, and was initiated by fisheries biologists in the southeastern United States who felt the need to meet other scientists that were using histology as a tool to investigate reproduction in fishes. The second workshop occurred in July 2005 at the Florida Fish and Wildlife Research Institute in St. Petersburg, Florida, and the third workshop was held in July 2006 immediately prior to the Joint Meetings of Ichthyologists and Herpetologists in New Orleans, Louisiana. Attendance as well as geographical representation continued to grow with each subsequent workshop. Topics of presentation and discussion at the first two workshops focused on marine teleosts, and included ovarian cycling, reproductive classification, sex transition in hermaphrodites, distinguishing immature vs. inactive mature specimens, and identification of histological artifacts such as postmortem decay of postovulatory follicles. Topics at the third workshop broadened to include freshwater fishes and elasmobranchs, and a major focus of this workshop was presentations and discussions leading to the development of a standardized terminology for describing the reproductive cycle in fishes.

The Gonadal Histology Workshops became truly international at the 4th workshop in Spain, with 100 participants from 23 countries participating in presentations and discussions. Oral and poster presentations centered around four major themes (reproductive strategies and gametogenesis, fecundity and skip spawning, temporal patterns, and terminology and methodology), and plenary discussions each day highlighted the importance of histology in achieving an understanding of complex aspects of fish reproductive biology.

One of the goals of the 4th Workshop on Gonadal Histology of Fishes was a broad dissemination of information based on the presentations at the workshop. Previous workshops produced a printed booklet of extended abstracts of each presentation that was distributed to all workshop participants. However, this information was not in a citeable format, and was unavailable to non-participants. Thus, the Organizing Committee of the 4th Workshop decided to compile the extended abstracts of the 2009 Workshop into an editor-reviewed Proceedings, and post this Proceedings on the internet so that the articles are widely available and fully citeable. These Proceedings present a summary of each oral and poster presentation, and it is hoped that their wide distribution will advance the broad field of fish reproductive biology. In addition to the Proceedings, full papers based on 11 presentations from the workshop will be published in the on-line, peer reviewed journal *Marine and Coastal Fisheries* in a special section "Emerging Issues and Methodological Advances in Fisheries Reproductive Biology."

The Workshop was held at *Centro de Investigación y Formación Pesquera y Acuícola El Toruño* (IFAPA). The success of the 4th Workshop on Gonadal Histology of Fishes was due to the hard work and dedication of the 2009 Organizing Committee (Fran Saborido-Rey, Chair, Nancy Brown-Peterson, Sue Lowerre-Barbieri, Hilario Murua, Jonna Tomkiewicz and David Wyanski), Local Arrangements Chair María Ángeles Bruzón, and the help of staff and

students from the Institute of Marine Research (CSIC) and IFAPA,Spain. The Workshop was co-sponsored by COST Action FA0601: Fish Reproduction and Fisheries (FRESH) and the Marine Fisheries Section of the American Fisheries Society. The Organizing Committee is grateful to COST for their financial support of the Workshop and the publications resulting from the Workshop.

David M. Wyanski and Nancy J. Brown-Peterson, Co-Editors
Sonia Rábade, Editorial Assistant
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AMERICAN FISHERIES SOCIETY
Marine Fisheries Section

FRESH and COST

FRESH, Fish Reproduction and Fisheries, is the COST action FA0601. This Action has provided funds both to hold the workshop, for scientists to participate and for publishing this module.

COST- the acronym for European Cooperation in Science and Technology- is the oldest and widest European intergovernmental network for cooperation in research. Established by the Ministerial Conference in November 1971, COST is presently used by the scientific communities of 36 European countries to cooperate in common research projects supported by national funds.

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Fran Saborido-Rey, chair of COST Action FRESH

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Opening Keynote

Session 1 - Female and male reproductive strategies: Differences in gametogenesis across species.

Session 2 - Characterization and differentiation among reproductive phases with an emphasis on fecundity types, skipped spawning and abnormalities.

Session 3 – Determination of temporal patterns of reproductive activity.

The Utility of Gonadal Histology in Studies of Fish Reproduction and the Subsequent Management of Fisheries and Ecosystems

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Introduction

Histology is the most common and often the most reliable technique to assess the reproductive strategy and tactics of fish species and is therefore applied in a large number of marine laboratories. In this respect those laboratories aiming to provide management advice use histology for 1) maturity classification to separate the sexually immature component from the sexually mature component, 2) fecundity studies to be used in either recruitment-related studies or in Egg Production Methods (to estimate spawning stock biomass) or 3) to monitor prevalence of any type of reproductive disturbance or failure to spawn regularly. By necessity these different overall aims require different methodological approaches although in many cases the histological protocol as such is reasonably similar. In summary, histology is considered to be an important tool to better understand the productivity of fish stocks and thereby in the end contributes to the internationally adopted principles of the precautionary or ecosystem approach to fisheries.

The knowledge about fish gametogenesis of relevance to applied fisheries reproductive biology (AFRB) (Kjesbu 2009) has increased substantially over the last decades, partly influenced by method development within other scientific disciplines, such as the stereological Disector method (Sterio 1984), but also by the implementation of advanced image analysis (Thorsen and Kjesbu 2001) in combination with histology, modern fish husbandry techniques to analyse samples of known history, dedicated workshops (such as the present one) and co-operation with experts on aquaculture and reproductive physiology. Some recent applications of histology within AFRB indicate where future research might lead us in terms of understanding the highly complex nature of reproductive dynamics of marine fishes.

Examples of utility of histology

As marine institutes mainly focus on regional resource management, their research is, as expected, undertaken on local species. In the present case this refers to arcto-boreal waters, where the harvested species are typically long-lived (iteroparous), spawn their eggs freely (oviparous) and show a determinate fecundity. Thus, their reproductive style and investment is less complicated to assess than for more southerly, indeterminate spawners. Nevertheless, there is still a range in complexity from total spawners (e.g. Atlantic herring, *Clupea harengus*) to multiple batch spawners (e.g. Atlantic cod, *Gadus morhua*)).

Maturity classification using 'markers'

These can be grouped into three types (Table 1), but their present names should likely be reconsidered following recommendations from this workshop (see Brown-Peterson et al., this workshop). The single or combined use of these markers is probably the best way in existence

today to classify a female into the right maturity stage or phase (sub-stage). Here the males are ignored, as often done, but there does also exist reliable histological classification schemes for this gender, although demanding a higher level of expertise.

Oocyte recruitment
Studies on oocyte recruitment are very limited in the fish literature, primarily because of the high complexity in enumerating these small cells. The most natural route to take is by using sophisticated stereology (see Hagstrøm Bucholtz et al. this WS), but the procedure is extremely laborious and is complicated by shrinkage/distortion problems along the so-called z-axis. Thus, we advocate also the use of advanced packing density formulae (Kurita and Kjesbu 2009) in combination with Delesse principle (see Korta et al. this WS).

The concept of natural down-regulation (Kurita et al. 2003) has opened up a completely new understanding of oocyte number regulation and thereby quantification and standardization. However, to understand the underlying processes, histology is clearly required to address atresia formation and fate.

Atresia
Studies on atresia have been ongoing for many years but only now one can say if it is being correctly quantified. The combination of image analyses (whole mount) using specific stains and validation by histology look very promising for further method development (Witthames et al. 2009).

Perspectives
There is a tendency in the literature that further development of macroscopic schemes should be based on the introduction of more stages, while the opposite is probably more correct, i.e., one should realize the limitation of the human eye. The use of gonadosomatic index should, however, be recommended as it has proven successful in separating sexually immature and mature specimens. Again, however, histology is required as a validation tool.

The combined used of histology and image analyses is clearly giving us new perspectives on different reproductive styles and the underlying, relevant oocyte recruitment mechanisms involved. It is foreseen that in the near future one will be able to convincingly demonstrate the formation of ‘waves’ of oocytes formed during the reproductive cycle and thereby will be more able to delimit the so-called ‘sensitive periods’, both in terms of environmental influences and throughout the life of an animal.

The present quantification of atresia raises concerns in terms of model assumptions (Kjesbu 2009). Future research should address these carefully.

In conclusion, the combination of histology and image analysis has proven to be an effective approach. Histology will still be the main validation tool for a series of maturity markers. There is a growing understanding of oocyte recruitment patterns, but there are so far many ‘black boxes’. The current estimation of atretic fecundity needs to be evaluated and likely revised.

Acknowledgements
I would like to thank the organizers for inviting me, and the following persons for giving valuable inputs: Merete Fonn, Anders Thorsen, Yutaka Kurita and Peter R. Witthames. A thank you also to the University of Bergen, an essential partner over many years.

A manuscript based on this presentation has been submitted to the journal Marine and Coastal Fisheries as part of the special module "Emerging Issues and Methodological Advances in Fisheries Reproductive Biology". Publication is anticipated in fall 2010. Web page: www.fisheries.org/mcf.

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Table 1. Overview of type of maturity markers and their sub-categories.

Type of marker	Category	Sub-category	Comment
Pre-spawning marker	Advanced previtellogenic oocytes	Various phases	Risky: might not proceed
	Cortical alveoli oocytes	Normally not	Risky: might not proceed
	Vitellogenic oocytes	Early to late	Normally safe, but massive atresia might occur
Spawning marker	Final maturation oocytes	Three phases	Both yolk uptake (early on) and proteolysis
	Hydrated oocytes	No	Collapse during histological dehydration
	Ovulated oocytes	No	Collapse during histological dehydration
	Post-ovulatory follicles	Short-lived type	Last few days, e.g. anchovies, clupeids
		Long-lived type	Last for months, e.g. gadoids
Post-spawning marker	Post-ovulatory follicles	Long-lived type	
	Previtellogenic oocyte size	Leading cohort	Smaller PVO in spent females
	Amount of ovarian stroma	Mostly connective tissue	More in spent-recovering females
	Ovarian wall thickness	Blood vessels and connective tissue	Thicker in spent-recovering females, but risky
	Encapsulated 'eggs'	Unovulated oocytes ('eggs') or large, abnormal atretic oocytes (cysts)	Might last many months
	Late atresia	Two phases	Shown for delta atresia

Female Reproductive Strategies: An Energetic Balance Between Maturation, Growth and Egg Production

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Introduction

Natural selection aims at maximizing the survival opportunities of the offspring until it reaches sexual maturity and reproduces successfully. In the context of reproductive biology of fishes, the term strategy emphasizes the co-relationships between reproductive traits so that the resulting strategy maximizes the production of reproductively active offspring in each generation (Wotton 1984). Therefore, traits are not linked together at random, but through the agency of natural selection. In consequence, female fish exhibit a wide range of reproductive strategies regarding gender definition (gonochorism or hermaphroditism), parental care (oviparous or viviparous), breeding opportunity (semelparous or iteroparous), oocyte recruitment (synchronous, group synchronous or asynchronous), fecundity type (determinate or indeterminate), and spawning pattern (total spawners or batch spawners).

While the fish is sexually immature, assimilated energy is fully allocated for survival and growth, but once maturation starts, a part of the energy is required for gamete production and reproductive behavior. The amount of energy allocated to growth and reproduction will depend on a number of factors, some of them intrinsic (genetic, physiological) while others are environmentally driven (temperature and feeding). Thus, a compromise on balancing the energy must exist, reflected in specific growth and reproduction dynamics in the lifetime of an individual fish.

All of the above reproductive strategies combined with different growth strategies and use of surplus energy (e.g., capital or income breeders, sensu Houston et al. 2007) results in different vital strategies. Most of the defined reproductive strategies account for only very specific reproductive parameters (Murua and Saborido-Rey 2003). However, an adaptive interpretation of the adjustment of growth and longevity might be explicable in simple Darwinian terms by supposing that it is an 'advantage' to the individual to complete as much as possible of its potential growth within its likely maximum life-span, thus best enabling its reproductive potential to be realized and so maximizing the contribution of its progeny to future generations (Beverton, 1963). At the same time reproductive effort beyond sexual maturation is not fully reflected in the above mentioned strategies, i.e. the way a fish produces eggs and uses available energy for such purpose. Schaffer (1974) argued that an optimal life history maximizes the reproductive value in terms of benefit and costs of Reproductive Effort (RE), and Ware (1984) defined two types of strategies, one assuming that age-specific reproductive rate (in energy units) is an increasing function of the supply of surplus energy and another assuming that reproductive rate is determined strictly by body size at each age.

Newly Proposed Reproductive Strategies

The models of Schaffer (1974) and Ware (1984) do not completely capture the use of the energy and allocation rules for growth and reproduction, especially in relation with reproductive modes (oocyte recruitment, fecundity and spawning). Therefore, hence we propose here the definition of five general strategies:

- Strategy A Fish invest in growth before and after maturation. Maturing at a low proportion of maximum size (50%), late maturation, long-lived, long reproductive life span. Reproduction mostly depending on energetic reserves, i.e. capital breeders, which lead to iteroparous, group-synchronous, batch or total spawners with determinate fecundity. Type: cold water species (*Gadus morhua*). Illustrated in Figure 1A.

- Strategy B Fish investing in growth before and after maturation. Maturing at a low proportion of maximum size (50%), late maturation, long-lived, long reproductive life span. Reproduction is intially financed from stored energetic capital, but energetic provision through concurrent feeding during reproduction contributes to oocyte development, i.e. a mixture of capital and income breeding. This strategy leads to iteroparous, asynchronous, batch spawners with determinate fecundity. Type: temperate-cold water (*Trisopterus luscus*). Illustrated in Figure 1B.

- Strategy C Fish investing in growth more before than after maturation. Maturing at a higher proportion of maximum size (70%), earlier maturation, medium-lived, medium reproductive life span. Reproduction does not depend on energetic reserves accumulated at an earlier time but in concurrently energy gained, but the capacity to obtain such energy is related to fish size, so fish prefer grow before maturation. This strategy shows iteroparity, asynchronous, batch spawners with indeterminate fecundity. Type: temperate (*Merluccius merluccius*). Illustrated in Figure 1C.

- Strategy D Fish investing in growth before maturation, with little or no growth thereafter. Maturing at almost the maximum size, very early maturation, short-lived, short reproductive life span. Reproduction is financed exclusively using current energetic income (income breeders). Very high fecundity, iteroparous, asynchronous, batch spawners with indeterminate fecundity. Type: temperate-tropical (*Sardina pilchardus*). Illustrated in Figure 1D.

- Strategy E Fish investing in growth before maturation, with no growth thereafter. Maturing at maximum size, but not necessarily early, medium-lived, extremely short reproductive life span. Reproduction always depend on energetic reserves. Pure capital breeders. Type: Semelparous (*Oncorhynchus*). Illustrated in Figure 1E.

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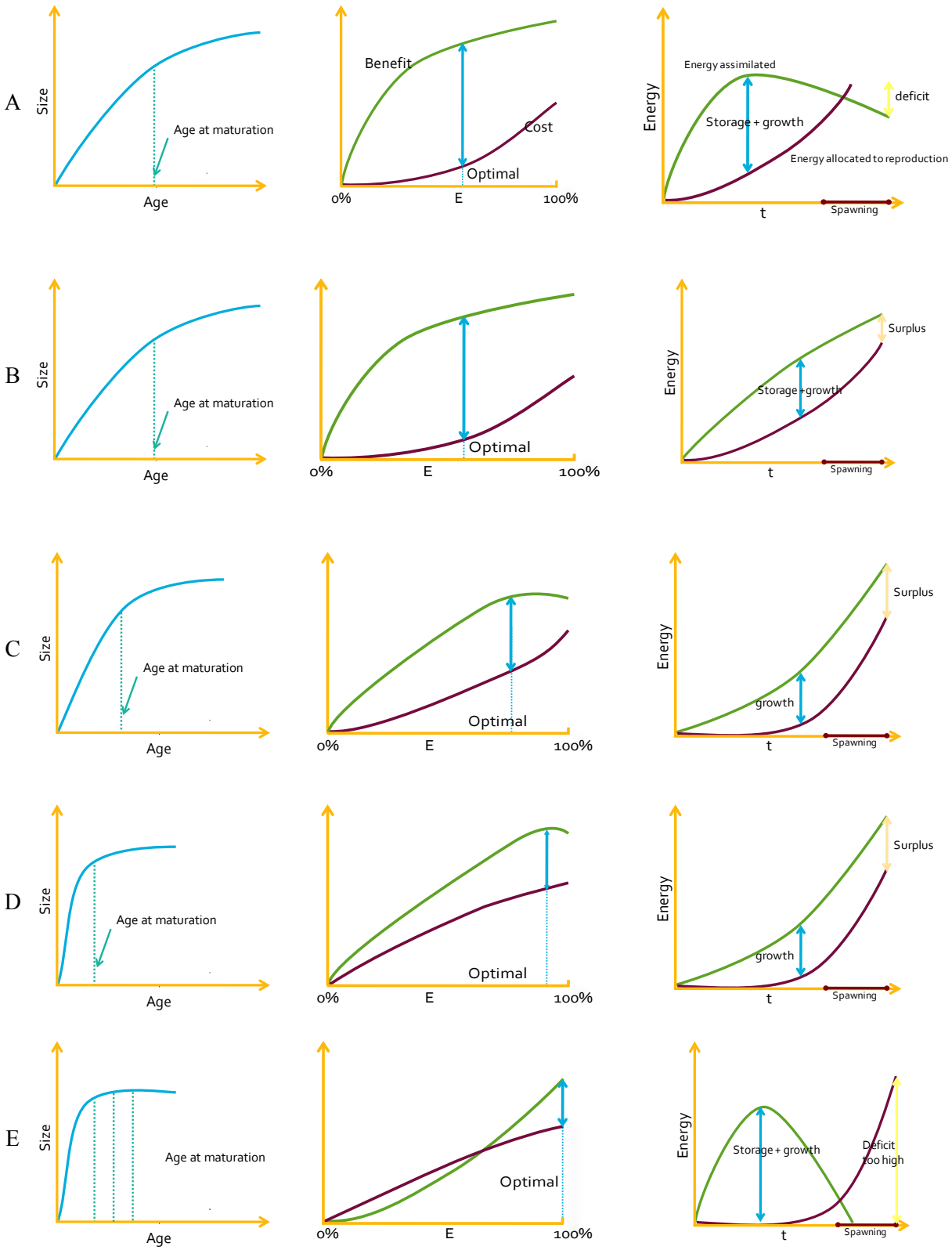

Figure 1. Summary of proposed strategies (A-E) about the use of the energy and allocation rules for growth and reproduction in relation to reproductive modes. Left Panel—Age at maturation schedule related to growth trajectory. Middle Panel—Reproductive value in terms of benefit and costs of Reproductive Effort (RE). Right Panel—Energy investment along the breeding season.

Comparative Analysis of Reproductive Strategy in Three Demersal Species from the Galician Shelf

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Introduction

The present study is focused on three demersal species from the Galician Shelf with high commercial interest: four-spot megrim (*Lepidorhombus boscii*), pouting (*Trisopterus luscus*) and European hake (*Merluccius merluccius*). The study area is part of the Lusitanian ecoprovince, a transition zone between the temperate North Atlantic and the tropical Atlantic (Spalding et al. 2007). The Galician Shelf is characterized by geographical complexity of coastal shape and temporal changes of hydrographical conditions (upwelling events and sub-superficial warm water poleward current). Megrim inhabits sandy bottoms (200 - 600 m) and pouting lives in rocky and sandy bottoms (30 – 100 m) on the continental shelf. European hake is a bathypelagic species that makes daily vertical migrations and inhabits muddy-sandy bottoms on the continental shelf and slope (30 - 1000 m). Reproductive strategies of these species have been analyzed to study the relationship with different ecosystem roles adopted by each species and the effect of ecosystem heterogeneity on reproductive biodiversity.

Methods

Sampling was carried out on the Galician Shelf between 2003 and 2008. Length and gutted weight of females was recorded. Ovaries were removed, weighed and fixed in 3.6% buffered formaldehyde. The central portions of the fixed ovaries were dehydrated, embedded in paraffin, sectioned at 3 µm and stained with haematoxylin-eosin for microscopic analysis. Hepatosomatic index (HSI), gonadosomatic index (GSI) and condition factor (K) were estimated. The number of developing oocytes/g female gutted weight (NDOrel) was analyzed as well as oocyte size distribution in the ovary based on image analysis techniques (Domínguez-Petit 2007). Ovaries were microscopically classified into three maturity stages: pre-spawning, spawning and inactive mature. These variables were analyzed to establish spawning season duration, oocyte development dynamics, fecundity type, and energy distribution pattern.

Results and Discussion

Four-spot megrim: Most spawning activity occurred in February and March (Table 1), while in summer months almost all females were inactive; i.e. spawning season duration is 2 months. Reproductive activity at the population level was synchronous. The GSI fluctuated between 0.3 and 23.5%; the highest values were reached in March, indicating there is a single peak of spawning in this month. Primary growth oocytes reached a maximum of 180 µm; oocytes develop from cortical alveoli stage to vitellogenic stage between 180 and 750 µm, while hydrated oocytes are > 1 mm. Only one mode was detected in the mature oocyte group in addition to a small group of hydrated oocytes. Thus, four spot megrim is a batch spawner with group synchronous oocyte development. NDOrel decreased significantly ($F = 11.13$; $p < 0.01$) from the beginning to the end of spawning season (Figure 1A, left). This suggests there is no recruitment of new oocytes from primary growth to the maturing pool, thus indicating

determinate fecundity. HSI and K decreased considerably from pre-spawning to inactive mature females (Figure 1A, right), which could indicate that gonadal development depends directly on body energy reserves accumulated both in liver and muscle.

Pouting: The spawning season is approximately 3 months from January to March, but some spawning females are observed in summer-early autumn (Table 1). Thus, population synchrony is not so marked as in four-spot megrim. GSI fluctuates between 0.05 and 18.1%, with maximum values recorded in January-February. Primary growth oocytes are < 150 µm and hydrated oocytes are > 800 µm. Within the maturing group of oocytes (150-750 µm), 2 groups of oocytes are observed; one group around 300 µm (cortical alveoli and early vitellogenic) and the group around 500 µm (advanced vitellogenic). Pouting is a batch spawner but with asynchronous oocyte development. NDOrel decreased significantly ($F = 15$; $p < 0.01$) from the beginning to the end of spawning season (Figure 1B, left), indicating determinate fecundity. HSI decreased throughout the spawning season whereas K increased in inactive mature females, following the opposite pattern of GSI (Figure 1B, right). This could indicate that gonadal development depends directly on liver energy reserves and not on muscle energy.

European hake: High spawning activity was observed year round (Table 1), probably because of two factors: the existence of a protracted spawning season and population reproductive asynchrony. Two peaks of GSI were observed, the main one in February and a secondary peak in late spring-summer. Individual spawning season duration could be not established because it is not known if the same females are spawning in both peaks or if they are different sections of the spawning stock. Multimodal distribution within the maturing oocyte group was observed. All oocyte developmental stages are present in the ovary at the same time, i.e. it is batch spawner with asynchronous oocyte development. Although mean NDOrel decreased from January to May (Figure 1C, left), this was not significant ($F = 1.01$, $p = 0.43$). Indeed, NDOrel increased slightly in April, indicating that recruitment of new oocytes to the maturing pool occurs during the spawning season, indicating indeterminate fecundity. HSI decreased from pre-spawning to spawning females and remained constant in inactive mature fish while K increased progressively throughout the spawning season (Figure 1C, right). Thus, we could not verify that gonad development depends directly on body energy reserves.

Previous studies reported that biodiversity declines when latitude increases (Hillebrand 2004); this concept can also be applied to the variety of reproductive strategies. According to Southwood (1977), spatial and temporal heterogeneity of habitat partially determines fish reproductive strategies, with maximization of reproductive fitness through phenotypic plasticity. The studied area is a transition zone between high and low latitudes with spatial and temporal heterogeneity, so we can hypothesize that in the Galician Shelf a high diversity of reproductive strategies should exist. As shown here, in the demersal continental shelf there is a gradient of reproductive strategies from the determinate fecundity, short and population-synchronous spawning season with group synchronous oocyte development (four-spot megrim) to indeterminate fecundity with long and population-asynchronous spawning season and asynchronous oocyte development (European hake). The next step would be to analyze to what extent this diversity of fish reproductive strategies has been determined by habitat characteristics, and how this information could be incorporated into assessment and management procedures.

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Table 1. Monthly percentage of females in different maturity stages for the three studied species. (-) No data available.

		Jan	*Feb*	*Mar*	*Apr*	*May*	*Jun*	*Jul*	*Aug*	*Sept*	*Oct*	*Nov*	*Dec*
Four-spot megrim	*Prespawning*	-	23.7	1.8	14.9	3.8	1.2	0	-	-	13.6	92.3	33.3
	Spawning	-	73.7	91.2	52.9	35	8.2	3.8	-	-	13.6	7.7	2.4
	Inactive	-	2.6	7	32.2	61.2	90.6	96.2	-	-	72.7	0	64.3
Pouting	*Prespawning*	33.3	0	5.6	0.9	21.6	13.3	8.6	17.7	44.9	29.6	58.8	80.7
	Spawning	66.7	100	93.3	52.8	30	36.1	7.4	5.9	6.1	1.2	0	12.9
	Inactive	0	0	1.1	46.3	48.4	50.6	84	76.5	49	69.1	41.2	6.5
European hake	*Prespawning*	50	46.2	22.1	27.1	41.5	21.4	30.6	-	-	0	-	57.9
	Spawning	50	53.9	75	66.1	26.8	28.6	22.2	-	-	100	-	10.5
	Inactive	0	0	2.9	6.8	31.7	50	47.2	-	-	0	-	31.6

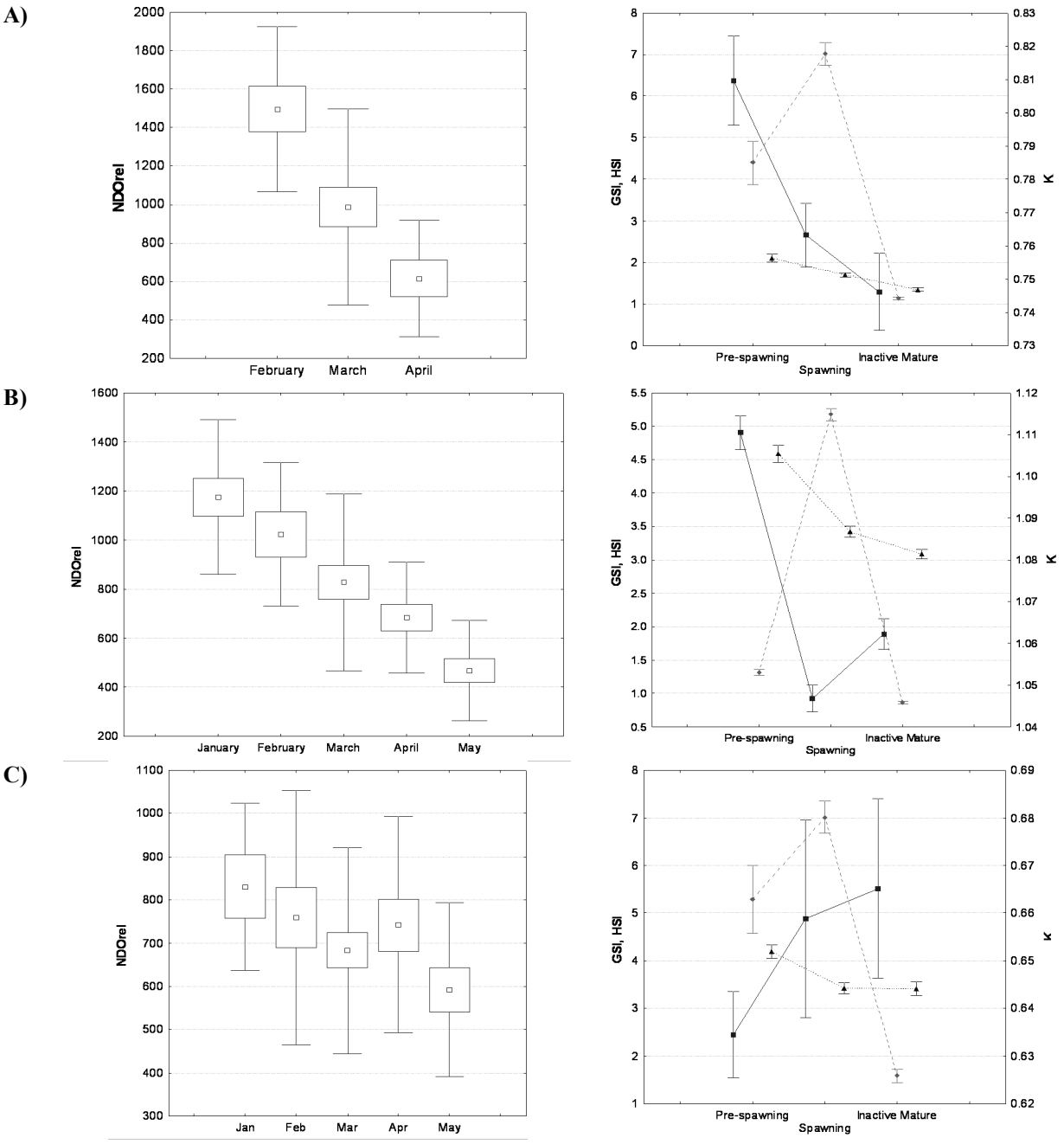

Figure 1. In left panel: monthly variation of mean relative number of developing oocytes (NDOrel); box=standard error, whisker=standard deviation and in right panel: monthly variation of mean ±standard error of GSI (dashed line), HSI (dotted line) and K (solid line) for A) four-sport megrim, B) pouting and C) European hake.

Three Different Hermaphroditic Sexual Patterns on the Balearic Islands Shelf (North-Western Mediterranean Sea)

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Introduction

To study reproductive biology it is essential to understand fish population dynamics. Sexual pattern is defined as the typical expression of sexuality exhibited by individuals of a population or species in a strictly functional sense of individual fish expressed within their lifetime (Sadovy and Shapiro 1987). Proper sexual pattern classification should be fundamental to the selection of fisheries conservation strategies since sex-changing species may be particularly prone to overexploitation by size-selective fishing (Molloy et al. 2007). In the case of hermaphroditic fish, histology appears as a crucial tool for proper identification since histological examination of specific gonadal features must be used to confirm the sexual pattern.

We examined in detail three species from the same ecosystem with different sexual patterns: *Coris julis*, *Serranus scriba* and *Diplodus annularis*. The purpose of this study was to delineate the seasonal cycle of sexual maturation, timing and rhythm of spawning, ovarian development organization and to establish the functional sexual pattern for each species using a histological approach.

Materials and Methods

The sampling method was based on experimental fishing sessions conducted in the Mallorca Bay and at the National Park of the Cabrera Archipelago located in the south coast of Mallorca Island, from December 2005 through August 2008. A total of 1221 *C. julis*, 802 *S. scriba* and 918 *D. annularis* were sampled and their total length (mm), gonad weight ($\pm$0.01g) and age were recorded for each individual. Gonadosomatic index (GSI) was estimated considering only mature fish. Central portions of the ovaries were fixed in 3.6% buffered formalin and then dehydrated, embedded in paraffin, sectioned and stained with haematoxylin-eosin following standard histological techniques. The follicles were classified into stages of development using histological criteria (Tyler and Sumpter 1996). Female and male gonadal development was based on Brown-Peterson et al. (2007). Estimation of length and age at sex change was conducted using Generalized Linear Models (binomial).

Results

Coris julis

Histological examination of the ovaries revealed that *C. julis* exhibits an asynchronous ovarian development organization (Figure 1A). Study of ultrastructure of the testes showed unrestricted spermatogonial testicular type and that spermatogenesis is cystic in this species (Figure 1B). The seasonal variability in mean GSI values and proportion of actively spawning

females indicated a peak of reproductive activity during May and June. Testes of males presented spermatozoa from March to August.

Intersexual individuals were clearly identified after histological examination. The first evidence of sex-change was the presence of degenerating ovarian tissue with the presence of many atretic follicles and spermatogonial nests spread within the gonad. The highly prevalence of eosinophils in intersexual individuals could indicate an association of these cells with the sexual transition process (Figure1C). Thus, *C. julis* is identified as a diandric protogynous hermaphrodite.

Figure 2A shows separate frequency distributions for females, primary males, secondary males and intersexual individuals. A bimodal frequency distribution by length and age is clear, with a smaller and younger mode for females. The estimated length and age at sex change is 132 mm TL and 4 years respectively.

Serranus scriba
In this species, the ovary and testis are present and mature at the same time in the same individual (Figure 1D). Asynchronous ovarian development organization was present and testes are characterized by cystic spermatogenesis and unrestricted spermatogonial testicular type. Based on the proportion of spawning capable fish and GSI values, the spawning season extends from late spring through mid summer. Spermatozoa were present in testicular tissue from March to August. There were no distinct modes in size or age frequency distributions (Figure 2B), and this species is classified as a simultaneous hermaphrodite.

Diplodus annularis
In individuals with clearly identified ovotestes, only one of the tissues (either testis or ovary) was functional, and no intersex individuals with degenerating tissue were found. Ovarian organization was asynchronous (Figure 1E) and the testes were of unrestricted spermatogonial testicular type with cystic spermatogenesis (Figure 1F). May and June showed the highest reproductive activity during the year. The size frequency distribution for both sexes was basically the same (Figure 2C). Based on size frequency distribution and histology, this species was classified as a rudimentary or non-functional hermaphrodite.

Discussion
Available reproductive biology information for *Coris julis* and *Serranus scriba* is poor and in the case of *Diplodus annularis* the sexual pattern classification is still controversial (Buxton and Garrat 1990). Managing fishing on stocks of sex-changing species requires considering the sex-change pattern (Molloy et al. 2007), thus, this study provides scientific evidence to improve the efficacy of all management tools. Histology is a crucial tool for proper identification since histological examination of specific gonadal features must be used to confirm sexual patterns (Sadovy and Shapiro 1987).

Histological examination of the ovaries revealed that all species posses asynchronous ovarian development and testicular tissue showed unrestricted spermatogonial testicular type and is characterized by cystic spermatogenesis. All three species presented a peak of spawning activity from late spring through early summer. The fact that spermatogenesis is completed prior to spawning guarantees reproductive success.

Determination of sexual pattern requires the application of several types of diagnostic criteria (Sadovy and Shapiro 1987). In *C. julis*, diandric protogynous hermaphroditism was clearly identified by the presence of intersexual individuals and bimodal size and age frequency distribution. *Serranus scriba* presented functional testicular and ovarian tissue at the same time, conclusive proof for a simultaneous hemaphoditism diagnosis. This species spawns almost daily which may increase the chances of self-fertilization. However, as in most simultaneous hermaphroditic species, it is likely that each partner alternates its sexual role and successively releases eggs or sperm in successive spawning sequences.

The presence of non-functional ovarian tissue in functional testes is not a clear feature diagnostic of sequential or simultaneous hermaphroditism (Sadovy de Mitcheson and Liu 2008); thus, *Diplodus annularis* was considering a rudimentary or nonfunctional hermaphrodite since no histological evidence of functional simultaneous hermaphroditism or sex inversion was found. Additionally, a similar population structure exists in both sexes by length and age.

In summary, the present study confirmed diandric protogynous and simultaneous hermaphroditism in *C. julis* and *S. scriba* respectively, and establishes rudimentary hermaphroditism for *D. annularis*. This study provides scientific evidence to improve the efficacy of all management tools and selection of fisheries conservation strategies.

A manuscript based on this presentation has been submitted to the journal Marine and Coastal Fisheries as part of the special module "Emerging Issues and Methodological Advances in Fisheries Reproductive Biology". Publication is anticipated in fall 2010. Web page: www.fisheries.org/mcf.

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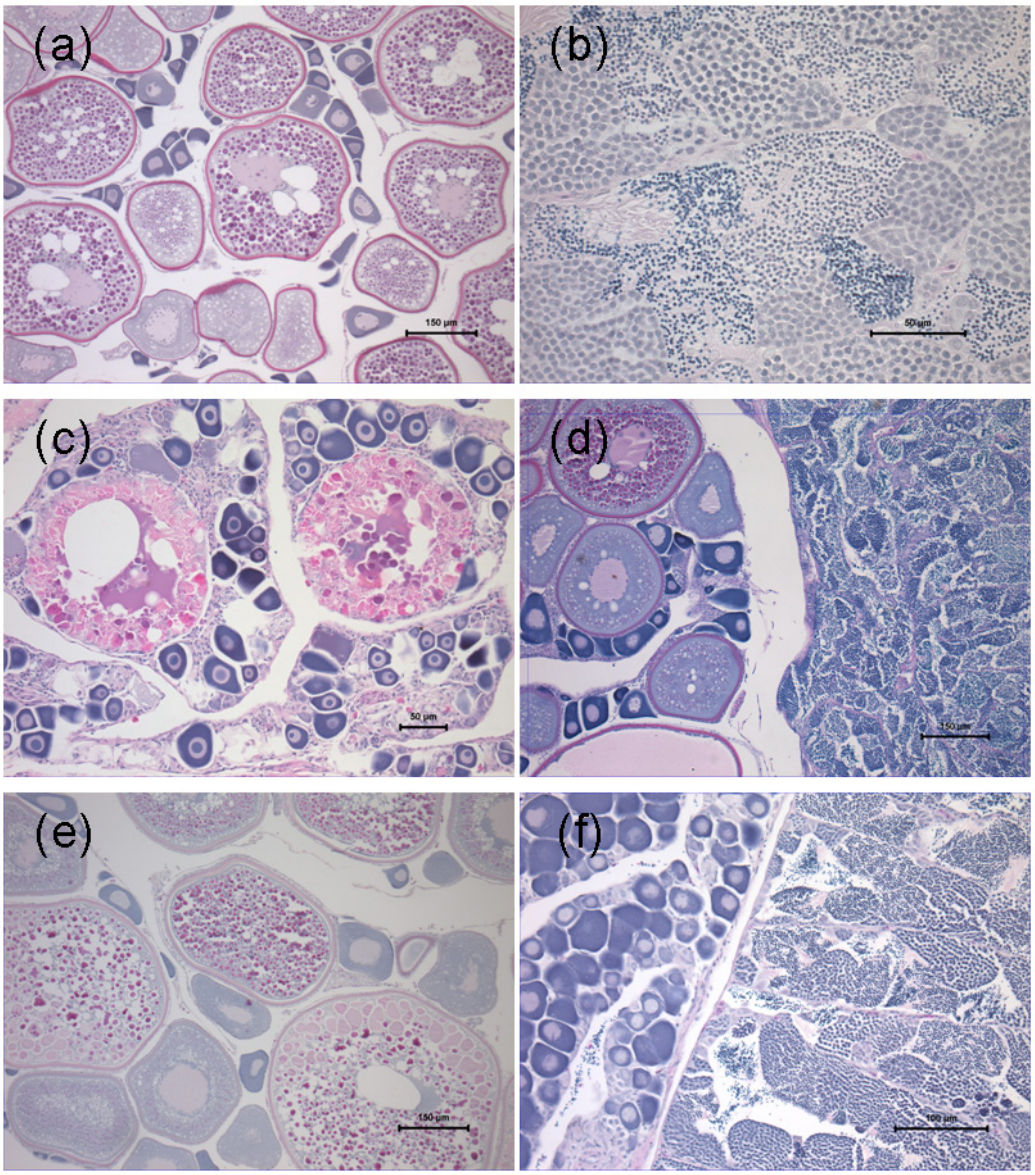

Figure 1. Transverse sections of gonads of fishes from the south coast of Mallorca Island. (a) Actively spawning female of *Coris julis* (b) Mature male of *Coris julis* (c) Intersexual specimen of *Coris julis* (d) Ovotestis of *Serranus scriba* (e) Actively spawning female of *Diplodus annularis* (f) Functional male of *Diplodus annularis*.

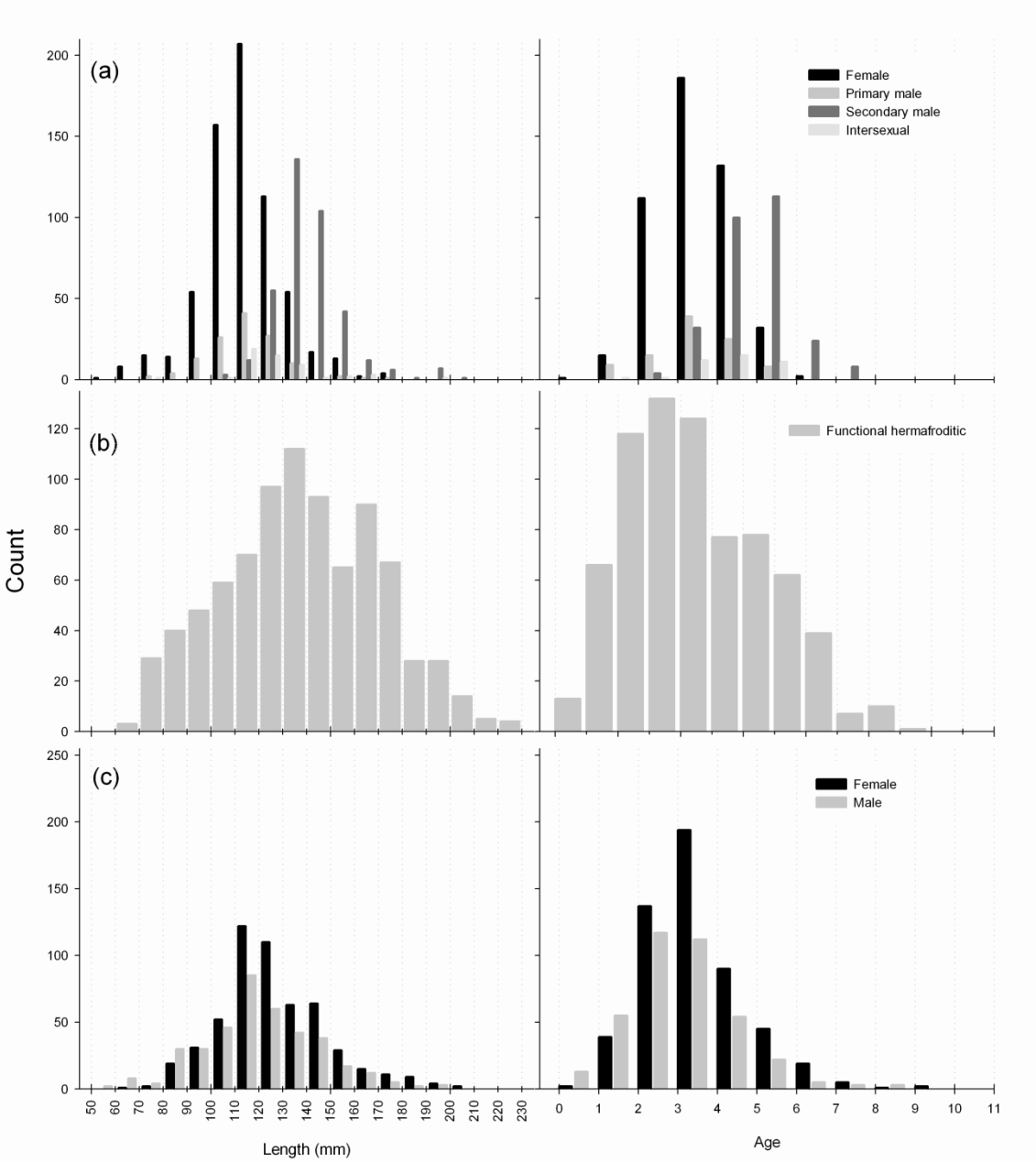

Figure 2. Size and age frequency distributions of *Coris julis* (a), *Serranus scriba* (b) and *Diplodus annularis* (c) from the south coast of Mallorca Island (NW Mediterranean).

Oviparous Elasmobranchs: How Different is their Reproductive Cycle from Teleosts – The Case-Study of the Thornback Ray, *Raja clavata*

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Introduction

Rays are oviparous elasmobranchs with internal fertilization. They produce eggs enclosed in hard egg capsules. The spawning period varies between geographical regions. For the species under study, *Raja clavata*, in Portugal, extrusion of the eggs (here referred as spawning) is continuous throughout the year. As a k-strategist, this species has: late maturation, around 80% of the maximum size (Walker 1999); slow growth (k = 0.117 year^{-1}; Serra-Pereira et al. 2008); large maximum sizes (females attain 91 cm and males 87 cm); and low fecundity (140 eggs/female/year; Holden 1975). Studies on the reproductive cycle of elasmobranchs are considerably fewer than those on teleosts. The reproductive cycle of teleost is commonly divided into six phases: immature, developing, spawning capable, spawning, regressing (ending of spawning season), and regenerating (preparation for the next season) (Brown-Peterson et al. 2007). In oviparous elasmobranchs the reproductive cycle is divided into ovarian stages: immature, maturing, mature; and uterine stages: active, advanced and extruding (Stehmann 2002).

The main objectives of this study were: i) to make a histological characterization of the macroscopic scale of thornback ray; ii) to propose a new scale using the Brown-Peterson et al. (2007) terminology; and iii) to summarize the main differences of the reproductive cycle of oviparous elasmobranch in relation to teleosts.

Materials and Methods

Samples were collected along the Portuguese continental coast (n = 135). The reproductive system of both females and males were removed and fixed in 10% buffered formalin. Paraffin embedding was used and the following staining techniques were applied on 3-5 µm sections: i) Hematoxylin and Eosin (H&E); ii) Toluidine blue; iii) Periodic Acid-Schiff (PAS), to identify neutral mucins; iv) combined Alcian blue and PAS (PAS/AB), to identify sulfated acid and neutral mucins; and v) Van Gieson, to identify collagen.

Results and Discussion

Female ovaries had an asynchronous development of the follicles, which suggests that *R. clavata* is a serial spawner (Figures 1A-B). In the immature phase and throughout the gonadal development, early follicles (this term includes oocytes and involving membranes) were connected to the germinal epithelium and to a thin layer of connective tissue, the tunica albuginea (Figure 1C). In the developing (formerly maturing) phase, follicles (diameter ~2 mm) entered into vitellogenesis, including formation of yolk platelets, pseudostratified

follicular epithelium and and increased peripheral vascularisation related to the transport of yolk precursors into the oocyte (Figure 1D). In this phase, the oviducal gland started to differentiate: gland tubules differentiated, beginning from the lumen and expanding to the entire gland. In the late developing phase, the oviducal gland was fully developed and four secretory zones were distinguished (Figure 1E). Ovulation occurred with ~3 cm follicles. The egg after fertilization was surrounded by a series of envelopes produced by the oviducal gland: i) sulphated acid and neutral mucin secreted by the club zone (hydrodynamic support); ii) second layer of jelly secreted by the papillary zone, composed by sulphated acid and neutral mucins; iii) third layer of jelly, a sulphated acid mucin secreted by the papillary zone (lubricant and bounding layer); iv) hard egg capsule, probably proteic, secreted by the baffle zone; and v) surface hairs (chemically similar to the capsule), coated with mucous secretions that cover the exterior of the capsule (sulphated acid mucins), produced by the terminal zone. In spawning capable (formerly mature) and spawning (formerly active/advanced/extruding) phase females, the gland tubules of the four zones were filled with secretory materials. The spawning phase included all the uterine stages, since no histological differences were observed between them. Also in spawning females the uterus had a great contribution to the capsule surface structure and chemistry (Hamlett 2005), by producing sulphated acid and neutral mucins (Figure 1F). The highly vascularised folds facilitate biochemical processes associated with capsule polymerization, including provision of oxygen and absorption of water (Hamlett 2005). No structures of the reproductive system were observed that can be assigned to a regenerating phase. Postovulatory follicles are present in the spawning capable females, that have developing oocytes and enlarged oviducal gland and uterus (Figure 1D).

Males had a compound testis with radial and diametric sperm development inside the lobes (Figures 2A-B). In each lobe spermatogenesis started in the germinal zone located in the center of the lobe and dorsally in the testis (Figure 3C). In immature males, only primordial germ cells, gonocytes, spermatogonia and spermatocytes were observed. After that, from developing to spawning, all spermatogenic stages, including spermatids and spermatozoa (Figure 2D) coexisted at the same time. The last stages were more abundant during the spawning phase. In the spawning phase, the sperm was transported to the claspers, through the epididimus (Figure 2E) and vas deferens (Figure 2F) to the seminal vesicles. The sperm was transferred to the female during copulation through claspers. Sperm bundles were observed beginning in the late developing phase deep inside the baffle gland tubules, near the muscle layer. Latter in the spawning phase, the sperm was observed isolated near the lumen. No specimens were collected that indicate the presence of regressing and regenerating phases. In fact, absence of males with fully developed claspers and “developing” testes reinforced the idea of continuous spawning capable/spawning phases in males after attaining maturity.

Comparing this oviparous elasmobranch to gonochoristic, oviparous teleosts, egg production undergoes a series of more complex processes than those involved in the egg production of teleosts. The elasmobranch reproductive cycle is not only restricted to the female and male gonads, but also to other organs, like the oviducal gland and uterus in females, and the sperm ducts and claspers in males. The egg is surrounded by a series of mucins and by a proteic capsule, production of which is triggered by a complex hormonal regulation (Hamlett 2005). Additional to the internal fertilization, these species are able to store sperm inside the oviducal gland of the females during several months. This complexity is translated into an extended reproductive cycle, to which is necessary to allocate a large quantity of energy, that is crucial to the success of fish reproduction.

A manuscript based on this presentation has been submitted to the journal Marine and Coastal Fisheries as part of the special module "Emerging Issues and Methodological Advances in Fisheries Reproductive Biology". Publication is anticipated in fall 2010. Web page: www.fisheries.org/mcf.

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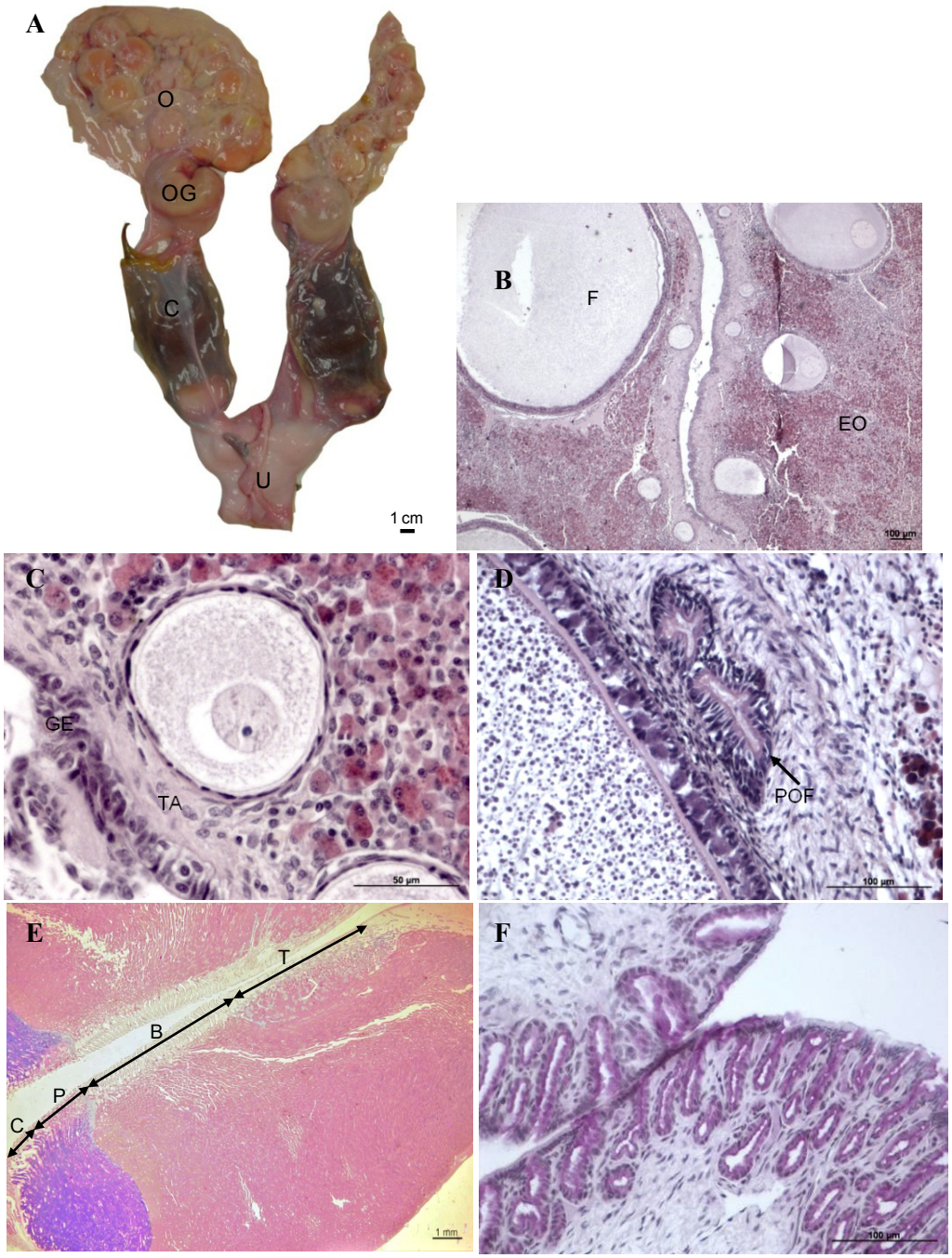

Figure 1. Female reproductive system. **A.** Reproductive system in the spawning phase; O: ovary, OG: oviducal gland, C: egg capsule, U: uterus (scale bar = 1 cm). **B.** Ovary: developing phase, showing follicles (F) in different stages of development, surrounded by the epigonal organ (EO) (H&E, scale bar = 100 µm). **C.** Ovary: primary follicle with 100 µm diameter, closely attached to the germinal epithelium (GE) and tunica albuginea (TA) (H&E, scale bar = 50 µm). **D.** Ovary: follicle in the spawning capable phase with 6000 µm diameter and a postovulatory follicle (POF) (H&E, scale bar = 100 µm). **E.** Oviducal gland: late developing phase with differentiated zones (C: club, P: papillary, B: baffle and T: terminal) (PAS/AB, scale bar= 1 mm). **F.** Uterus: spawning female with high levels of neutral mucins (pink) (PAS, scale bar= 100 µm).

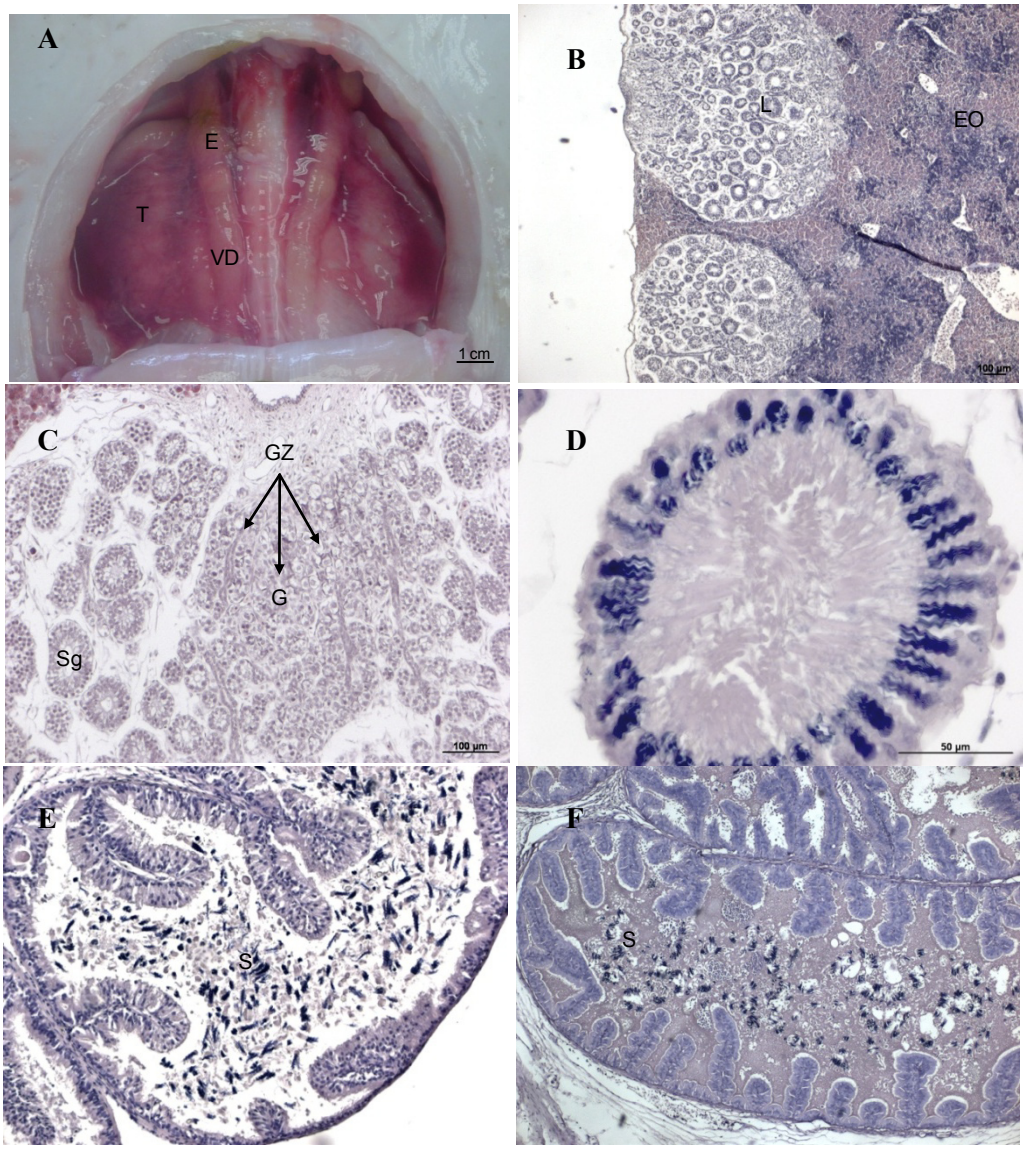

Figure 2. Male reproductive system. **A.** Reproductive system in the spawning phase (T: testis, E: epididimus, VD: vas deferens). **B.** Testis: immature phase with lobes (L) starting to differentiate, surrounded by epigonal organ (EO) (H&E, scale bar = 100 µm). **C.** Testis: lobe in the immature phase; proliferation of spermatocysts from the germinal zone (GZ) from gonocytes (G) into spermatogonia (Sg) (H&E, scale bar = 100 µm). **D.** Testis: spermatocyst in the spawning capable phase with visible sperm bundles arranged near the periphery (H&E, scale bar = 10 µm). **E.** Epididimus: in the spawning phase; sperm bundles (S) surrounded by a sparse seminal liquid (H&E, scale bar = 100 µm). **F.** Vas deferens: in the spawning phase; sperm bundles (S) surrounded by a dense seminal liquid (H&E, scale bar = 100 µm).

Ovarian Development and the Assessment of Maturity Stage in Greenland Halibut

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Introduction

The calculation of spawning stock biomass requires the correct identification of the maturity stage of the fish within this stock. The current method for identification of maturity stages in Greenland halibut is through macroscopic evaluation which is known to be inaccurate in other species (Gerritsen and McGrath 2006). This study involves the assessment of maturity stage of Greenland halibut (*Reinhardtius hippoglossoides*) caught in the Barents Sea between January and December 1997 by means of both macroscopic and histological methods. The ovary maturation cycle was examined with respect to the development stage and size of the oocytes. The current method and maturity scale used to assign a maturity stage to Greenland halibut was assessed to see if it was appropriate for the separation of mature and immature fish.

Methods

Ovaries from Greenland halibut were collected every month for one year during surveys in the Barents Sea. The ovaries were examined histologically in the laboratory. The macroscopic stage was assessed using the information from the histology; all the oocytes present on the slides were measured and staged according to the 8 stage scale of Federov (1968). The ovary was then given a histological stage (H) which corresponded with the group of the most advanced oocytes present in the ovary.

Results and Discussion

Ovaries were found to contain between one and 3 cohorts of oocytes; previtellogenic (PV), corticol alveoli (CA) and vitellogenic oocytes. Both the vitellogenic and CA oocytes increased in size as the ovary developed (Figure 1) and reached a maximum size at stage H7 and H6 respectively. The decrease in the size of the CA oocytes between stage H6 and H7 indicates that these oocytes were becoming vitellogenic. It therefore appears that the CA oocytes which were present in the ovaries alongside the vitellogenic oocytes were being developed for spawning the following year. At stage H3, the vitellogenic oocytes were smaller than 1 mm, with a large overlap in the size range of CA oocytes.

There were two distinct groups of fish in respect to oocyte development (Figure 2). One cohort contained vitellogenic oocytes which increased in size through the year, reaching a maximum in December/January, indicating spawning occurred at this time. The second cohort contained only PV and CA oocytes, and the latter did not increase in size until October. A previous analysis of Greenland halibut survey data taken between October 1997 and May 1998 using log transformed GSI values also showed two distinct groups of 'maturing' females, one with small oocyte size, another close to spawning (Albert et al. 2001). The GSI of the group with the small oocytes increased in May and it was concluded that this group consisted of first time spawners which would go on to develop their ovaries for spawning in

the next spawning season (Albert et al. 2001). The group seen in the present study with only CA oocytes probably corresponds to this 'maturing' group with smaller oocytes. This group was significantly smaller than the group with vitellogenic oocytes supporting the conclusion that these are fish which were developing their gonads for the first time. This two year development time is supported by Junquera et al. (2003), who found that there was more than one year between the mean age of the females that are at the onset of ovarian development and the mean age of the females that are actually spawning.

As the first-time development of the ovary took place over two years and CA oocytes are developed alongside vitellogenic oocytes, it appears that the development of oocytes from PV to the completion of vitellogenesis can take up to two years. This is probably due to the large size of the oocytes and the cold temperatures in the habitat of Greenland halibut.

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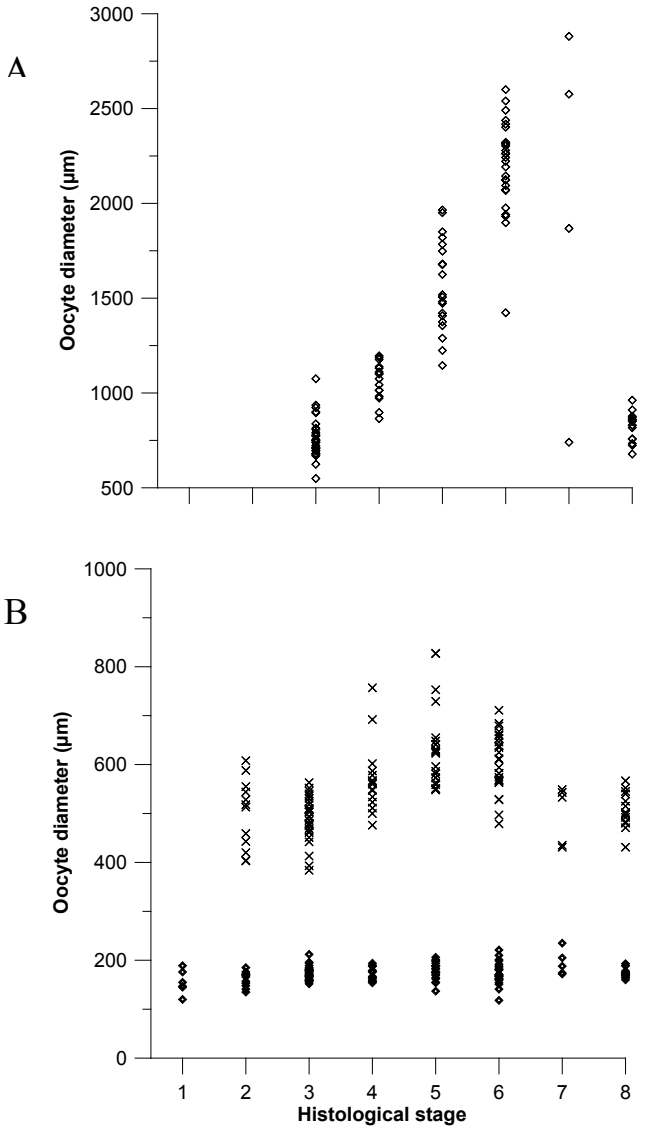

Figure 1. The average oocyte diameters for the 3 types of oocytes present in Greenland halibut ovaries at each histological stage. A. Vitellogenic oocytes. B. Previtellogenic and cortical alveoli oocytes. Key: previtellogenic oocytes (♦); cortical alveoli oocytes (x); vitellogenic oocytes (◊).

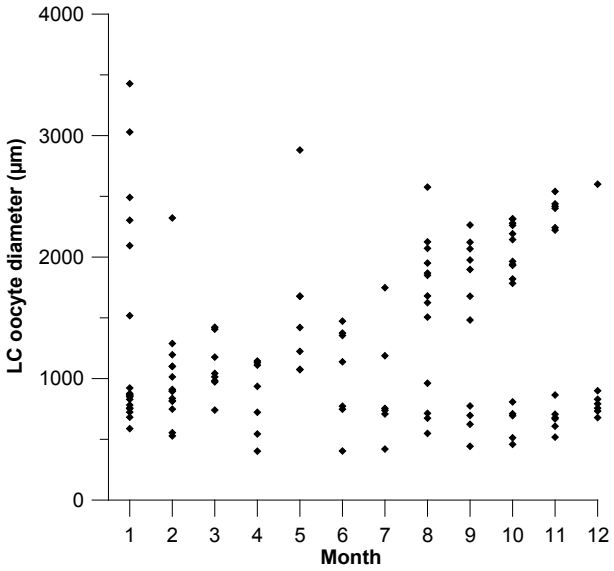

Figure 2. The Leading Cohort (LC) oocyte diameter of Greenland halibut caught in each month.

Macroscopical and Microscopical Investigation in Kattegat Cod (*Gadus morhua*)

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The cod *Gadus morhua* stock living in the Kattegat (Eastern North Sea, ICES Subdivision 21) has been estimated to be currently at its lowest level since 1971 and the biomass of reproducing fish (spawning stock biomass, SSB) has been reduced by 95%. The whole stock is compressed to a few age classes and the reproduction is mainly dependent on first spawners. Despite rigorous catch limitations, there are no signs of recovery and since the year 2000 this stock has been considered outside safe biological limits (ICES 2009).

Assessment and management of fish populations currently rely on estimations of SSB, which in turn is based on the proportion of mature fish within age classes in the population (i.e. maturity ogives). A proper identification of mature individuals in the population is thus a crucial step for a precise estimation of SSB, and ultimately for evaluating the status of the stock and establishing harvest levels. Although maturity ogives are routinely calculated through the macroscopic evaluation of the gonads, histology is generally considered to be more unambiguous and objective (Murua et al. 2003). In this study, the gonadal development of cod in the Kattegat was studied by investigating ovarian histological structure on a temporal scale. To obtain a good temporal resolution, fish sampling took place on a monthly basis, from September 2002 to May 2003 (with the exception of April 2003) and from December to February the following three years (2004, 2005 and 2006). Individual sexual maturity was at first classified according to a 4-stage macroscopic scale (ICES 1999). The individual maturity status was subsequently reassigned based on the characteristics observed histologically. The earliest signs of maturation were observed at the end of October, whereas the peak occurred in February-March when individuals in spawning stage were predominant.

Starting from existing maturity criteria, a modified system based on histological features was developed to emphasize crucial steps in the developmental process. The seven-stage scale produced in this study underlines the importance of the passage from endogenous to exogenous vitellogenesis, which coincides with the beginning of yolk production in the oocytes. Previous studies have identified the threshold between mature and immature fish in the endogenous vitellogenesis or even at an earlier stage of gonadal development. However, finding ovaries with oocytes in endogenous vitellogenesis does not necessarily imply that the fish will be reproductively active in the forthcoming spawning season (Burton et al. 1997). Hence, only fish containing oocytes which have developed up to the exogenous vitellogenesis phase and therefore containing yolk ought to be considered as sexually mature, i.e. they are likely to spawn in the forthcoming season. These two phases are hardly discernible by the naked eye and consequently the most susceptible to misclassification. Further sources of error were the misclassification of immature individuals and inactive individuals omitting spawning as regressing, and their consequent inclusion as mature in the calculation of SSB.

Errors in estimating maturity ogives will therefore lead to spurious SSB estimates, distorting the relationship between stock and recruitment (Murawski et al. 2001), and thereby increasing the variability of assessment results. This is especially problematic at low levels of SSB, because the precision in assessment projections is usually reduced at low stock levels.
In the present study, comparisons between histological and routinely used macroscopic (visual) maturity assessments revealed consistent discrepancies. The outcomes show that the visual analysis consistently overestimates the proportion of mature females in all age classes (Figure 1).

A recalculation of the historical female spawning biomass (FSSB) for the period 1991-2007, applying the bias obtained from the comparison between the two staging methods, showed a consistent overestimation of the proportion of mature females. The re-estimated FSSB was always lower than the historical FSSB, revealing an overestimation ranging from 12 to 20% (Figure 2). The overestimation is more severe for first-time spawners, due to a decreasing error with increasing age (Figure 1). It is therefore obvious that the risk is amplified in stocks such as Kattegat cod, where the spawning biomass is skewed towards younger and smaller individuals, likely due to overfishing. According to present results, the female spawning biomass of Kattegat cod may have been overestimated by up to 20% for more than 20 years. As a consequence the overestimation of the stock reproductive potential may have led to the implementation of regulating measures far above the stock capacity, masking the need for more drastic catch control. This may partially explain the lack of recovery despite catch restrictions.

On the whole, histology allows an unambiguous interpretation of the maturity status and ought to be used to enhance maturity determinations, thereby avoiding overestimation of the stock reproductive capacity. The use of more accurate methods for estimating individual maturity will complement and reinforce the routinely used methodology during research surveys. Improving the methods for correct and objective estimates of SSB is an important step for a sustainable fishery and recovery of overexploited fish stocks.

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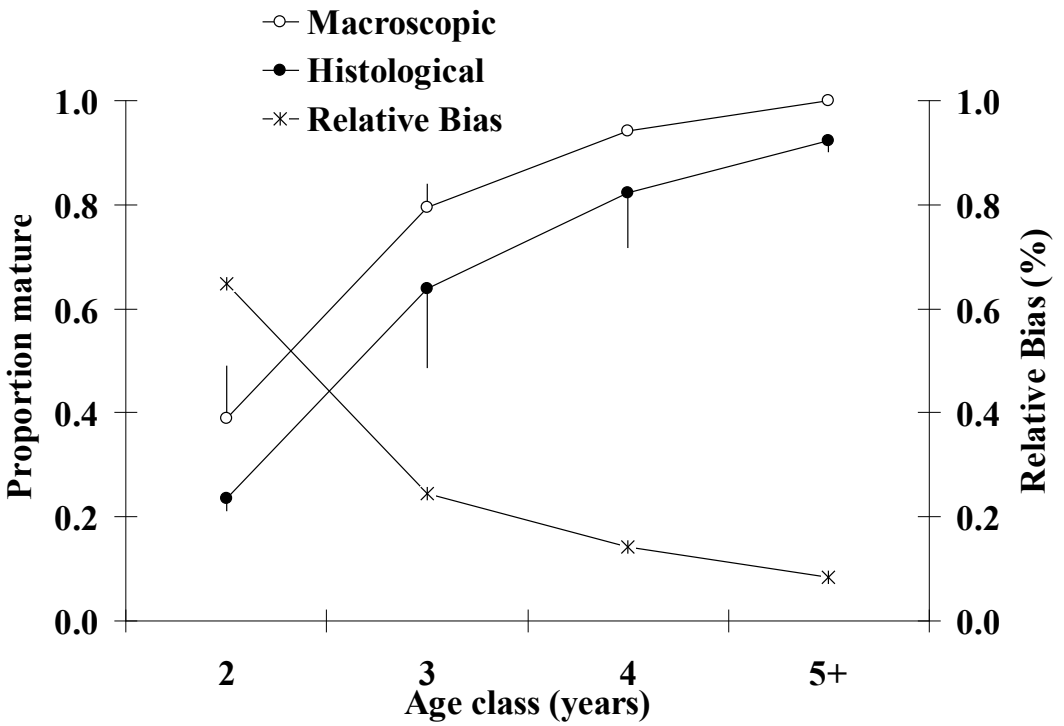

Figure 1. Comparison between histological and macroscopic staging of the gonads. The Relative Bias (%) is calculated per age class. Bars represent standard errors.

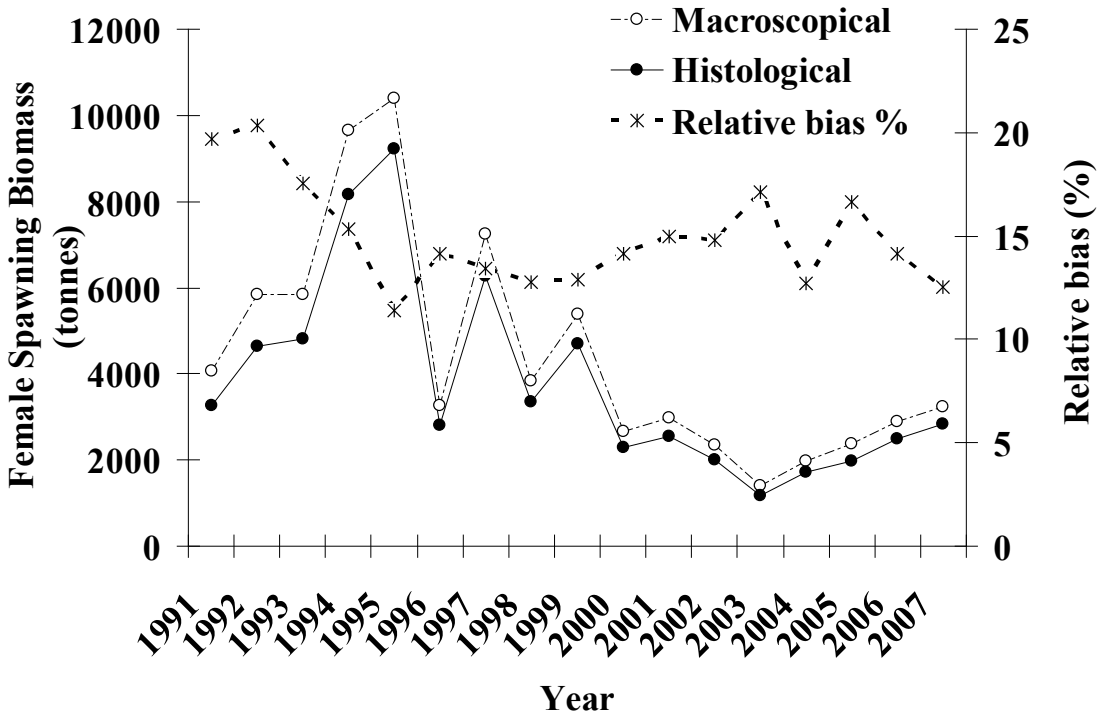

Figure 2. Comparison between the Relative Bias (%) between the re-estimated (through histological analysis) and the historical values of female spawning biomass.

Ovarian Structure and Oogenesis in Viviparous Teleosts: Poeciliids and Goodeids

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Introduction

Viviparity in fishes is an essential process to understand viviparity in vertebrates since fishes are the first viviparous vertebrates and they manifest the maximum diversity in the degree of maternal-embryonic relationships (Wourms and Lombardi 1992). All teleosts lack Müllerian ducts from which oviducts are formed in other vertebrates. Therefore, in teleosts, the communication between the ovarian lumen and the exterior occurs in the caudal region of the ovary, by the gonoduct, which should not be confused with oviducts. The ovaries of viviparous teleosts are modified for intraovarian gestation that is unique among the vertebrates (Wourms et al. 1988). Intraovarian gestation in teleosts involves morphological and physiological characteristics in the ovary that change as embryos develop during gestation. Considering that viviparity in teleosts occurs as intraovarian gestation, the ovary of viviparous teleosts is the site, not only for oogenesis, but also for insemination, internal fertilization, gestation and parturition (Wourms et al. 1988; Uribe et al. 2009).

Results and Discussion

Viviparous poeciliids and goodeids have a single ovary of the cystovarian type. The ovarian wall surrounds a central lumen or ovocoel (Figures 1A-C) (Uribe et al. 2005, 2009). In goodeids, the ovocoel is completely or partially divided by a septum (Figures 1A, B) which is an extension of the ovarian wall; this does not occur in poeciliids. The ovarian wall of poeciliids and goodeids contains germ cells and somatic tissues. The germ cells are oogonia and oocytes in different stages of development (Figures 1A-C). The somatic tissues are the mucosa (formed by somatic cells of the germinal epithelium), stroma and the peritoneum at the periphery of the ovary. The ovarian mucosa forms folds, the lamellae that extend from the periphery of the ovary into the ovarian lumen (Figure 1A). The surface of each lamella in viviparous fishes is lined by the germinal epithelium (Grier et al. 2005) in which oogonia and early stages of oocyte development are observed (Figure 2A). Germ cells frequently occur in cell nests during folliculogenesis (Figure 2A). A basement membrane supports the germinal epithelium and is continuous around cell nests which are connected to the germinal epithelium. The basement membrane separates the epithelium from the subjacent stroma (Figure 2A) (Grier et al. 2005, 2009).

Oogenesis is the process that includes a sequence of stages through which germ cells undergo morphological and physiological changes prior to becoming a fertilizable egg. These stages are initiated by oogonia and end with ovulation in oviparous teleosts. However, in viviparous poeciliids and goodeids fertilization is intrafollicular, and ovulation does not occur (Uribe et al. 2009). As with other teleosts, oogenesis in poeciliids and goodeids is divided, according to Grier et al. (2009), into 5 stages: oogonial proliferation (OP), chromatin-nucleolus (CN), primary growth (previtellogenesis; PG), secondary growth (vitellogenesis; SG) and oocyte maturation (OM). During OP the germ cell population is renewed in the ovary. Oogonia

initiate meiosis, becoming primary oocytes during CN (Figure 2A) and advance until early diplotene when lampbrush chromosomes and a single, distinct nucleolus form. Early diplotene oocytes advance to PG when their ooplasm becomes basophilic (Figures 2A, B). During CN and early PG, oocytes are progressively encompassed by a single layer of prefollicle cells, derived from the germinal epithelium. These cells become follicle cells at the completion of folliculogenesis when the oocyte and follicle cells are encompassed by a basement membrane. During PG, the size of the oocyte increases. The nucleus, now called germinal vesicle, contains several nucleoli which progressively become situated at the periphery of the germinal vesicle (Figure 2B). The ooplasm becomes basophilic due to the accumulation of, primarily, ribosomes. Cell organelles begin to appear during PG including mitochondria, endoplasmic reticulum and multiple Golgi apparatus. Oil droplets and cortical alveoli gradually form in the ooplasm (Figure 2B).

During SG, vitellogenin, a hepatic yolk precursor, is internalized by the oocyte and processed into yolk globules which continuously coalesce to form fluid yolk (Figures 2C, D). This is a character of all Atherinomorpha, a taxon to which poeciliids and goodeids belong. In contrast, yolk globules persist during SG in perciform fishes and only become fluid during OM. At the end of SG in poeciliids and goodeids, vitellogenesis is completed. The oocyte is full-grown. During OM the germinal vesicle migrates to the animal pole, breaks down and the first meiotic division is completed. Meiosis is arrested in metaphase of the second meiotic division in the later portion of the OM stage. Then, the oocyte can be fertilized within the follicle. It is questionable if hydration of fluid yolk also occurs during OM in poeciliids and goodeids. In poeciliids, embryos emerge into the ovarian lumen from the follicle. Birth shortly ensues. In goodeids, embryos emerge into the lumen during early embryogenesis (blastodisk or neurula) where they continue their development until birth (Figure 1B). Embryonic nutrition during intraovarian gestation includes maternal-embryonic metabolic interchanges in addition to yolk stored in the egg during oogenesis. Intraovarian gestation involves two types of embryonic nutritional patterns, lecithotrophy and matrotrophy. Lecithotrophy occurs when embryonic nutrition is dependent upon energy reserves laid down in the oocyte during oogenesis. Matrotrophy is more complex and depends on maternal provisions that are transferred to the developing embryo after fertilization when the yolk stored in the oocyte during oogenesis is spent (Figure 1B). Most poeciliids are lecithotrophic with an example of a matrotrophic species being *Heterandria formosa* (Figure 1C) which has the smallest egg (mean diameter of 0.4 mm) among the poeciliids. The goodeids are matrotrophic, having reduced vitellogenesis during SG, but embryos develop trophotaenia for maternal/fetal nutrient exchange.

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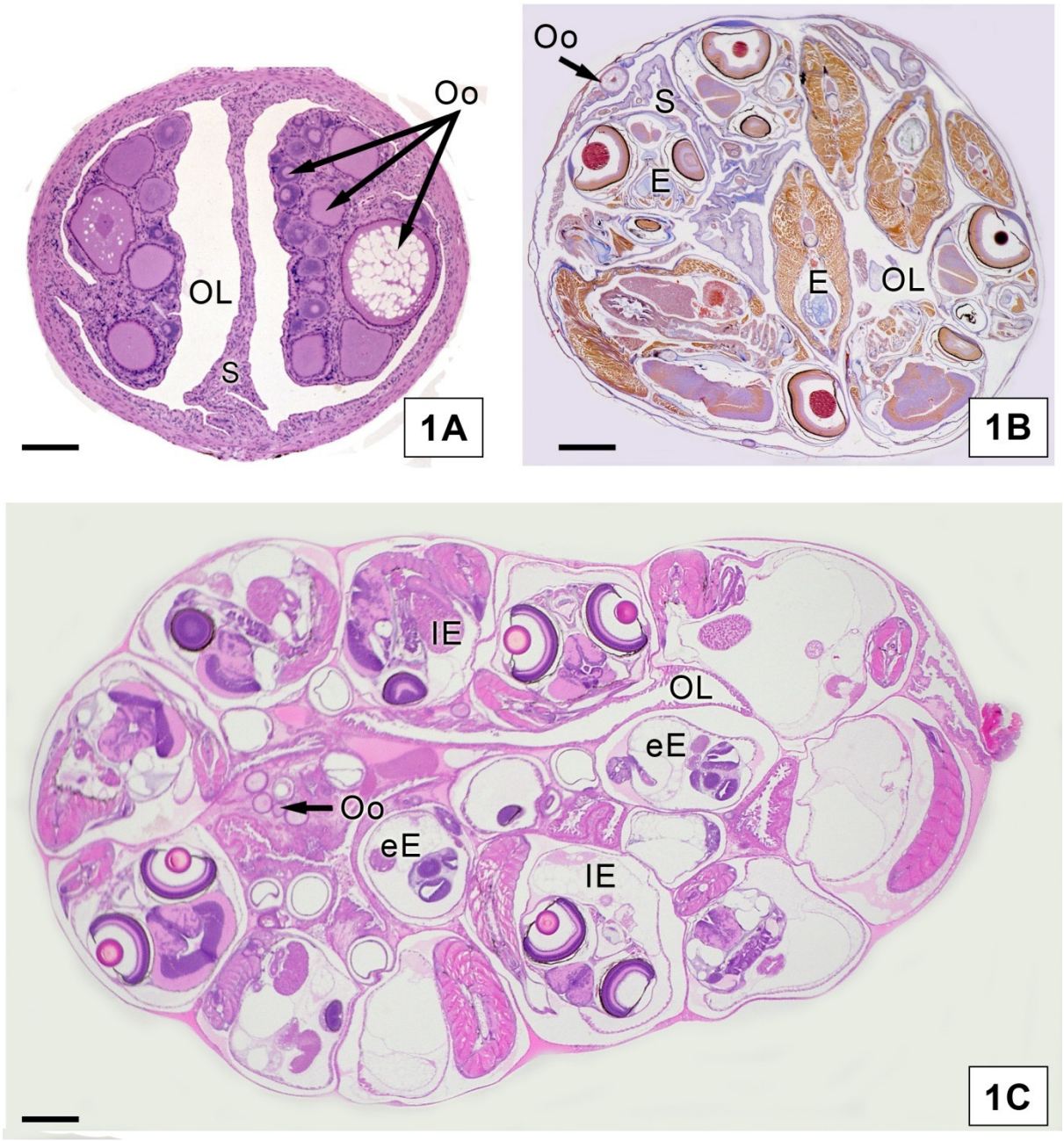

Figure 1. Panoramic views of ovaries in non-gestation and gestation. A. Ovary of the goodeid *Ilyodon whitei* in non-gestation. The germinal tissue containing oocytes (Oo) in different stages of development is observed in two lateral folds, one along each side of the ovary. The septum (S) divides the ovarian lumen (OL). Bar = 50µm. B. Ovary of the goodeid *Skiffia multipunctata* during gestation. Oocytes (Oo) in a fold are seen at the periphery of the ovary. Several developing embryos (E) are in the ovarian lumen (OL). The septum (S) is folded. Bar = 100µm. C. Ovary of the poeciliid *Heterandria formosa* during gestation in intrafollicular position. Oocytes (Oo) are observed. Superfetation is evidenced by several embryos in different stages of development, early embryos (eE) and late embryos (lE). The ovarian lumen (OL) lacks septum. Bar = 100µm.

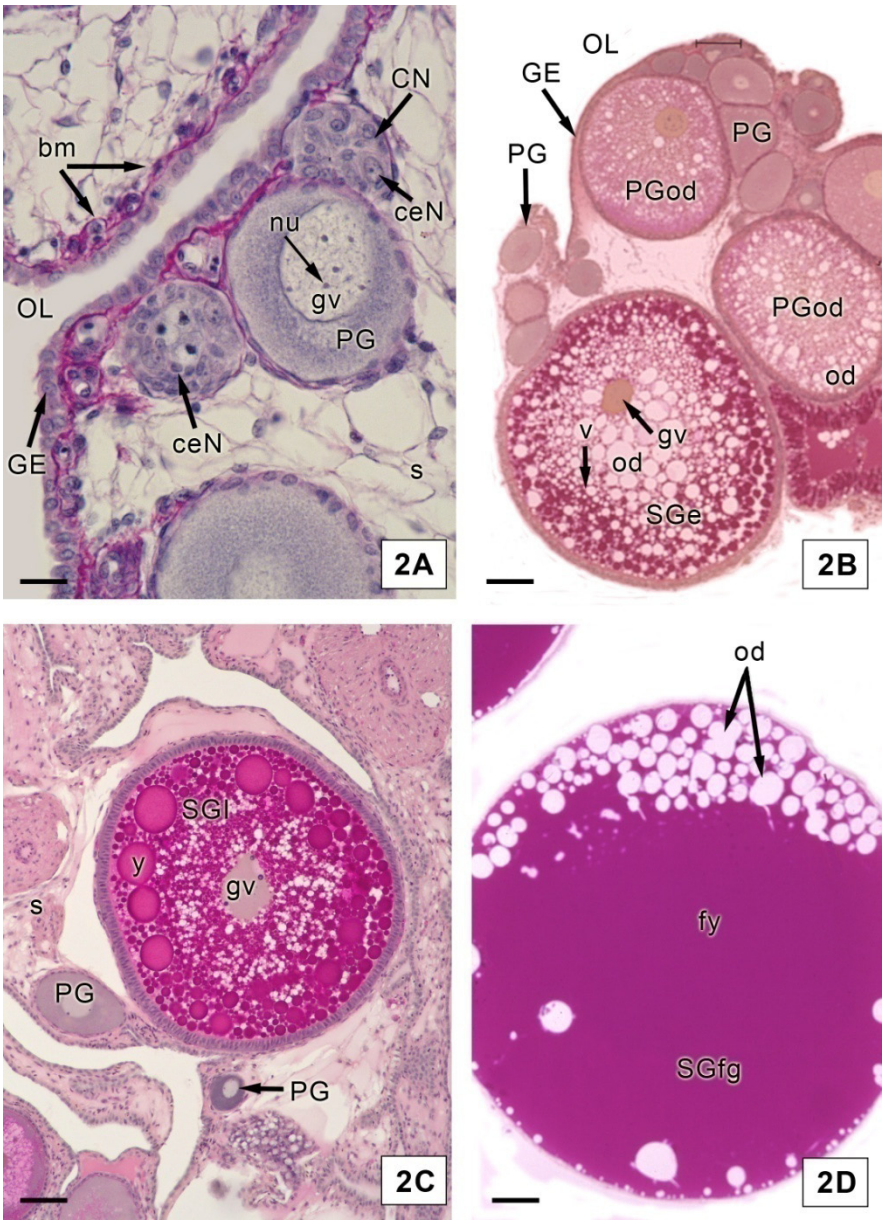

Figure 2. Oocytes in different stages of oogenesis. A. Ovary of the goodeid *Ilyodon whitei*. The germinal epithelium (GE) lines the ovarian lumen (OL). A periodic acid Schiff-positive basement membrane (bm) is subjacent the germinal epithelia of two lamellae. Cell nests (ceN) contain oocytes in the chromatin nucleolus (CN) stage. The oocyte in the primary growth (PG) stage has multiple nucleoli (nu) in the germinal vesicle (gv) and the ooplasm is basophilic. Bar = 10µm. B. Ovary of the poeciliid *Poecilia latipinna*. Early in primary growth (PG), oocytes have basophilic ooplasm. Later, oocytes in primary growth (PGod) are larger and contain numerous oil droplets (od). The initial deposition of periodic acid Schiff-positive yolk (v) is seen in oocytes during early secondary growth (SGe). Bar = 20µm. C. Ovary of the goodeid *Xenotoca eisenni*. Small yolk gloubules coalesce and increase in size, forming larger yolk gobules (y) during late secondary growth (SGl). Primary growth oocytes (PG) are also seen for growth comparisons. Bar = 30µm. D. Ovary of the poeciliid *Poecilia latipinna*. In a full-grown oocyte (SGfg) the fluid yolk (fy) has become a single mass and yolk globules are no longer present. Oil droplets (od) are primarily at the periphery of the oocyte. (gv) germinal vesicle; (s) stroma. Bar = 50µm.

Characterisation of the Reproductive Trait of Female Birdbeak Dogfish, *Deania calcea* (Lowe, 1839), in Portuguese Continental Slope

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Elasmobranchs species show wide reproductive strategies, including oviparity, aplacental viviparity and placental viviparity. Most viviparous species may have either aplacental yolk sac or aplacental development with uterine villi or throphonemata. In aplacental yolk sac species, the embryos derive their nutrition solely from yolk reserves while, in aplacental development with uterine villi or throphonemata species, the embryos supplement the yolk reserves by obtaining maternally derived nutrients during gestation.

The reproductive strategy of birdbeak dogfish was investigated based on structural specialisations of the ovary, shell gland and uterus during 4 phases of the sexual cycle: immature, developing, expecting and spent. The reproductive tract was preserved in 10% buffered formaldehyde and slices were taken from each structure, dehydrated with ethanol and embedded in resin. Two 3 µ sections were cut from each slice and stained with toluidine blue.

Ovary follicles development is more related to its size than to the maturity stage. The follicle appears completely formed at 400 µ and until its maturity size (5.4 mm), most differences are related to the different width of the granulosa and basement and vitelline membranes.
The shell gland, inexistent in the immature phase, showed an increase of the number of the tubules, which are completely full of secretion products, at the end of the developing stage. In the expecting and spent phases the tubules are empty and in decreasing number. The uterus was investigated based on the type of epithelium, the development of the muscle layers and the form and size of the uterine ville, which attain the larger development in the expecting phase.

The study of the reproductive trait is fundamental to characterise the type of viviparity present in this species

Histological Evaluation of Gametogenesis in Blackspot Sea Bream, *Pagellus bogaraveo*.

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Introduction

Blackspot sea bream, *Pagellus bogaraveo*, a marine protandrous hermaphrodite in the family Sparidae, is a species with a high market value. In the last two decades this species has been subject to intense over-fishing and nowadays is considered a particularly interesting species in commercial aquaculture. A good understanding of the reproductive biology is of paramount importance for management of fisheries, conservation of the species and to develop reproduction techniques in captivity.

Culture and ongrowing of *P. bogaraveo* in captivity was reported in previous studies (Olmedo et al. 2000; Peleteiro et al. 2000; Micale et al. 2002; Olmedo et al. 2002; Bruzón et al. 2006). However, relatively little is known about its gametogenesis, which is a biological event of the greatest possible significance and interest. In the present study, histological examination of gonads, is used to present the main characteristics of oogenesis and spermatogenesis to provide an understanding of their reproductive biology.

Methods

Male and female blackspot sea bream were dissected in different stages of their reproductive cycle and the gonads were removed. The mid-portion of the lobe of the gonad was fixed in formaldehyde (pH 7.2). Tissue samples of gonads were dehydrated in increasing concentrations of ethanols, infiltrated and embedded with paraffin wax. Sections were cut at 3 µm and stained with haematoxylin/eosin and haematoxylin/VOF (Gutiérrez 1967) for histological observation under light microscopy to determine the stages of gametogenesis according to the presence of the most developed type of germ cell in the sections. The diameter of 100 randomly chose oocytes were measured in slides from each individual by an image analyser system.

Results

The specimens of blackspot sea bream were separated as: females (with ovaries or ovotestes with functional ovary), males (with ovotestes with functional testes), hermaphrodites (with ovotestes at the same maturity stage) and immature (with gonads not identifiable). The gonads were composed of 2 unequal lobes. They were located in the posterior portion of the abdominal cavity and suspended by mesenteries.

Gametogenesis is a continuous process, but germ cells development could be divided into 6 stages in females and 5 stages in males. In the blackspot sea bream, the oocyte development was synchronous. The earliest stage in oogenesis was represented by oogonia (stage I). They were spherical and the smallest in size cells. Oogonia had a large nucleus and clear cytoplasm. Oogonia developed into previtellogenic oocytes (43-180 µm, stage II; Figure 1A). These oocytes had a homogeneous and strongly basophilic cytoplasm and a large nucleus with a small number of nucleoli. By the end of this stage the oocyte increased in size and the

cytoplasm appeared less basophilic. Numerous nucleoli and lampbrush chromosomes were present in the nucleus. Additoinally, each oocyte was surrounded by a layer of follicle cells.

As oocytes began vitellogenesis, early vitellogenic oocytes (140-390 µm, stage III) were characterised by the accumulation of cortical alveoli in the inner cytoplasm and the appearance of clear lipid vacuoles close to the nucleus. The cytoplasm was granular and lightly basophilic. The zona radiata became visible for the first time. During the second part of vitellogenesis, late vitellogenic oocytes (270-640 µm, stage IV; Figure 1B) were characterised by the appearance of small yolk granules scattered in the cytoplasm. They were clearly distinguishable from the cortical alveoli for their very dense and intensely stained content. The zona radiata was thickened and appeared bipartite and striated. The oocyte size increased considerably and lipid vacuoles around the nucleus began to fuse. Yolk granules increase in number and size until the ooplasm was completely filled by acidophilic yolk granules. Cortical alveoli were located in the peripheral region of the ooplasm.

At the attainment of the maximum size, the oocytes entered the maturation stage. Large lipid vacuoles and yolk granules coalesced in the post-vitellogenic oocytes (540-720 µm, stage V). In this stage occurred the peripheral migration of the nucleus and dissolution of its membrane occurred. These oocytes turned into hydrated oocytes (710-950 µm) by a rapid increase in the size due to hydration of oocytes. Finally, evidence of spawning activity was revealed by the presence of post-ovulatory follicles (stage VI; Figure 1C). The remaining post-vitellogenic oocytes, which failed to ovulation, degenerated and underwent atresia. However, atretic follicles could be observed in any stage of development.

Pagellus bogaraveo males displayed an asynchronous pattern of spermatogenesis, where all germ cell stages were present at the same time. Within the lobules spermatogenesis occurred in cysts and germ cell development occurred synchronously in each cyst. Spermatogenesis was divided into five stages according to the most advanced type of germ cell present in the sections. Lobules of the testes classified in stage I (immature) contained spermatogonia and small cysts of spermatocytes (Figure 1D). Spermatogonia were the largest cells and they were round cells with a relative large nucleus. Spermatocytes decreased in size due to meiosis. In stage II (developing) the testes increased in size compared with the previous stage. Spermatogonia, spermatocytes and spermatids were present, though spermatozoa sometimes were present with their heads oriented towards the cyst walls. When the testes were mature (stage III), germ cells at all stages of spermatogenesis were present and there was an increase in the number of spermatocytes and spermatids (Figure 1E). Likewise, the spermatozoa appeared free in the lumen of the lobule. As spermatogenesis proceeded, the spermatozoa were more abundant in the lumen and in the deferens duct. Cysts of spermatogonia were observed in the testicular periphery, and successive stages of spermatogenesis occurred in the lobules, which extended from the periphery to the central region of the gonad where they opened in a network of deferent ducts. At this time the males were spawning (stage IV; Figure 1F). Finally, spent males (stage V) were characterised by testes with large empty lobular spaces, except for some residual sperm.

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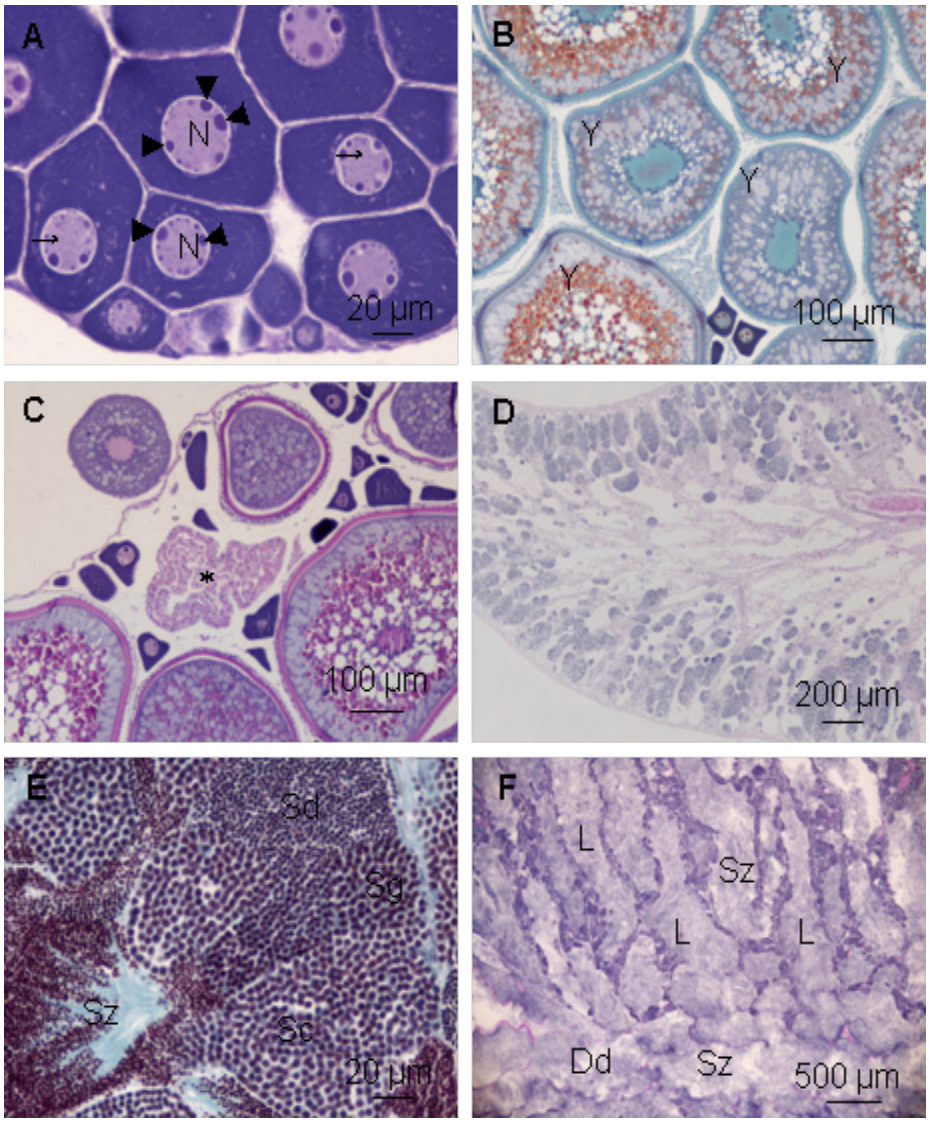

Figure 1. (A-C) Histological sections showing different oocyte developmental stages in a female gonad of *P. bogaraveo*. (A) Previtellogenic stage oocytes. The oocyte nuclei (N) contain multiple nucleoli (▶) and lampbrush chromosomes (→). H & E. (B) Vitellogenic oocytes at different development stages. Yolk granules (Y) were increased in number. H & VOF. (C) Post-ovulatory follicle (∗). H & E. (D-F) Photomicrographs of sections of *P. bogaraveo* testis at different stages of germ cell development. (D) Immature stage. Cysts of spermatogonia and spermatocytes in the periphery of the testis. H & E. (E) Mature stage. Major density of spermatocytes and spermatids, and spermatozoa adhered to the lobular walls. H & VOF. (F) Spawning stage. Transverse section of the deferent duct with spermatozoa. H & E.

Oogenesis and Spermatogenesis in the Common Sole *Solea vulgaris* (Quensel, 1806) in the Gulf of Cádiz (SW Spain)

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The common sole *Solea vulgaris* (Quensel, 1806) is a species of high economic interest in Spain, where it is exploited by different fisheries. We have studied oogenesis and spermatogenesis of *S. vulgaris* in the Gulf of Cádiz (SW Spain) by means of histological techniques.

A total of 176 males and 184 females of *S. vulgaris* were collected during monthly sampling in the fish markets of Sanlúcar de Barrameda and El Puerto de Santa María (both in Cádiz) from November 2003 to September 2004. Gonads were fixed in 10% buffered formalin, processed, embedded in paraffin, and sectioned (3 µm thick) with a microtome. Sections were then stained with Haematoxylin/Eosin and Haematoxylin/VOF (Light Green, Orange G and Acid Fuchsin) (Gutiérrez, 1967).

According to Yamuza et al. (2007) and Weltzein et al. (2002), five gonad development classes were microscopically defined for both ovaries and testis: I Immature, II Maturing, III Mature, IV Spawning, V Post-spawning. Oogenesis and spermatogenesis have been morphologically differentiated into five stages. Oogenesis: I Pre-vitellogenesis, II Early vitellogenesis, III Late vitellogenesis, IV Ripening, V Post-spawning. Spermatogenesis: I Resting, II Development, III Mature, IV Spawning, V Post-spawning.

Oocyte development stages were defined in connection with vitellogenesis, according to changes observed in the ooplasmic inclusions, the nucleus morphology, and the follicular cells. Spermatogenesis stages were defined according to the presence and relative frequency of the different germ cells: spermatogonia, spermatocytes, spermatids, and spermatozoa.

The ovaries were in the mature class from December to March, and females spawned mainly in April. Post-spawning occurred from May to June. Ovaries showed asynchrony, as they contained oocytes at different classes of development all year long. Post-spawning percentages were highest from May to July, when non-spawned oocytes were resorbed (atresia).

In oogenesis (Figure 1), pre-vitellogenesis was the prevailing stage followed by early vitellogenesis. Oocytes in both stages were found in almost every month. Pre-vitellogenesis (Figure 1A) included oocytes in Central Nucleolus Stage (CNS) and in Perinuclear Nucleoli Stage (PNS); isolated oogonia could also be detected. Oocytes in CNS had a small nucleus, rather oval in shape, a unique central nucleolus, and a thin nuclear membrane. Oocytes in PNS had a larger spherical nucleus, a larger ooplasm, and various nucleoli arranged peripherally in the nucleus. During pre-vitellogenesis the ooplasm was clearly basophilic, and mean oocyte diameter was 64 ± 21µm. In early vitellogenesis (Figure 1B), the ooplasm had increased in size and was slightly basophilic. In this stage, we first observed the Yolk

Inclusions (YI), and the Lipid Vesicles (LV), as well as the Zona Radiata (ZR). Oocyte nuclei were elliptical-spherical, multiple nucleoli were observed peripherally in the nucleus, and mean oocyte diameter was 206 ± 47µm. In late vitellogenesis (Figure 1C), Yolk Granules (YG) were first observed, these were rather round in shape, and displaced the YI to the periphery. The Theca (Th), and the Granular Layer (GL) were also observed, indicating development of the Follicular Layer (FL). The ZR was so highly developed and thickened that striae were recognisable, as well as the inner and the outer layers. Oocyte nuclei (sometimes amoebic-shaped) migrated to the animal pole, and their membrane underwent breakage. There were multiple peripheral nucleoli, the ooplasm was eosinophilic, and mean oocyte diameter was 295 ± 41µm. In ripening (Figure 1D), oocytes had considerably increased their volume, and gradually became Hydrated Oocytes (HO). The LV had increased both in size and number, the YG occupied most of the ooplasm, and the YI were arranged around the oocyte membrane. Oocyte nuclei were of irregular shape, and mean oocyte diameter was 540 ± 62 µm. Post-Ovulatory Follicles (POFs) were sometimes observed, indicating that spawning had taken place. In Post-spawning (Figure 1E), the ovary was flaccid, POFs were still present, oocytes underwent atresia, and Pre-Vitellogenic Oocytes (PVO) could be observed in the ovary again.

Spawning class males were found from December to March. Immature and post-spawning males occurred during almost every month. Maturing males were only noted in January (in a low percentage), whereas mature males were not recorded at all.

In spermatogenesis (Figure 2), resting and post-spawning were the prevailing stages. In resting (Figure 2A), the Seminiferous Tubules (ST) were forming, and Spermatogonia (Sg) and Primary Spermatocytes (PS) were already present. In development, the ST were completely formed, and Sg, PS, Secondary Spermatocytes (SS), and Spermatozoa (Sz) were already present, indicating that spermiogenesis had begun. In spawning (Figure 2B), the Sz filled the ST and the Ductus Deferens (DF), and the Stroma (St) gradually becomes more vascularized. In post-spawning (Figure 2C), we observed empty Lumina (L), some residual Sz, and Sg.

The stages of oocyte development described here correspond to those reported by Ramos (1983), although this author divides pre-vitellogenesis into three different stages, and Jiménez-Tenorio et al. (2009). According to Ramos (1983), oocyte development can be divided into two growth phases during oogenesis. The first is a phase of primary growth, and corresponds to oocytes in pre-vitellogenesis. The latter is a phase of secondary growth which includes all stages of vitellogenesis.

Because maturing males were only noted in January and mature males were not noted at all, this suggests that development and maturation of spermatozoids occurs rapidly, just preceding spawning, as has been already described for other Teleostei (Jiménez-Tenorio et al., 2008). The high incidence of post-spawning males could indicate a long-lasting post-spawning stage or, most probably, the existence of a spawning period longer than that observed, which fits better with most male reproductive strategies within the Teleostei (Jiménez-Tenorio et al., 2008).

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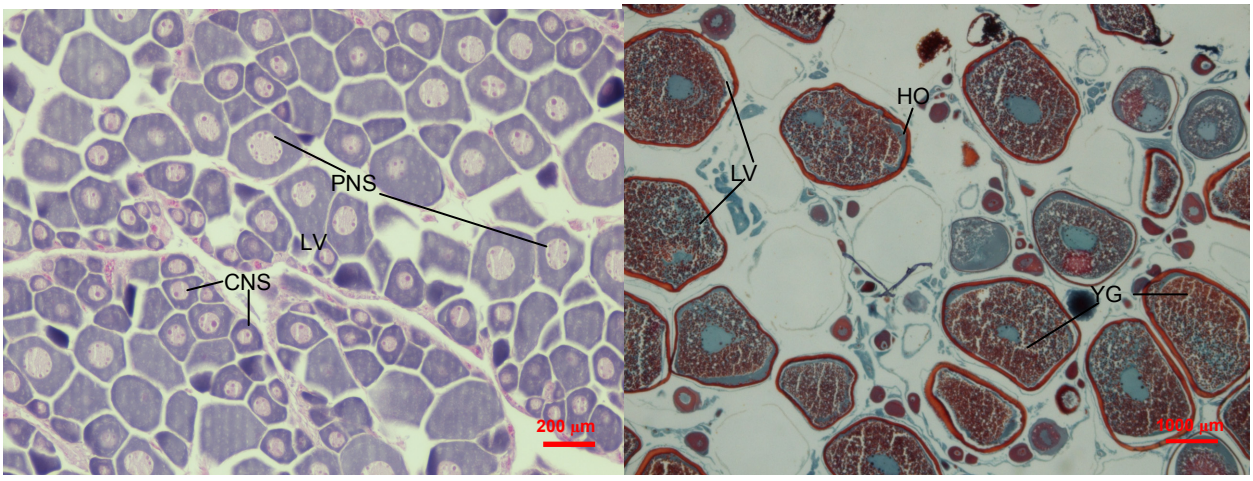

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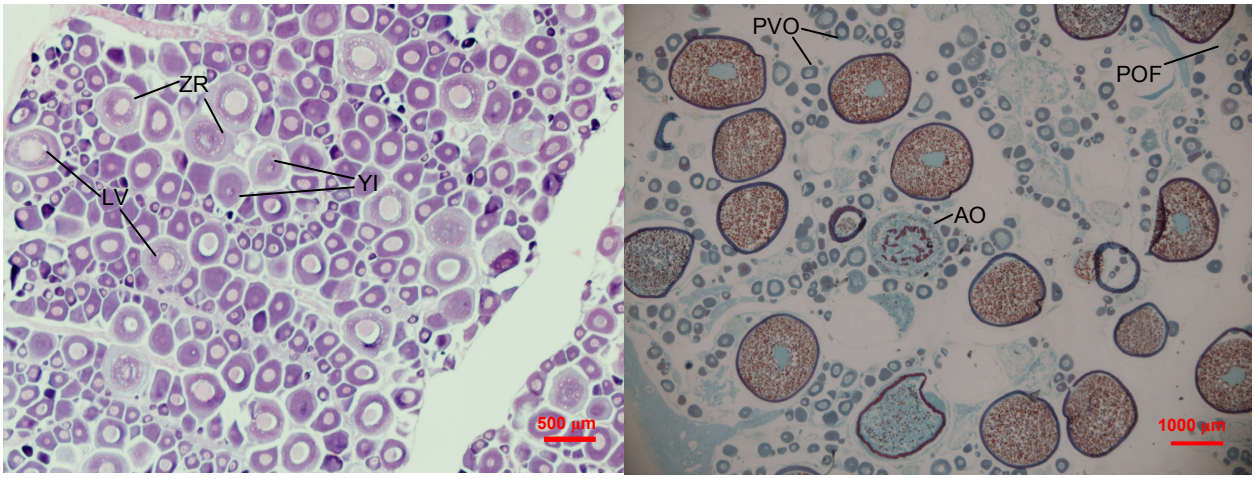

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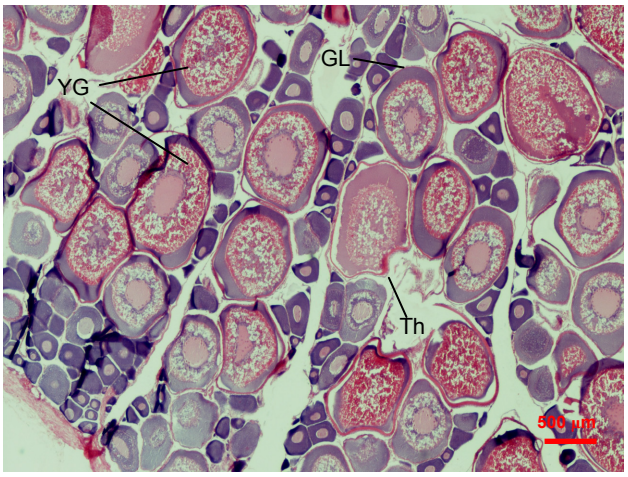
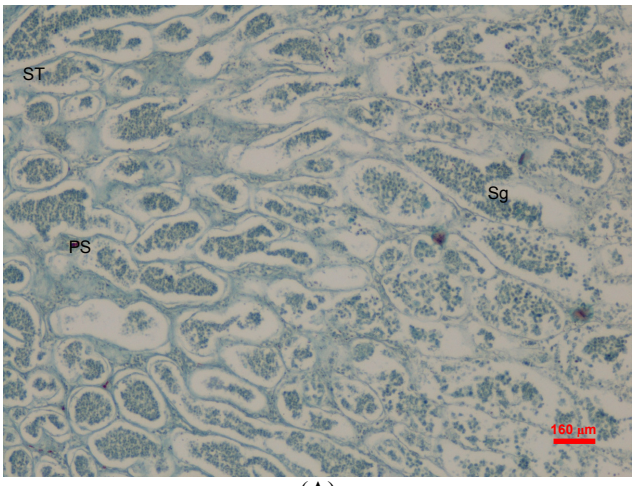


(C)

Figure 1. Oogenesis**. (A)** Pre-vitellogenesis with oocytes in Central Nucleolus Stage (CNS) and Perinuclear Nucleoli Stage (PNS). **(B)** Early vitellogenesis showing Lipid Vacuoles (LV), Yolk Inclusions (YI), and the Zona Radiata (ZR). **(C)** Late vitellogenesis showing Yolk Granules (YG), the Theca (Th), and the Granular Layer (GL). **(D)** Ripening showing the LV, the YG, and the Hydrated Oocytes (HO). **(E)** Post-spawning showing an Atretic Oocyte (AO), a Post-Ovulatory Follicle (POF), and Pre-Vitellogenic Oocytes (PVO).



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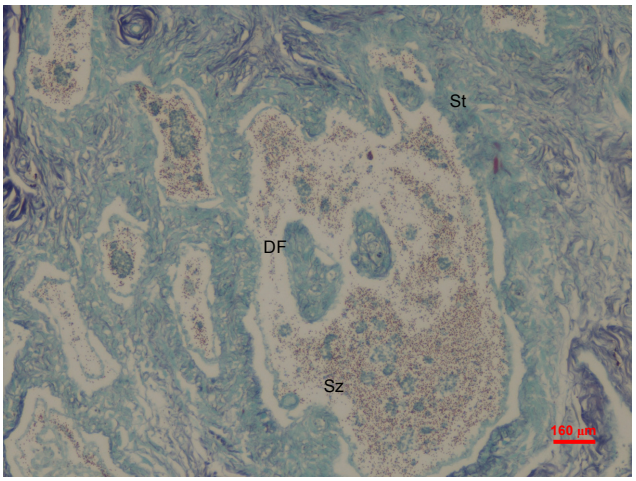

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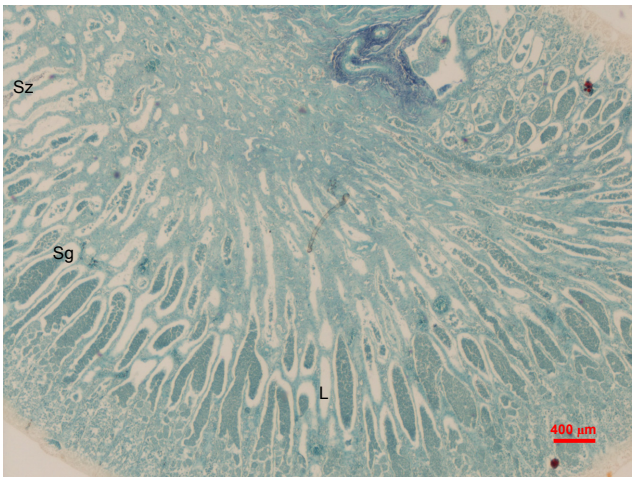

(C)

Figure 2. Spermatogenesis**. (A)** Resting showing the Seminiferous Tubules (ST), Spermatogonia (Sg), and Primary Spermatocytes (PS). **(B)** Spawning showing the Ductus Deferens (DF), the Stroma (St), and Spermatozoa (Sz). **(C)** Post-spawning showing Sg and Sz.

Reproductive Biology of Garfish, *Belone belone* (Linnaeus, 1761), in the Middle Eastern Adriatic Sea

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Introduction

Garfish, *Belone belone* (L. 1761), is an epipelagic and commercially important migratory species widely distributed in the north eastern Atlantic, Mediterranean and Black Sea. In the Adriatic Sea this species is found mainly in the offshore areas but migrates towards coastal waters during the spawning period. The reproductive biology of garfish, in particular the sexual pattern, was studied in order to determinate the time and duration of the annual spawning activity as well as the size at first sexual maturation and fecundity of this species.

Materials and methods

Garfish specimens were collected monthly from December 2003 to December 2008, except June, in the area of the eastern mid-Adriatic Sea. Their total lengths (TL) were measured (nearest mm) and weighed (body and gonad weight; nearest 0.01 g) and sexed. Overall gonads were dissected immediately after capture and the assessment of gonad maturity stages was determined macroscopically using an empiric scale described by Sinovčić (1978). A sub-sample of 100 gonads was processed for histological analysis by excising the central portion of one lobe of each gonad, for which the oocytes were classified according to their morphology and the presence and position of lipid droplets, yolk vesicles and granules (Yamamoto 1956), whereas the spermatogenic cells were classified according to Grier (1981). Additional information of the reproductive cycle was obtained from the ratio of gonad weight and total weight of each individual, using the gonadosomatic index (GSI). Specimens with the most progressive gonad stages (ripe, spawning, spent - with hydrated and semi hydrated oocytes) were used for the calculation of maturity curve and size at first maturity. For the estimation of minimal length at 50% and 100% maturity of garfish population, the logistic function was applied. The gravimetric method was used to estimate fecundity, where counts were made from oocytes present in an ovarian subsample of known weight (Hunter et al. 1985). Namely, three pieces of ovary of approximately 0.1 g each were subsampled from the anterior, middle and posterior section of each gonad and put in Gilson's solution for 4 weeks. After that the number of oocytes was counted. The fecundity was the result of the mean count of the three subsamples. A power model was fitted to the relationship of fecundity versus total body length.

Results and Discussion

A total of 2,776 garfish were collected during the study including 1,166 males (49.4%) and 1,195 (50.6%) females. The TL of all observed specimens ranged from 27.2 to 75.4 cm, the mean value was 38.3 ± 7.94 cm. The males ranged from 27.7 cm to 62.6 cm (mean TL = 37.4 ± 5.37 cm) and females from 27.2 cm to 75. 4 cm (mean TL = 43.6 ± 9.12 cm). Overall sex ratio was 0.98, which insignificantly deviated from the hypothetical distribution of 1:1 ($\chi 2$ =

0.332, d.f = 1, p>0,05); an insignificant preponderance of females over males was observed throughout the investigated period.

The reproductive cycle of garfish was analysed by the maturity stages of gonads as well as by gonad weight, gonadosomatic ratio and gonad tissue histology. According to the monthly percentage composition of gonad maturity stages, the most advanced stages (V, VI, VII) were dominant during the spring season for both sexes (Figure 1). Mean values of GSI ranged from 0.03% (August) to 30.94% (April) in males, and 0.02% (October) to 26.1% (May) in females, showing distinct seasonal changes. For both sexes, the GSI started increasing in January, reached the peak in April for males and in May for females, and then dropped sharply in August. The GSI remained very low thereafter until the following January. The seasonal variation of GSI is consistent with the percentage composition of each maturity stage of gonads, suggesting that the spawning season of garfish is from January to May. Histological examination of gonad tissue confirmed the spawning pattern of garfish - all stages of oocyte development were present in mature ovaries, but during the peak of spawning migratory nucleus and hydrated oocytes were the largest group present in the ovary, whereas spermatid cells and spermatozoa were dominant in testes (Figure 2).

Both ripe males and females (corresponding to stages V, VI and VII from the maturity key of Sinovčić 1978) were collected during the March - May period. The smallest male with the most developed gonad was 28.0 cm TL and the smallest female was 30.0 cm TL. Fifty per cent of garfish population becomes mature at 28.5 cm TL ($r^2 = 0.929$), with males achieving sexual maturity at a smaller size (28.0 cm TL; $r^2 = 0.915$) than females (31.5 cm TL; $r^2 = 0.897$).

The fecundity ranged from 8,320 to 53,534 eggs per individual; there were no significant differences in number of oocytes between the anterior, middle and posterior region of gonad (ANOVA, F = 0.59, d.f. = 2, p = 0.555). The total number of eggs per ripe ovary increased with TL in accordance with the equation: $F = 19.713TL^{1.844}$ ($r^2 = 0.8570$).

Conclusions

The number of *B. belone* females is insignificantly greater that than of males (♂/♀=0.98). Females attained 50% sexual maturation at 28.5 cm TL while males began maturing at a smaller size than females. Temporal evolution of gonad masses, gonadosomatic index, stage of gonads as well as the gonad histological analysis showed that the spawning season of garfish ranged from January to May. Average fecundity was 23,595.62 ± 10,318.339 and could be described as a power function of total length.

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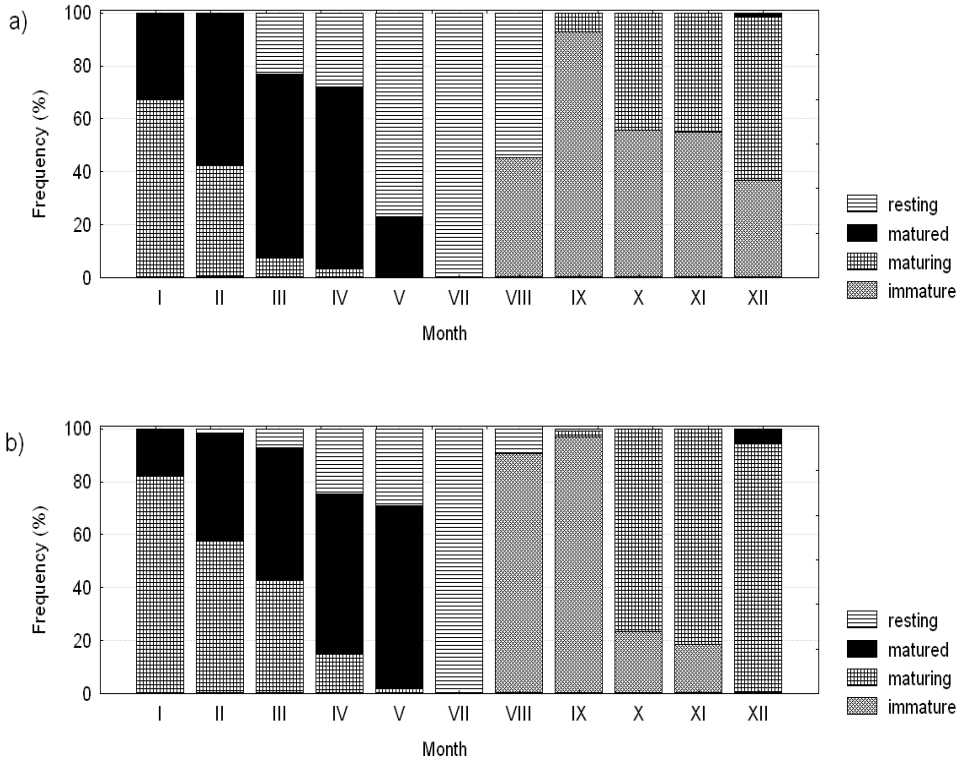

Figure 1. Monthly variations in the frequency of gonad maturity stage of male (a) and female (b) garfish specimens collected in the Middle eastern Adriatic Sea, 2003 - 2008

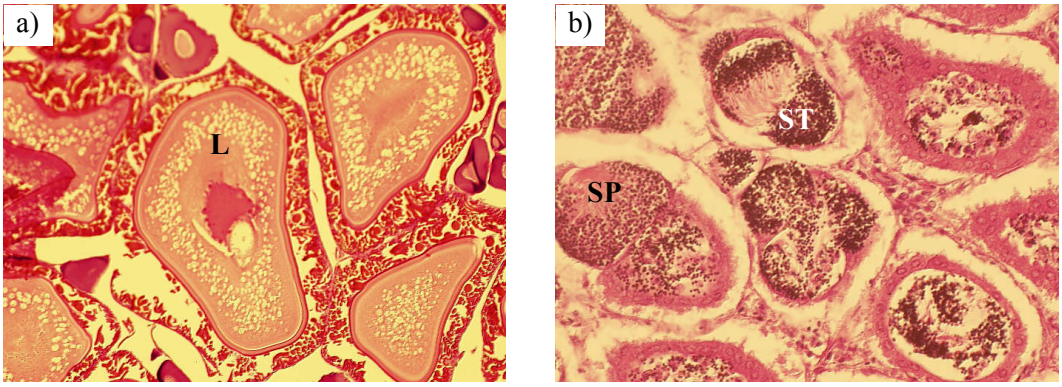

Figure 2. *Belone belone*. Section of gonad tissue: (a) ovarian tissue with oocytes from second growth phase (L- stage of late- vitellogenesis), (b) testicular tissue with some stages of spermatogenesis (SP- spermatocytes, ST- spermatides) (magnification 100x)

Reproductive Characteristics of the Black Anglerfish, *Lophius budegassa* (Spinola, 1807) in the Western Mediterranean

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Introduction

Black anglerfish, *Lophius budegassa* (Spinola, 1807) is a demersal fish distributed in the Mediterranean Sea as well as in the eastern north Atlantic from the British Isles to Senegal at depths ranging from 70 to 800 m. *Lophius budegassa*, together with its conspecific, the white anglerfish *L. piscatorius*, have an important role in the fisheries economy of the Mediterranean Sea, reaching catches of 4700 t in the last 8 years, representing 33 million Euros. Despite that, studies on the reproduction of these species are scarce, especially for *L. budegassa*. In the present study, the reproductive cycle of the black anglerfish was analysed from specimens caught by commercial trawls in the western Mediterranean (Catalan coast) from June 2007 to March 2009.

Methods

Sex and maturity stages (I-V) were determined through macroscopic examination of the gonads in accordance with Afonso-Dias and Hislop (1996). In addition, microscopic histological analyses were performed in order to validate the macroscopic scale and classify oocyte development stages (Murua et al. 2003; McMillan 2007). Histological samples were prepared using standard techniques and chemicals.

Results

Mean gonadosomatic index (GSI) for females caught during winter time (January-March) was significantly higher than in other periods of the year (Student's *t*-test; $p < 0.05$). Mean GSI for males did not differ significantly through the year. The inverse relationship between GSI and hepatosomatic index (HSI) was observed.

Macroscopic analysis of the gonads revealed an unusual structure in this species. At all developmental stages, males had tubular testes that showed a bean shape in transverse section. Females had ovaries consisting of a flattened band made up of a single layer of oocyte clusters which contained a group of oocytes usually in different stages of maturation. The ovaries were anchored to the peritoneum by a mesovarium. The ovary maturity stages were determined through the macroscopic description as follow: Stage I. Immature: The ovaries were narrow and translucent. No visible oocyte clusters; Stage II. Developing/Resting: The ovaries, less translucent, increased in length and width. Oocyte clusters not visible yet; Stage III. Mature: The ovaries increased considerably in length and width. Opaque oocyte clusters were visible; Stage IV. Spawning: The ovaries were extremely long and wide. Orange oocyte clusters were embedded in a transparent gelatinous matrix; Stage V. Spent: The ovaries were very wide and vascularized. They were opaque and flaccid with striations. No oocyte clusters were visible.

The microscopic analysis of the oocyte development in each ovary maturity stage revealed that in the immature stage (Figure 1a) the oocytes had a nucleus surrounded by a thin layer of

cytoplasm and contained a big nucleolus (oocytes in primary growth stage: chromatin nuclear and perinucleolar). In the developing/resting stage (Figure 1b), oocytes at the beginning of vitellogenesis (cortical alveoli oocytes) and oocytes in the initial phase of growth were observed. In the mature stage (Figure 1c), the oocytes were characterized by yolk granules and oil droplets present in the cytoplasm. The nucleus was still central. In the spawning stage, oocytes have a nucleus migrating to the animal pole or oocytes are hydrated; however, in our samples, only oocyte clusters situated on the ovary membrane were available at this stage (Figure 1d). In the spent stage (Figure 1e), atretic oocytes were observed together with postovulatory follicles (POF) and oocytes in the vitellogenic stage.

The size frequency distribution of oocyte diameter (Figure 2) showed that oocytes in different stages of development were found at each maturity stage. During Stage I, only oocytes in primary growth (chromatin nuclear and perinucleolar) with a narrow diameter range distribution were present. In Stage II, oocytes in primary growth and cortical alveoli with noteably larger diameters were present. A wider oocyte diameter range distribution was observed. In Stage III, yolk granule stages are present along with the previous oocytes types. In Stage IV five types of oocytes should be observed, although we found only two. Since spawners are extremely scarce, hydrated oocytes were not available in this study. In Stage V, oocytes in primary growth were observed along with atretic oocytes.

Conclusions

An analysis of the seasonal variations of gonadosomatic and hepatosomatic indexes for both sexes of *Lophius budegassa* showed a preferred spawning period during winter time (January-March) for females, while males did not show a preferred season. The frequency distribution of oocyte diameter and the presence of different developmental oocyte stages within oocyte cluster corroborates that the black anglerfish is a total spawner with group-synchronous oocyte development.

Acknowledgments

This study has been funded by the Project “Monitoratge dels recursos pesquers i marisquers al litoral català” (Direcció General de Pesca i Afers Marítims, Generalitat de Catalunya).

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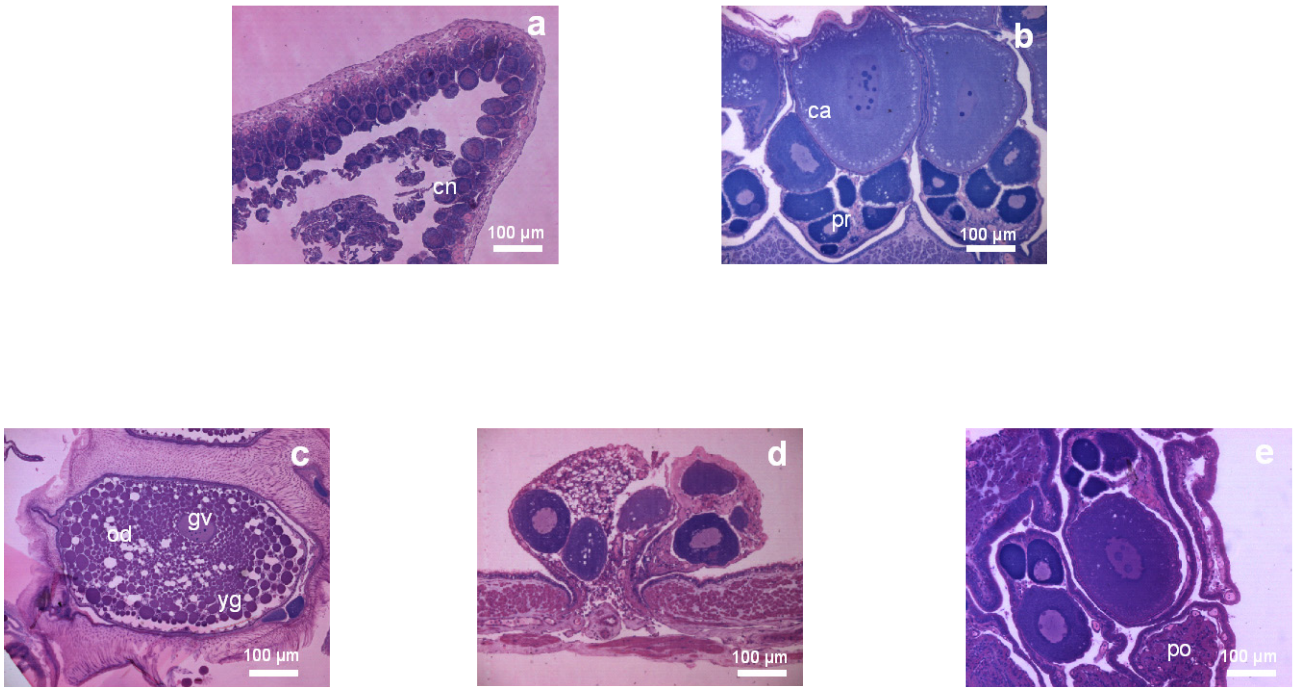

Figure 1. Ovaries at different maturity stages. a) Immature (Stage I); b) Developing/Resting (Stage II); c) Mature (Stage III); d) Spawning (oocyte cluster situated on the ovary membrane); e) Spent (Stage V). ca, cortical alveoli; cn, chromatin nuclear; gv, germinal vesicle; pr, perinucleolar, od, oil droplet; po, postovulatory follicle; yg, yolk granule.

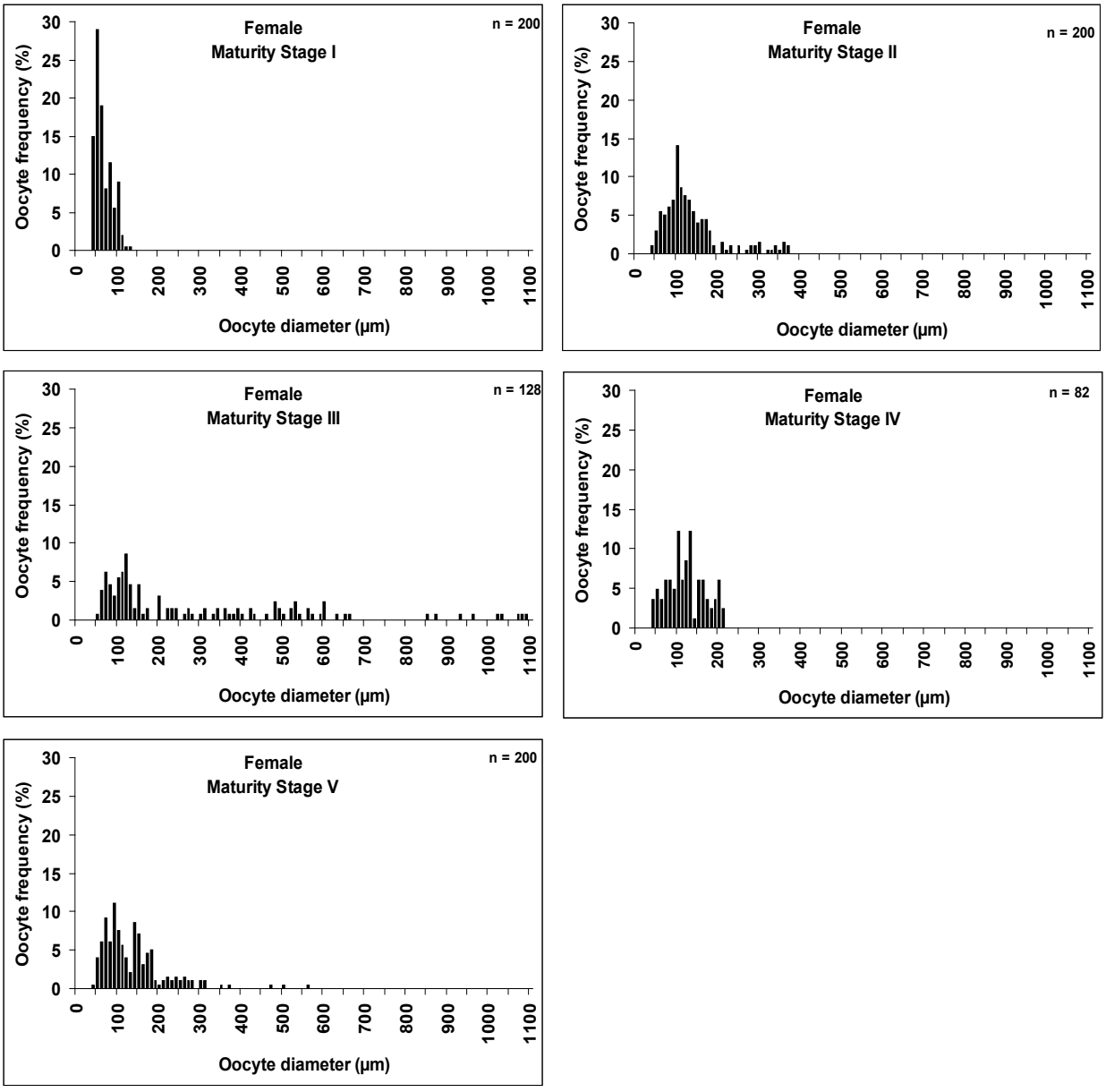

Figure 2. Distribution of oocyte diameter in black anglerfish at each maturity stage.

Histological and Macroscopic Analysis of Horse Mackerel *Trachurus trachurus* (Linnaeus, 1758) Ovaries from M´diq Region (Mediterranean of Morocco)

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Horse mackerel (*Trachurus trachurus* Linnaeus, 1758) were captured monthly between May 2005 and October 2006 from the M'diq region of the Mediterranean Sea off Morocco. The gonads of 372 females have been analyzed macroscopically and the histological characteristics of 192 of them were compared. The analysis confirmed that this species has asynchronous ovarian development and is capable of multiple spawns during the reproductive season.

Reproductive Strategy of the Mesopelagic, Synchronous Hermaphroditic Species *Chlorophthalmus agassizii* (Bonaparte, 1840) in the Greek Ionian Sea

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The reproductive strategy of the mesopelagic fish *Chlrorophthalmus agassizii* has been studied from monthly samples taken from the Greek Ionian Sea with a commercial trawl between December 1996 and November 1997. A total of 658 individuals were used for the study of the reproductive biology and 433 were histologically processed.

Histological analysis of the gonads of *C. agassizii* revealed that female and male tissues occurred contemporaneously in the same gonad as two distinct components separated by the connective tissue. The ovarian and testicular tissue matures simultaneously, indicating that *C. agassizii* is a synchronous hermaphroditic species. All reproductive studies were based on histological examination of the female and male component of the gonads.

Oocyte size distribution in pre-spawning and spawning ovaries showed that no hiatus was present between previtellogenic and vitellogenic oocytes. The hiatus was only present in the ovaries just before ovulation, thus separating the batch of oocytes to be released (Figure 1). One indicator that *C. agassizii* is believed to have an indeterminate fecundity is the lack of a distinct hiatus separating the yolked oocyte stock from the reservoir of the unyolked stocks (Hunter et al. 1992). Since a lack of such a hiatus does not necessarily indicate that fecundity is indeterminate (Hisplop and Hall 1974), another indicator is the observed high prevalence of atresia at the end of the spawning season, which has been shown to be consistent with indeterminate fecundity (Gordo et al. 2008).

The male component of the gonad was always found to mature earlier than the female one. First maturity for the female component was always later than first maturity for the male component, indicating that female and male components do not start the spawning phase at the same time. All fish < 90 mm TL had immature ovaries. Intra-annual variations of the proportion of mature individuals per length class size indicated that the spawning extent and the timing of peak spawning were size-dependent, in accordance with the trade-off between somatic and gonadal growth.

Analysis of the seasonal reproductive cycle and the gonadosomatic index indicated a very protracted spawning period, a reproductive feature typical for batch spawners, from the beginning of April through October. For the female component, the main spawning season was between May and September while for the male component the spawning period was more extensive (April to November). The extensive atresia observed at the end of the spawning season confirmed the rapid decrease of GSI values, suggesting that the GSI index represents well the dynamics of ovarian development.

The GSI values showed monthly fluctuations during the protracted spawning season. Highest values of GSI were observed in May (1.38 ± 0.15), July (1.42 ± 0.17), August (1.87 ± 0.17) and September (1.27 ± 0.21), which coincided with the main spawning season of the species. The two peaks observed on May and August may be related to different spawning timing between the several length/age classes. After September, the end of the spawning season, a rapid decrease in GSI was observed until March, the month when oocytes begin secondary developmental growth. The lowest GSI values were observed in the immature phase, while the highest GSI values were observed in the spawning phase. A rapid decrease of GSI index was observed following spawning. GSI values during the spent phase (VII) were very similar to those of the resting/recovering phase. ANOVA showed significant differences among GSI values for maturity phases, and the Tamhane's post-hoc tests confirmed that the immature and spawning phases are well defined and significantly different from the rest of the phases (Table 1).

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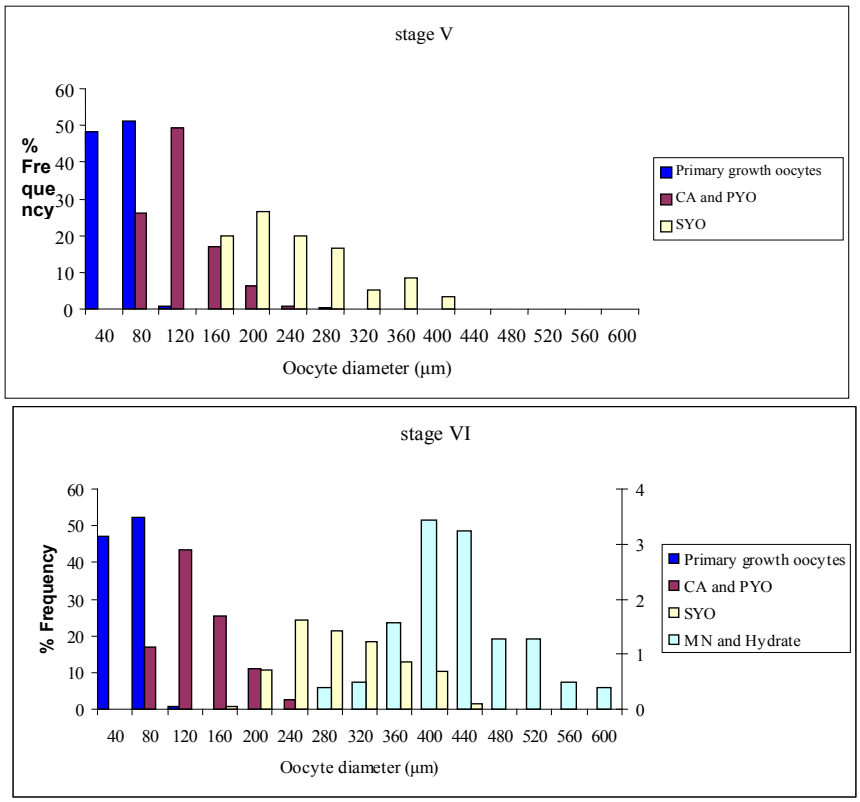

Figure 1. The oocyte size frequency (in µm) distribution in pre-spawning (stage V) and spawning (stage VI) ovaries. (CA=cortical alveoli oocytes, PYO=primary yolk oocytes, SYO=secondary and tertiary yolk oocytes, MN=migratory nucleus oocytes).

Table 1. Matrix with Tamhane test results for ANOVA post hoc comparisons of GSI (natural log transformed) between the maturity phases of the female gonad component of *C. agassizii*. The mean differences between Ln (GSI) values are given below the diagonal, while the significance levels are given above the diagonal. (MSF: maturity stage of female component, I: index for lines, J: index for columns, bold letters indicate significance values at a=0.001). Phases I and VI indicate immature and spawning, respectively.

	Mean Difference (I-J)						
	(J) MSF						
(I) MSF	I	II	III	IV	V	VI	VII
I		**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**
II	**2.6173**		0.7139	**0.0000**	**0.0000**	**0.0000**	1.0000
III	**2.8299**	0.2126		**0.0001**	**0.0000**	**0.0000**	0.8715
IV	**3.3427**	**0.7254**	**0.5128**		0.0573	**0.0000**	**0.0001**
V	**3.6253**	**1.0080**	**0.7954**	0.2826		**0.0000**	**0.0000**
VI	**4.6948**	**2.0775**	**1.8649**	**1.3521**	**1.0695**		**0.0000**
VII	**2.5469**	0.0704	0.2831	**0.7958**	**1.0784**	**2.1479**	

Formation of the Germinal Epithelium during Gonadal Differentiation in *Cyprinus carpio* (Teleostei: Ostariophysi: Cypriniformes)

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Introduction

During embryogenesis in Teleostei, primordial germ cells (PGCs), coming from an extra-gonadal region, migrate to the genital ridge and associate with somatic or epithelial cells from the mesothelium or coelomic epithelium (Nakamura et al. 1998). The PGCs and somatic cells form the gonadal primordium (Nakamura et al. 1998). Gonadal development and sexual differentiation involves changes in somatic and germ cells. It includes divisions and differentiation of the gonocytes, proliferation of the somatic cells, germinal epithelium differentiation, and formation of the gonads (Devlin and Nagahama 2002).

Material and Methods

In order to study the morphological changes of germ and somatic cells, and formation of the germinal epithelium during gonadal differentiation, gonadal tissues from larvae and juveniles of *Cyprinus carpio* were fixed in Karnovsky´s solution, processed and embedded in historesin. Serial sections were stained with Hematoxylin/Eosin (HE) and Metanil Yellow + Periodic Acid Schiff´s + Hematoxylin (MY).

Results and Discussion

In *C. carpio*, the gonadal primordium is an elongated structure with individual PGCs scattered among somatic cells. As described (Meijide et al. 2005; Le Menn et al. 2007), the PGCs divide and then organize into continuous cords that are delimited by the somatic cells (Figure 1A). Then, somatic cells move into the cords, wrap around and individualize the PGCs that subsequently differentiate into oogonia (Figure 1B). Each oogonium proliferates by mitosis or they enter into meiosis, becoming oocytes (Figure 1C). When the oocytes enter primary growth, they are subsequently surrounded by somatic cells, now called prefollicle cells and then becoming follicle cells at the completion of folliculogenesis (Figure 1D) when the follicle is completely surrounded by a basement membrane (Grier 2000; Grier et al. 2009).

The differentiating gonad of *C. carpio* maintains a compact structure, continues elongating, and becomes larger. Invaginations develop ventrally in the ovary as the ovigerous lamellae develop (Figure 1D). At this time, primary growth oocytes are observed. Mesenchymal cells, scattered inside the lamellae, spread apart forming the extravascular space in the developing stroma (Figure 1E). Mesenchymal cells also surround the ovarian follicles and become the theca (Figure 1E), completing formation of the follicle complex composed of the ooycte, its surrounding follicle cells, basement membrane and theca (Grier et al. 2009). As this occurs, epithelial cells from the gonad periphery, and present in the ventral invaginations, associate

with oogonia to form the germinal epithelium. On either side of the developing ovary, a coelomic epithelial cell proliferation forms a laminar tissue that grows ventrally (Figure 1F), then they both extend beneath the developing ovary and fuse to form the central lumen of the carp ovary, a cystovarian type (Nakamura et al. 1998).

The somatic cells, derived from the coelomic epithelium, appear to be involved in the process of reorganization of germ cells during gonadal differentiation, especially when the germ cells of undifferentiated gonads begin to form continuous cords and establish new relationships during both development of the germinal epithelium and stroma.

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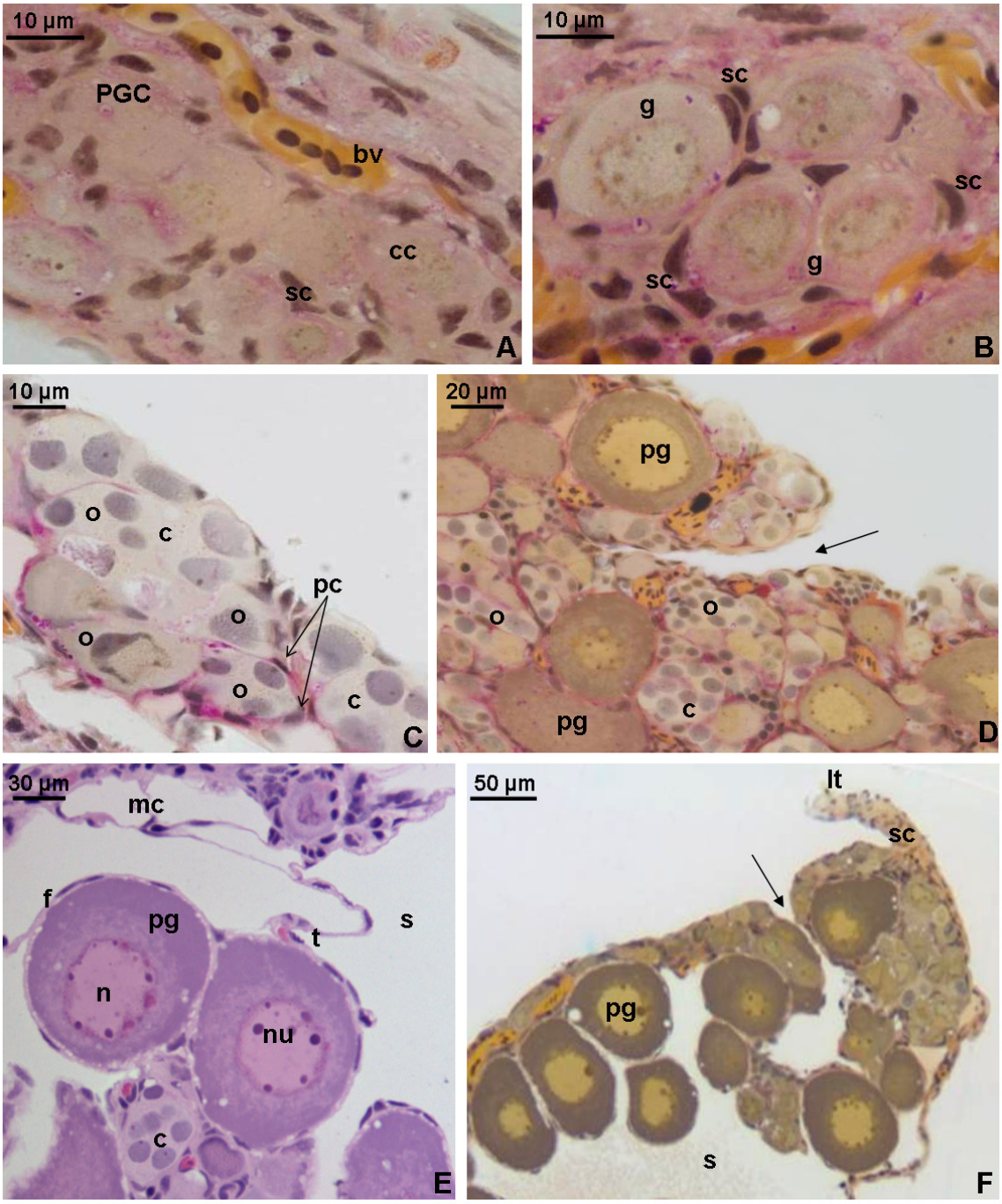

Figure 1. Germinal epithelial formation in *Cyprinus carpio.* **A.** undifferentiated gonad - formation of continuous cords (MY stain); **B.** early differentiated gonad - individualization of the oogonia (MY stain); **C.** differentiated gonad - clusters of oogonia and prophase oocytes (MY stain); **D.** early primary growth of the oocytes and formation of the ovigerous lamellae as outgrowths along the ventral surface of the developing ovary (MY stain); **E.** formation of the theca by mesenchymal cells of the stroma (HE stain); F. cross section of the ovary - formation of the cystovarian type by the laminar tissue and invaginations from ovigerous lamellae (MY stain). bv - blood vessels; c - clusters; cc - continuous cords; f - follicle cell; g - oogonia; lt - laminar tissue; mc - mesenchymal cells; n - nucleus; nu - nucleoli; o - prophasic oocytes; pc - prefollicle cell; pg - primary growth oocyte; PGC - primordial germ cells; s - stroma; sc - somatic cells; t - theca; arrow - invaginations from ovigerous lamellae.

Characterization of Zebrafish (*Danio rerio*) Females as Recipients for Spermatogonial Stem Cell Transplantation: a Model to Study Stem Cell Plasticity in Teleosts.

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Fish present an enormous plasticity regarding reproduction. As illustrated by different reproductive strategies or by naturally (e.g. protogynous and androgynous) or experimentally induced sex change (e.g. xenoestrogens). We speculate that this plasticity might be attributed, at least in part, to the comparatively undifferentiated state of oogonial and spermatogonial stem cells (SSCs). However there is no proof about it yet. Recently, germ cell transplantation in fish provided the possibility to characterize putative germline stem cells. The aim of this work is to characterize adult zebrafish females as recipients for germ cell transplantation, to establish a model for studying SSC plasticity. First of all, we characterized the transplantation pathway by analyzing serial sections of the genital tract. The female genital pore measures about 120x80µm and the glass capillary for cell transplantation has to be positioned at an angle of 63°. Moreover, another characteristic to be taken into account is the availability of niches, since transplanted SSC need to find a suitable niche in the recipient´s ovary. In this context, we analyzed postovulatory females which showed a large number of postovulatory follicles. This suggests "available follicle cells" that might be reused to support transplanted germ cell. Therefore, postovulatory females appear to be suitable recipients for transplantation. Since we intend to perform transplantations using SSC from *vasa::egfp* animals, it is necessary to evaluate the expression of *vasa* in ovaries as a control. As consequence *vasa::egfp* ovaries were submitted to immunofluorescence. *Vasa* expression is strong in cell "nests" where oogonia and early oocytes are located, which decreases in perinucleolar oocytes, and is undetectable in vitellogenic oocytes. In summary, we have taken an important step for establishing females as recipients for transplantation as a mean to study SSC plasticity in zebrafish.

An Immunohistochemical and Ultrastructural Study of the Spermatogenesis of *Helicolenus dactylopterus*

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Introduction

Helicolenus dactylopterus is a zygoparous species with intraovarian sperm storage for long periods of time. Because of its peculiar reproductive technique we studied its spermatogenesis in depth. Spermatogenesis of *H. dactylopterus* appears similar to most teleosts. Spermatogenesis is cystic (Mattei 1993): the whole spermatogenic process takes place within the cysts delimited by cytoplasmic extensions of Sertoli cells, which integrate the functional unit of the testis. Spermatogenesis and the spermatozoon ultrastructure of this species were studied using conventional microscopic and electronic microscope techniques.

Results and Discussion

Primary spermatogonia (Figure 1A) often appear in small groups, along the whole length of the tubule, and near the lobule periphery. They are individually rounded by Sertoli cells, which form the cyst. They are the biggest germinal cells. Secondary spermatogonia have a more round shape and they are grouped within the cyst delimited by Sertoli cells. Furthermore, they are more numerous than primary spermatogonia and, sometimes, intercellular bridges could be seen between them. Ultrastructurally, in the cytoplasm secondary spermatogonia have evident but fewer mitochondria, Golgi Apparatus, smooth and rough endoplasmic reticle, and ribosomes. Nucleus chromatin could be more or less condensed depending on the phase these cells are in. Primary spermatocytes are characterized by a progressive chromatin condensation and synaptonemal complexes formation (Figure 1B). Secondary spermatocytes cells are smaller in size and with a smaller nucleus than primary spermatocytes.

When cells reach the spermatid stage, centrioles move to the vicinity of the nucleus to organize a cytoplasm channel that will carry the flagellum and its surrounding membranes (Figure 1C). As a result of the centriole movement towards the nucleus, the cytoplasmic channel is formed. At the same time, an evident reduction of the nuclear volume is observed as the consequence of a progressive and heterogeneous chromatin condensation, being more intense at the central region of the nucleus. This fact provokes the nucleus to adopt a "U" shape. Despite this, in the centriolar complex area there is always less condensed chromatin (Figure 1D). Chromatin condensation leaves an enormous space in the nucleus because the nuclear membrane has not been reduced. The process of vesiculation allows the elimination of the remains of nuclear material.

As the spermiogenesis process advances, mitochondria which were concentred in the flagellar area, prepare to surround the first flagellum segment and shape the future middle piece of the spermatozoa, which in the spermatid stage already has four layers of mitochondria. *Helicolenus dactylopterus* spermatozoa belongs to the complex type of anacrosomal

introsperm (Muñoz et al. 2002) defined by Jamieson and Leung (1991), typical of species with internal fertilization (Figure 1E).

We used the HRP technique to confirm the existence of the "Sertoli cell barrier." This barrier is thought to be established when male germ cells are at the spermatid phase in order to protect spermatids and spermatozoa from external aggressions. Despite the fact that tight and desmosomal junctions between Sertoli cells have been demonstrated using optical and electronic microscopes, we still do not know in which moment of the spermatogenesis process this barrier is established.

The tracer HRP was used to analyse if tight junctions between Sertoli cells restrict the transport of substances from the interstitial area to the cysts and the lumen of seminiferous lobules, as has been demonstrated in other species (Lou and Takahashi 1989). Initially, Sertoli cells surround one spermatogonia and maintain this wrapper incompletely closed at the basal membrane side. As spermatogenesis advances, tight junctions are formed, setting up the "testis-blood barrier" which is thought to provide protection from immune cells of the male, permit the existence of a favourable microenvironment, and give cytostructural support to spermatids and spermatozoa inside the cyst (Figures 1F, 2A, 2B).

With the break up of the cyst, mature spermatozoa are first released towards the lobular lumen and then accumulate at the sperm duct (Figures 2C, 2D). While spermatozoa are still inside the testicular lobules, they are lectin negative. However, unlike the other male sexual cells that are within the testis, spermatozoa which already are at the spermatic duct were stained with the lectin *Triticum vulgaris* (WGA). Additionally, this staining process also intensely stained the cells of the walls of the sperm duct, colouring them intensely brown (Figure 2E). This staining methodology has been used by different authors, such as Walter et al. (2005) with the species *Leuciscus cephalus*.

Spermatozoa located at the posterior part of the testis, where the spermatic duct leaves the gonad, show a cytoplasmic bag located on one side of their heads (Figure 2F) and a middle piece, with some diminutive vacuoles inside (Vila et al. 2007a; 2007b). Thus, in the case of *Helicolenus dactylopterus* we found that it is in the sperm duct where spermatozoa acquire and store reserves inside their cytoplasm in order to resist the long intraovarian sperm storage period, which sometimes lasts for up to ten months.

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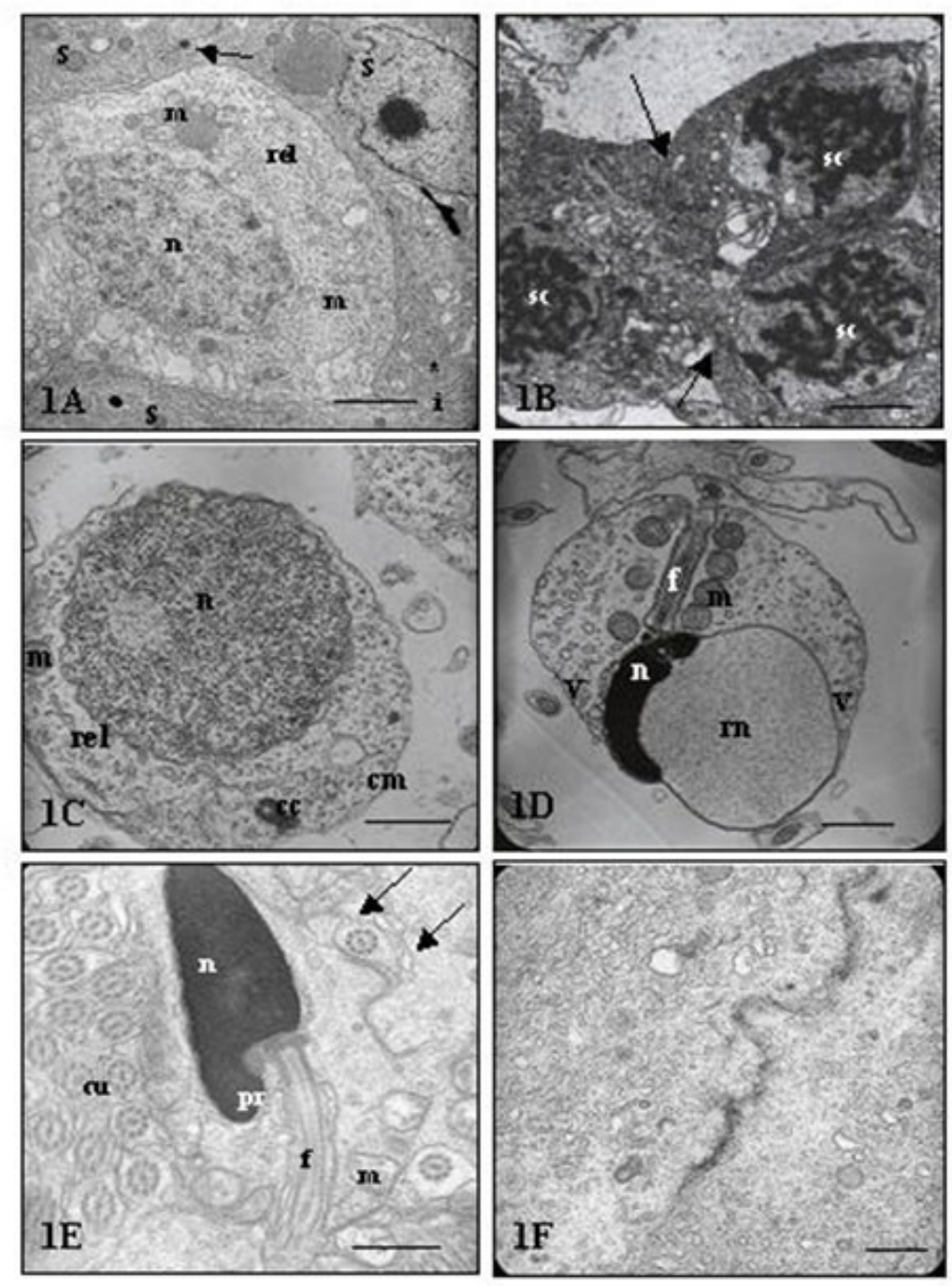

Figure 1. Electron micrograph images of spermatogenesis in *Heliocolerus dactylopterus*. **A**. Primary spermatogonia. Arrow indicates tight junction. Scale bar: 1 µm **B**. Primary spermatocytes, characterized by a progressive chromatin condensation. Arrow indicates intercellular bridges. Scale bar: 2 µm. **C**. Very early spermatid with the centriolar complex anchored to the plasma membrane. Scale bar: 1 µm. **D**. Spermatid, with the flagellar axis oblique to the spermatid nucleus. Scale bar: 1 µm. **E**. Spermatozoa. Arrows indicate lateral projections of the flagellum. Scale bar: 2 µm. **F.** Visulation of tight junctions between Sertoli cells using HRP tracer to analyze if substances are transported from the interstitial area to the cysts and the lumen of seminiferous lobules. Scale bar: 1µm. Key: cb - cytoplasm bag; cc- centriolar complex; cm-cytoplasm membrane; f – flagellum; h – spermatid or spermatozoa head; m- mitochondria; n – nucleus; rn – residual nucleus; S – Sertoli cell; sc – spermatocytes; ser – smooth endoplasmic reticulum; t – sperm tails; v – vacuoles.

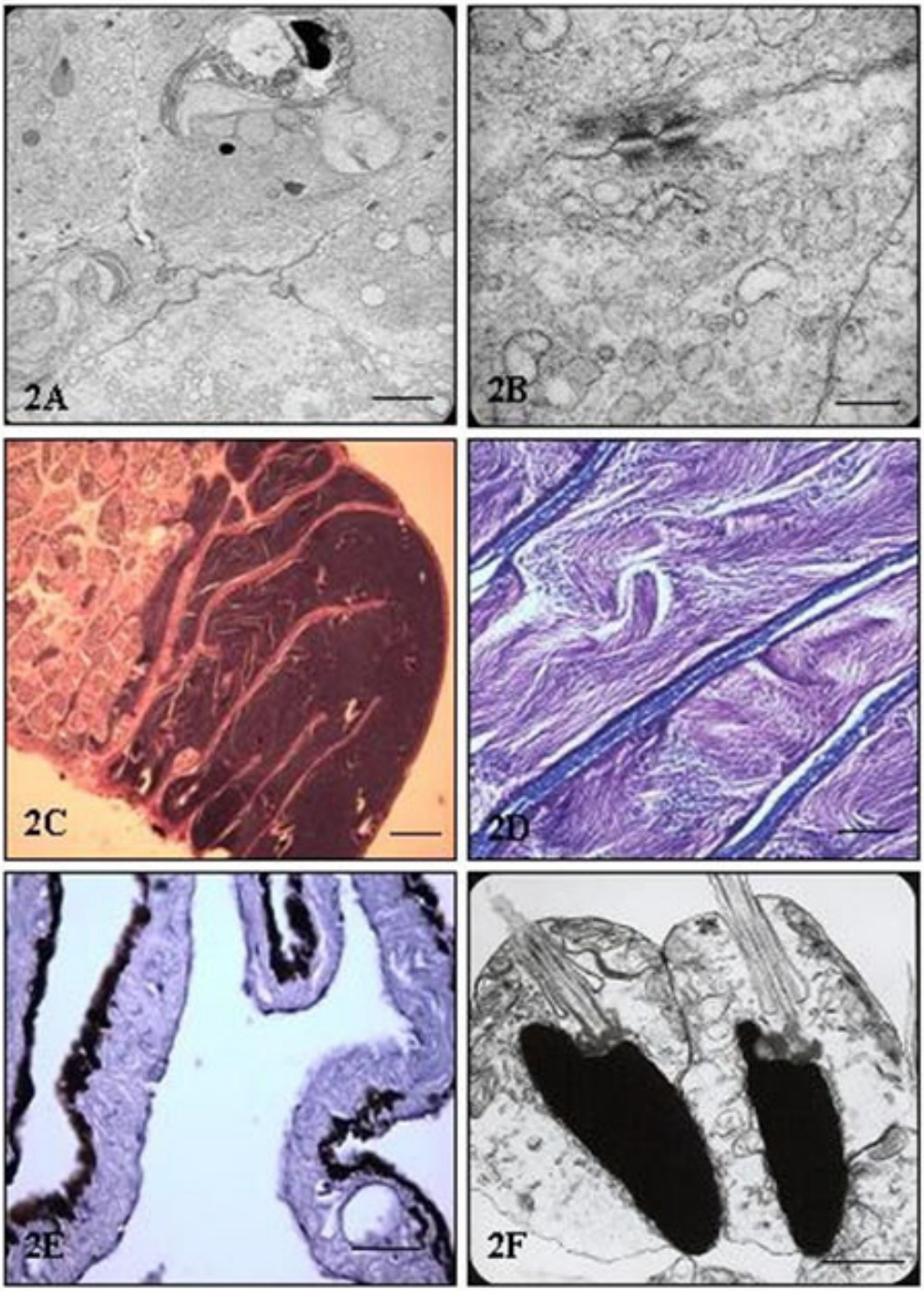

Figure 2. Spermatogenesis in *Heliocolerus dactylopterus.* **A. and B.** Tight junctions between Sertoli cells isolate spermatozoa (HRP tracer analysis). Scale bar: 1 µm. **C**. Mature, spermiated spermatozoa released towards the lobular lumen and accumulate at sperm duct. Scale bar: 20 µm. **D**. Spermatozoa bundles in efferent ducts during the spawning period. Scale bar: 20 µm. **E**. Intense brown staining of walls of the sperm duct using the lectin*Triticum vulgaris*. Scale bar: 15 µm. **F.** Spermatozoa in the sperm duct with cytoplasmic bag around heads. Scale bar: 2 µm.

Skipped Spawning: A strategy for Maximizing Reproductive Output in a Variable Environment

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The traditional view of iteroparity in fishes is one of an annual reproductive cycle that culminates each year in spawning. However, numerous reports suggest that mature iteroparous fish can abandon reproductive investment in any given year and thus fail to spawn. Skipped spawning, as this is usually called, does not interfere with the reproductive cycle and spawning in subsequent years (i.e. a single year interruption). The significance of these findings depends on the number of fish species capable of such reproductive plasticity and how frequently individuals skip spawning (i.e. how extreme conditions have to be in order to cause reproduction to be aborted). Not surprisingly the earliest reports of skipped spawning were of fish from northern lakes with low temperatures and an extremely short feeding season. Over the next 50 years there were sporadic reports of skipped spawning in various species, suggesting this plastic response to the environment might be widespread. In the past 5 years, perhaps reinforced by the need to explore alternative indices of fish stock reproductive potential, there has been a surge of research relating to skipped spawning in fishes. This research has demonstrated that (1) skipped spawning is perhaps even more widespread than previously believed and (2) skipped spawning is not just a response to extreme and unusual environmental conditions but rather a normal plastic response of fish to maximize lifetime reproductive output in a highly variable environment. Here I review our understanding of skipped spawning in fishes with an emphasis on current research directions.

A manuscript based on this presentation has been submitted to the journal Marine and Coastal Fisheries as part of the special module "Emerging Issues and Methodological Advances in Fisheries Reproductive Biology". Publication is anticipated in fall 2010. Web page: www.fisheries.org/mcf.

The Use of Histology in Defining the Pattern of Ovarian Development and Fecundity Type in Tropical Marine Teleost Fishes

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Introduction

In teleosts, three common modes of oocyte development are recognized (synchronous; group synchronous and asynchronous) which determine the type of seasonal fecundity presented by the species (determinate or indeterminate fecundity). In synchronous ovaries, one size-group of oocytes is present and recruitment of vitellogenic oocytes occurs as a one-time event before the breeding season. In group synchronous ovaries, at least two size-groups of oocytes are present and recruitment of vitellogenic oocytes occurs also as a one-time event before the breeding season. Finally, in asynchronous ovaries, several size-groups of oocytes are present and, depending on the species, recruitment of vitellogenic oocytes occurs as a one-time event before the breeding season or as a continuous ongoing process before and during the breeding season (Wallace and Selman 1981). Species with synchronous or group synchronous ovaries (total, isochronal spawners) shed their entire clutch of mature oocytes over a short period. They present a determinate fecundity and, in the case of group synchronous ovaries, the size distribution of vitellogenic oocytes is separated from previtellogenic oocytes prior to spawning. Species with asynchronous ovaries (partial; heterochronal; multiple; serial spawners) intermittently shed several clutches of mature oocytes during a protracted period. They can present a determinate fecundity or an indeterminate fecundity with a continuous size distribution of oocytes without separation between previtellogenic and vitellogenic oocytes, before and during spawning.

Methods

In the present work three commercially important fishes from the continental shelf of the Yucatan Peninsula (Campeche Bank), southeastern Gulf of Mexico; were studied: two gonochoristic snappers *Lutjanus synagris* (lane snapper) and *Ocyurus chrysurus* (yellowtail smaller) and a protogynous hermaphroditic grouper *Mycteroperca microlepis* (gag). The aim of the study was to characterize the ovarian development and fecundity type presented by these species by means of a simple method based on histological observations of their ovaries. This method is based on the estimation of relative numbers of the different oocyte stages, including postovulatory follicles (Pof) and atresia (At), present in the ovaries following N'Da & Déniel (1993). The terms "stage" and "class" (or "phase") were used for gamete and gonad development, respectively. Based on microscopic features of ovaries, six descriptive oocyte stages and six descriptive sexual classes were defined (Wallace and Selman 1981, Taylor et al. 1998). Pattern of ovarian development and spawning strategy were assessed during an annual reproductive cycle. For each collected female, the relative frequencies of developing oocyte stages were estimated by counting and classifying 200 oocytes, including Pof and At, per histological slide in randomly chosen fields. In a first step, the hypothetical succession of

oocyte maturation stages during the annual sexual cycle in a single female was reconstructed and illustrated using a pie diagram. Then, females with similar oocyte stage percentages within each month of the annual sexual cycle were grouped and the results shown in frequency distribution graphs. This produced a monthly view of female maturation stage diversity within the population, and helped to highlight the annual maturity stage succession. The presence or absence of a distinct hiatus between unyolked oocytes (previtellogenic) and the standing stock of yolked oocytes (vitellogenic), was the criteria used to distinguish between determinate and indeterminate fecundity, respectively (Hunter et al. 1985).

Results

In the case of *Lutjanus synagris*, the hypothetical annual cycle of ovarian development based on the recorded data showed that the ovaries of both immature (I) and regressed (Rd) females had only stage-I and -II oocytes (Figure 1). During the maturation process of sexually active females, the relative abundance of previtellogenic oocyte stages progressively decreased (from 74.5 to 52.5%) while that of vitellogenic stages increased. Stage-III vitellogenic oocytes (25.5%) appeared during early maturation, and stage-IV (11.5%) vitellogenic oocytes appeared in mid maturation. These stages remained in the ovaries into late maturation (LM), co-occurring successively with abundant, ready-to-ovulate stage-V oocytes (LM_1) (17%); postovulatory follicles (LM_2) (2.5%), indicating that a batch of stage-V oocytes had ovulated; and postovulatory follicles (1%) and stage-V oocytes (30%), indicating that a new batch of stage-V oocytes were in formation (LM_3). Ovaries moved from LM_1 to LM_3 repeatedly during the breeding season until spawning ended. Atretic oocytes (10-11%) and some residual stages-III oocytes (2%) remained in the ovaries of regressing females. As regression continued, residual stage-III oocytes progressively degenerated and only atretic oocytes were present in the ovaries at the end of the regression process. When degenerating oocytes had disappeared completely, females were considered to be fully regressed. Throughout the annual cycle, the ovaries of all sexually active females in early to late maturation contained populations of both previtellogenic oocytes (stage-I and -II) and vitellogenic oocytes in different development stages (Figure 2). Females in late maturation (from LM_1 to LM_3; N = 67) exhibited no gap in oocyte stage frequency distribution between previtellogenic and vitellogenic oocytes, or between vitellogenic oocyte development stages. Similar results were obtained for *Ocyurus chrysurus* and *Mycteroperca microlepis*.

Discussion

These results suggested that the three studied species are indeed asynchronous or heterochronal spawners with indeterminate annual fecundity. Conclusions on ovarian development agreed with observations by Claro and Lindeman (2004) for yellowtail snapper but differed from those obtained by the same authors for lane snapper. Conclusions on ovarian development and fecundity type agreed with observations of Collins et al. (1997) for gag.

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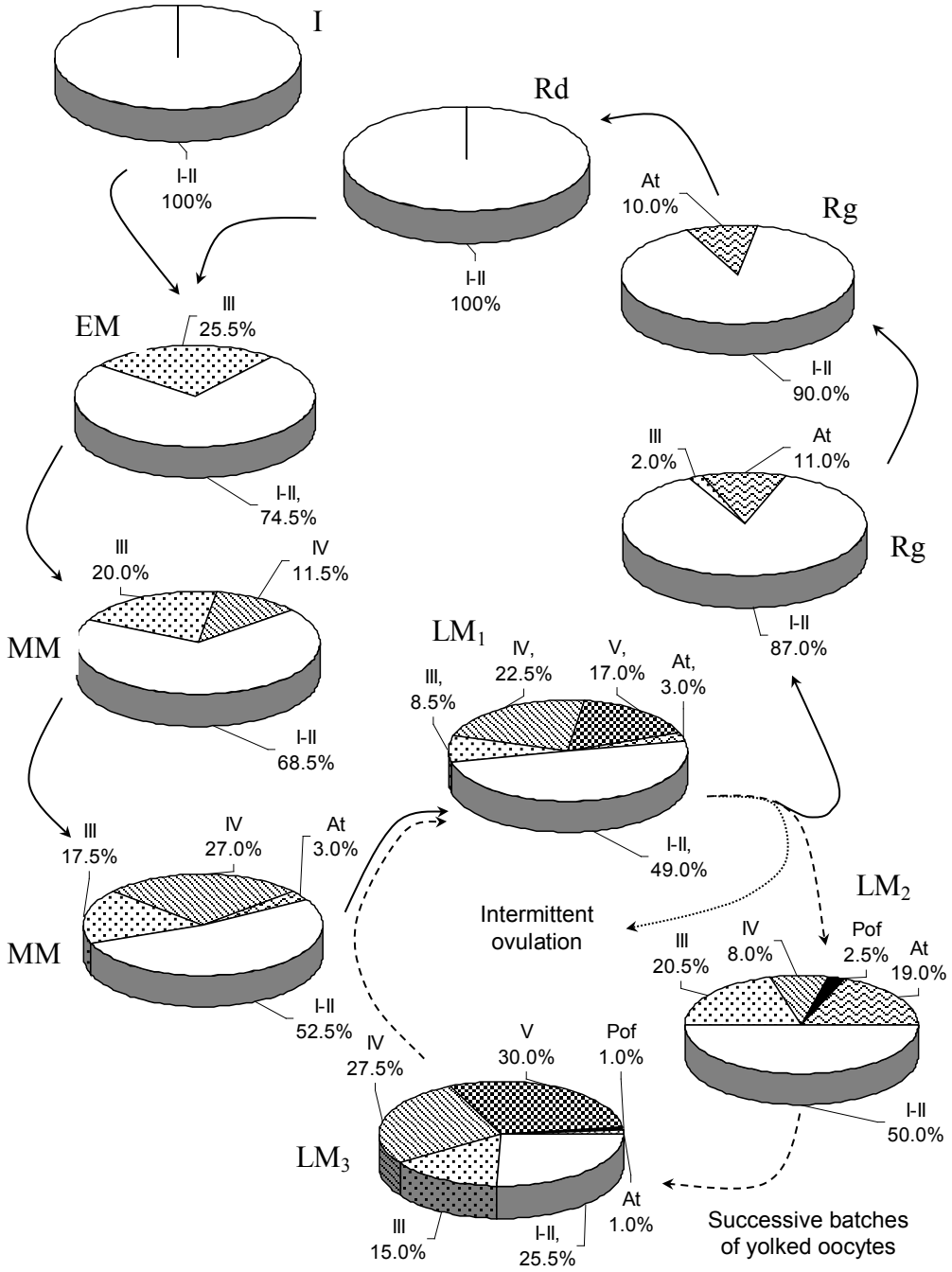

Figure 1. Development of oogenesis stages during a female lane snapper sexual cycle. Stage I; II; III; IV; and V oocytes follow Wallace and Selman (1981). At = atretic oocyte; Pof = postovulatory follicle. Sexual classes: EM = early maturation, MM = mid maturation, LM = late maturation, Rg = regressing, and Rd = regressed (Taylor et al. 1998).

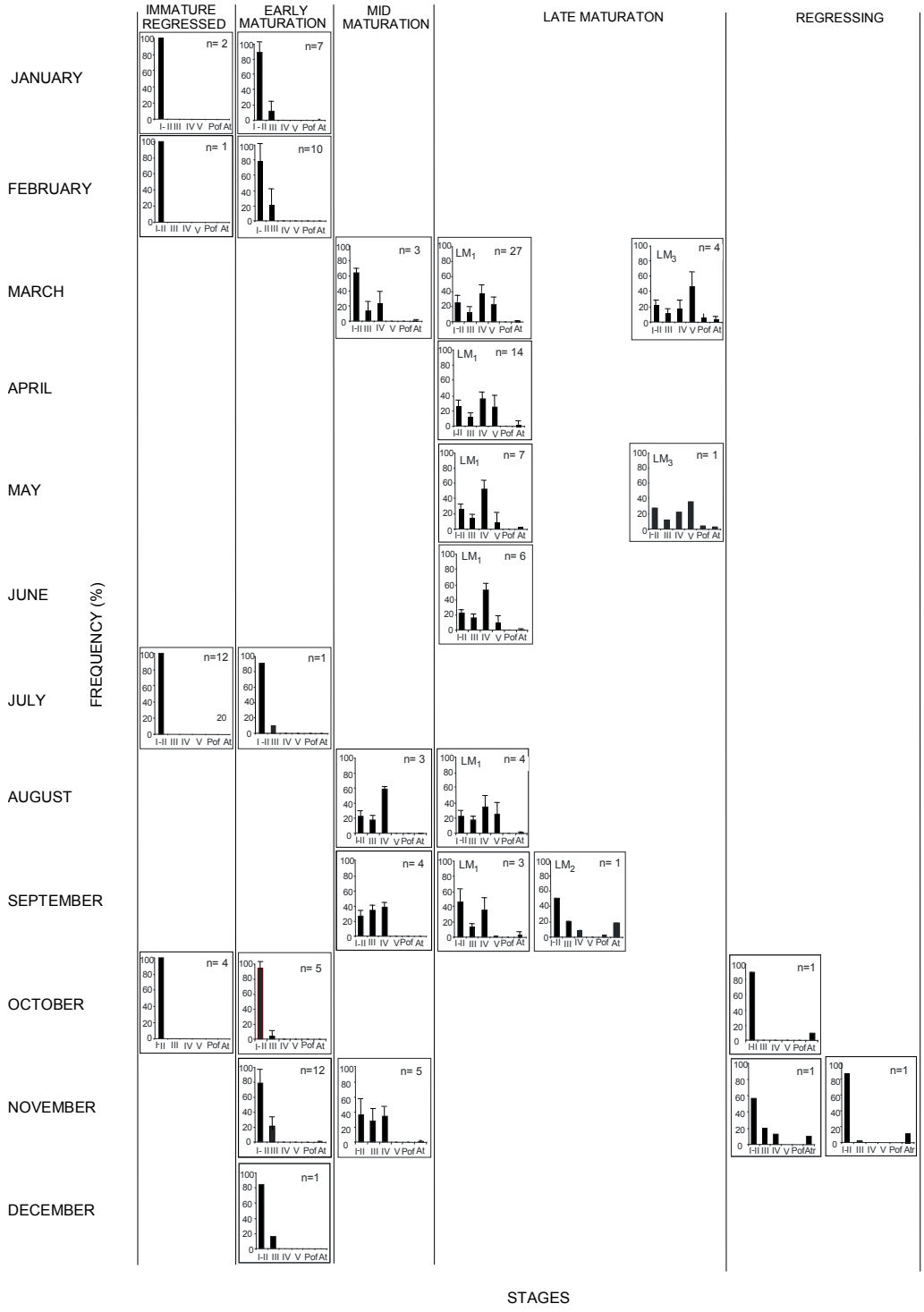

Figure 2. Monthly changes in relative oocyte stage frequency distributions for all female lane snapper collected on the Campeche Bank between March 2007 and February 2008. Females with similar oocyte percentages within each month were grouped to show monthly variations over a single year. Stage I; II; III; IV; and V oocyte following Wallace and Selman (1981). At = atretic oocyte; LM1,2,3 = late maturation sub-classes; Pof = postovulatory follicle. Number of fish sampled is given for each month.

Investigating the Fecundity Type of the Zygoparous Species *Helicolenus dactylopterus dactylopterus* (Delaroche, 1809)

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The bluemouth, *H. d. dactylopterus* (Pisces: Scorpaenidae), is a zygoparous species with internal fertilization that stores the spermatozoa in crypts within the female ovaries and releases embryos in initial development stages enclosed in a gelatinous matrix. It is a batch spawner species but the type of fecundity is unknown. This issue is investigated based on four criteria: (a) oocyte size frequency distribution, (b) the number of yolked oocytes in the ovary, (c) the mean diameter of the advanced vitellogenic oocytes, and (d) atresia, over the spawning season. To estimate the changes in size and number of yolked oocytes during the spawning season, the stereological method was applied. Atresia was analysed based on the incidence and prevalence. The incidence of alpha atresia stage in yolked oocytes was calculated as the percentage of alpha atresia stage oocytes in the total number of oocytes present. The prevalence of atresia (defined as the proportion of females with alpha atresia stage oocytes in the total number of females) was also determined. The results showed that (i) there is no distinct hiatus between pre-vitellogenic and vitellogenic oocytes, (ii) the number of the standing stock of advanced oocytes, and (iii) the mean diameter of the advanced vitellogenic oocytes stays constant, during the spawning season, (iv) a high incidence of atresia was found at the end of the spawning season. These results indicate that bluemouth has most probably an indeterminate fecundity.

Histological Study of Hormonally Induced Spermatogenesis in European Eel (*Anguilla anguilla*)

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Introduction

As part of an aquaculture reproduction study, spermatogenesis was induced in male European eel (*Anguilla anguilla*) through injection of hCG (human Chorionic Gonadotropin) once a week. A method to classify testes development from histological sections was established to allow accurate estimation of male development and comparison of different treatments. The method is based on estimation of the area fractions of various tissue types characterised by different gamete stages in histological sections of testes. The area fractions equalling the volume fractions, i.e. Delesse's principle (Weibel 1989), are used to establish a spermatogenic maturity index (SMI). The index weights the volume fractions of the different tissue types (gamete stages) based on a range of development from 0 to 1. The method provides a quantitative index that eliminates the difficulties finding valid cross-sections of seminiferous tubules for counting tubules containing different categories of germ cells (Neuenhagen et al. 2007). The SMI during testes development was compared to the gonadosomatic index (GSI).

Methods

Males receiving standard treatment (200 IU hCG per eel per week) were sampled weekly at the time of injection. Morphometric measures of the males that were sacrificed were obtained and the testes were preserved in buffered formalin for histological analysis. The remaining males in this and comparative treatments were stripped of semen 24 hours after injection from start of spermiation and onwards. At the end of the experiment, all males were sacrificed and testes development was compared histologically with each other and with a control group.

Histological sections of testis tissue were photographed and a point grid used to determine the area fraction of different tissues. The following tissue categories were distinguished: Testicular stroma (Ts), spermatogonia (Sg), spermatocytes (Sc), spermatids (St) and spermatozoa (Sz). The tissue at each point marked by the grid was identified (Figure 1). The area fraction (F) of each of the tissue categories was estimated by dividing the number of points hitting the particular category by the total number of points of the grid hitting testes tissue. Determination were made from one photograph for each male.

The spermatogenic maturity index (SMI) was calculated for each male (Equation 1) through summation of the area fractions of the different tissue categories weighted by a factor increasing with progressing maturity:

$$\mathrm{SMI} = 0F_{\mathrm{Ts}} + 0.25F_{\mathrm{Sg}} + 0.5F_{\mathrm{Sc}} + 0.75F_{\mathrm{St}} + 1F_{\mathrm{Sz}} \quad (1)$$

Results

The relative area fractions of the different cell types in the developing testes of males treated with hCG are illustrated for a period of seven weeks in Figure 2 in combination with the spermatogenic maturity index. The effect of the injected hormone was observed after the first week with a significant proliferation of spermatogonia. After the second week, spermatocytes had developed and after the third week, spermatids were present. In week 4 the first spermatozoa were observed in the histological sections, and the first semen (spermiation) appeared in week 5. The area fraction and thus volume fraction of tubules with spermatozoa increased gradually from weeks 4 to 7.

The SMI followed the gradual development of gametes towards spermiation and semen production starting with 0 in week zero and increasing to 0.9 in week 7. The SMI was similarly found useful in the comparison among treatments, where F_{SZ} varied among treatments and showed negligible development in the control group. In comparison, the GSI (not illustrated) showed little increase during the first weeks, followed by a step increase.

Discussion

The estimated area fractions of the various tissue types illustrated the rapid progression of gamete stages in the development of testes in response to the hormone injections. The established spermatogenic maturity index (SMI) provided a simple descriptor of the development by grading the volume fractions of the different tissues on a scale from 0 to 1. Using the estimate of area fraction equalling the volume fraction eliminated potential bias in counts of tubules containing different categories of germ cells, which may arise from differences in the orientation of the tissue in histological sections (e.g. Chang et al. 2002; Neuenhagen et al. 2007). The accompanying difficulties in finding valid cross-sections of seminiferous tubule for counting tubules containing different categories of germ cells may lead to subjectivity in the selection of counting areas and biased estimates and hence the index is more reliable (e.g. Chang et al. 2002).

The SMI increased proportionally with the development of the progressive stages of germ cells in the testes during treatment. In comparison, the GSI remained low during the early development of gametes without reflecting the onset of spermatogenesis. The graduation of SMI made it useful to follow and compare the efficacy of treatment, which was confirmed by the study of males, sacrificed by the end of the experiments. Similarly, the SMI may prove useful in the description and comparison of testes development of wild male fish, where spermatogenesis has received much less attention than oogenesis within and among fish species.

Further improvement of the sampling methodology can be made by improving the sampling within testes, ensuring that areas counted are appropriate, and that the number of points per grid is adequate.

A manuscript based on this presentation has been submitted to the journal Marine and Coastal Fisheries as part of the special module "Emerging Issues and Methodological Advances in Fisheries Reproductive Biology". Publication is anticipated in fall 2010. Web page: www.fisheries.org/mcf.

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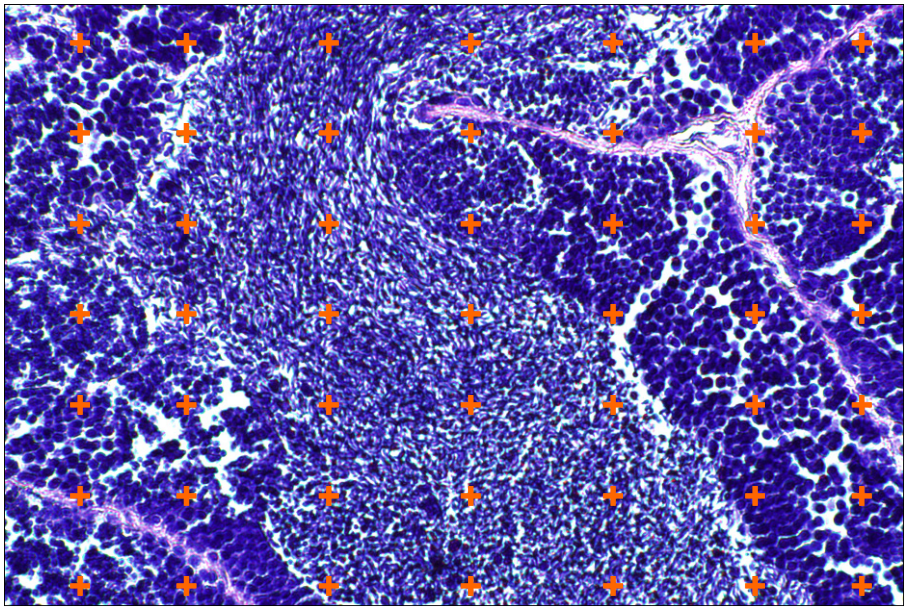

Figure 1. Image of a histological sectin of a developing testis of European ell with all tissue types present. The image is overlaid by a point grid in order to determine the area of fraction per tissue type.

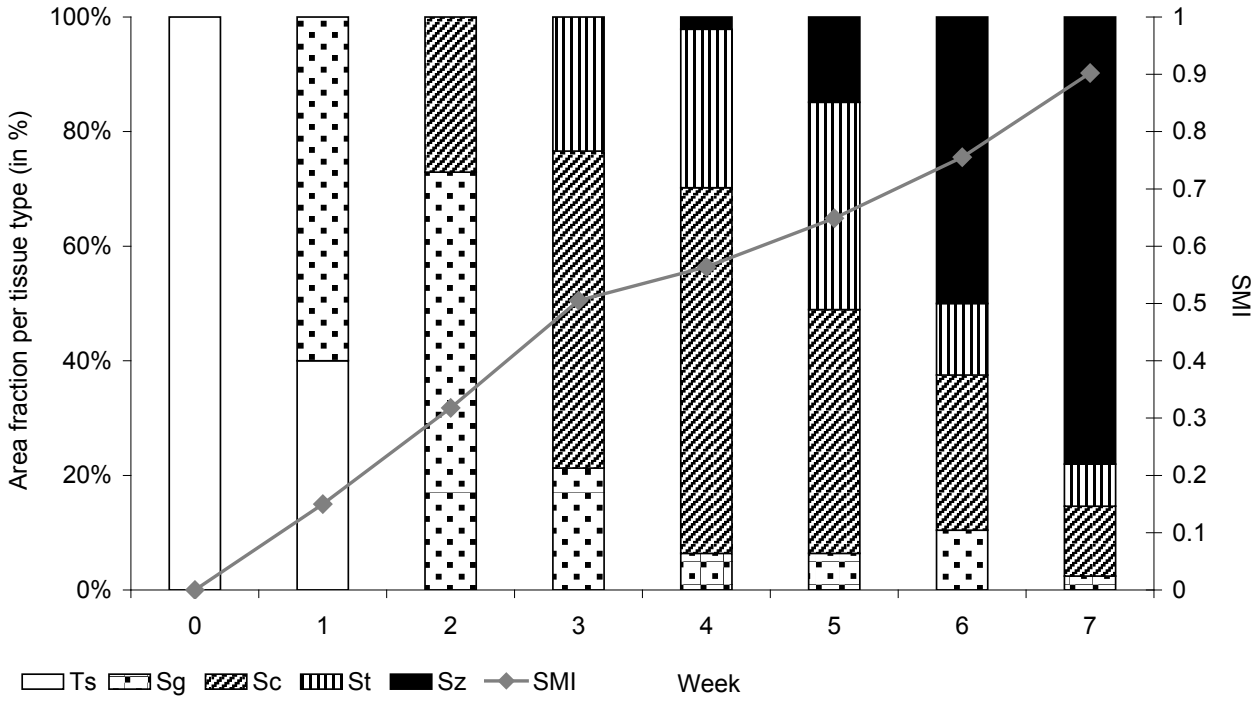

Figure 2. Histological changes of European eel testes during induced maturation from week 0 to week 7 exemplified by one male per week. Week 1 is prior to start of treatment. The columns illustrate the area fractions as percentage per tissue type i.e. Ts: testicular stroma, Sg: spermatogonia, Sc: spermatocytes, St: spermatids, Sz: spermatozoa. The solid line represents the spermatogenic maturity index (SMI).

Stereology as a Tool to Assess Reproduction Strategy and Fecundity of Teleost Fishes

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Introduction

Stereological methods have been applied in studies of fish reproduction to determine frequencies of developing oocytes, fecundity and atresia (e.g. Coward and Bromage 2002, Medina et al. 2002, Emerson et al. 2006). The field of stereology provides techniques for extracting quantitative information about three-dimensional structures from two-dimensional planar sections, in this case oocytes in histological sections. In combination with statistical sampling methods such as systematic random uniform sampling (SURS), absolute numbers of oocytes at all developmental categories in fish ovaries can be estimated, including accuracy of these estimates.

This study combines stereology and SURS, providing an improved tool to 1) describe the relative distributions of oocyte developmental stages, 2) identify reproduction strategies and 3) estimate fecundity. Herring (*Clupea harengus*) is used as a model for the application of unbiased stereological techniques to quantify the number of oocytes of different developmental categories in teleost fish gonads. Due to the relatively simple and well-known reproductive biology as well as their relatively small gonads, herring is ideal as a study object for method development. The objective of the study is to apply modern stereological methods to quantify oocytes in herring ovaries. This information is used to 1) describe oocyte recruitment and development dynamics using size frequency distributions and 2) estimate relative fecundity.

Methods

A random sample of female herring comprising 4 maturation classes, I: juvenile, II: early development, III: mid development and IV: late development was obtained during a research survey in the Baltic Sea. Total length of the fish was measured and the ovary was weighed before preservation in 4% buffered formaldehyde. A fractionator design (Gundersen 1986) was applied using the physical disector to quantify the total number of primary growth oocytes and developing oocytes. In this study, 8 ovaries including 2 per maturity class were analysed. From each fish, one lobe was selected randomly, embedded in paraffin and cut into eight similar sized slabs with a random starting point. From each slab, two disector pairs of 5 µm were sectioned for quantification of primary growth oocytes and developing oocytes, adapting the disector height to the size of the oocytes. Similarly, counting frame size varied depending on the diameter of the developing oocytes. The same frame size was used in both disector types. Depending on the size of the ovary, an area sampling fraction of 25 to 100% was used. Two-way counting was used and the area of the sampled oocytes was measured using the nucleator (Gundersen et al. 1988). The nuclei were used as the counting object. This sampling procedure is in accordance with systematic uniform random sampling (SURS).

For each ovary the number of oocytes of a particular developmental category was calculated using the fractionator equation:

$N_i = 1/osf * 1/ssf * 1/asf * 1/TW * 1/1+m * \sum Q-$ i=PG, CA, Vt_a, Vt_b

N_i: Number of oocytes of a particular developmental stage (i)
osf: Ovary sampling fraction
ssf: Section sampling fraction
asf: area sampling fraction
TW: 1 if one-way counting; 2 if two-way counting
m: Number of sections skipped in dissector
Q-: Number of oocytes counted
PG: Primary growth oocytes (maturity class I)
CA: Cortical alveoli stage (maturity class II)
Vt_a: Early vitellogenic (maturity class III)
Vt_b: Late vitellogenic (maturity class IV)

Additionally, the degree of oocyte shrinkage from fresh to embedded tissue was measured in order to correct the diameter of the embedded oocytes in the ovaries used to quantify primary growth oocytes and developing oocytes. Female fish comprising 4 maturation classes were randomly selected for this study. Fresh tissue was sampled from one lobe, while the other lobe was preserved in 4% buffered formaldehyde and embedded in paraffin. Oocytes areas from 8 random samples of each lobe were measured using the nucleator method. A grid was placed over smears of fresh tissue and oocytes hitting a grid point sampled, likewise for sections of embedded tissue. The areas were converted to diameters to compare with previous studies. In this study, 6 ovaries were analysed. Shrinkage due to histological processing was estimated and used to estimate fresh oocyte diameters from the processed tissues.

Results and Discussion

This study shows how the stereological methods can be successfully applied to illustrate oocyte recruitment and development dynamics using size frequency distributions and thereby describing the fecundity type and reproductive strategy (Murua and Saborido-Rey 2003). In Figure 1, the group synchronous development of oocytes and determinate fecundity of herring is clearly demonstrated. Furthermore, the numbers of oocytes per g ovary (Figure 2) was estimated for application in fecundity estimation and comparison among small and large herring specimens within maturity stages.

The applied stereological method allows accurate determination of the number of oocytes in ovaries. Stereological methods can be labor intensive. However, when using automated equipment and software for stereological analysis, design based stereology provides a useful supplement to gravimetric, volumetric or autodiametric quantification methods used for fecundity determination (e.g. Coward and Bromage 2002, Emerson et al. 2006).

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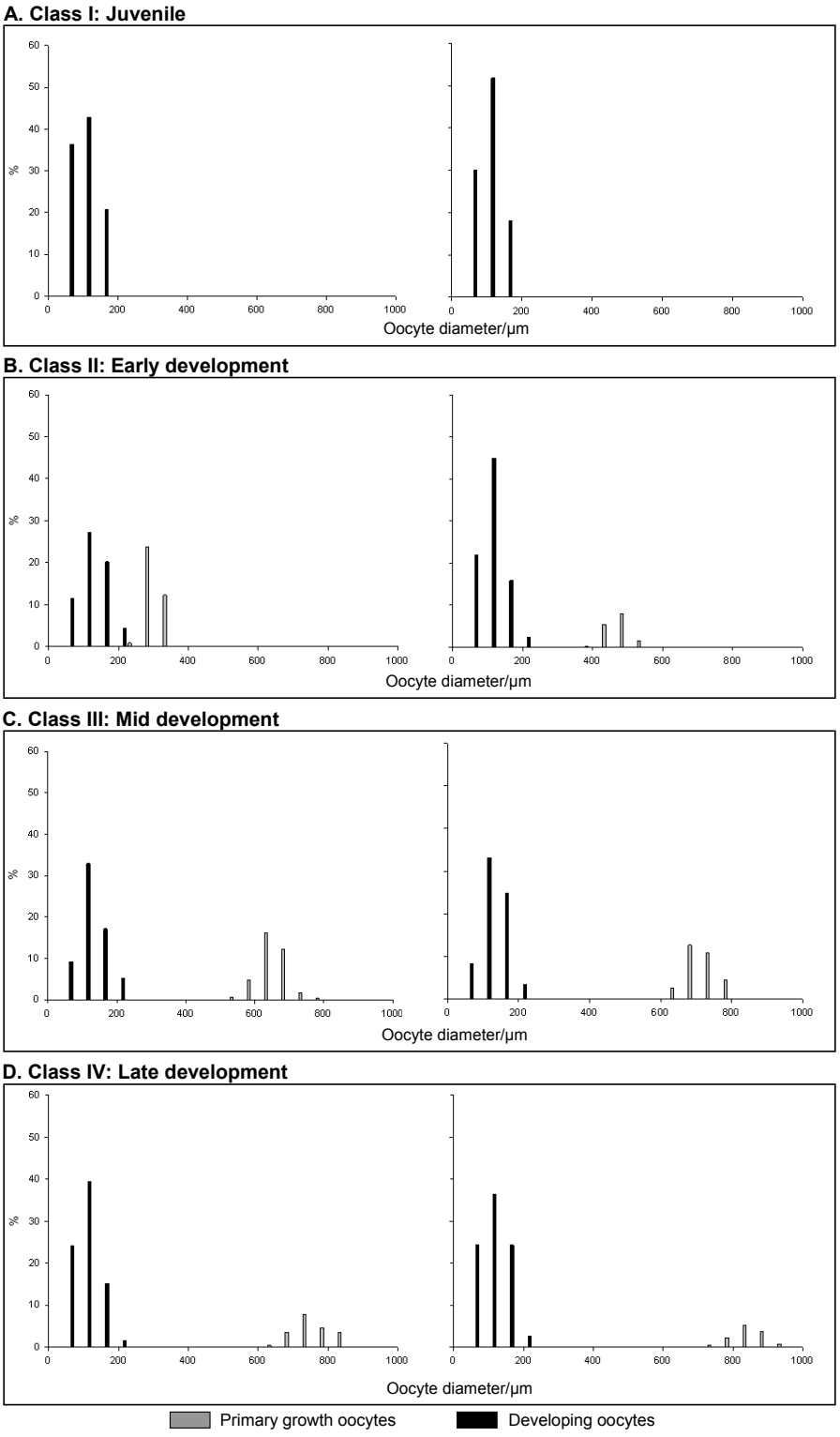

Figure 1. Oocyte size frequency distributions in developing ovaries of herring (*Clupea harengus*) illustrated as the relative abundance of primary growth oocytes and developing oocytes in 8 ovaries comprising 4 maturity classes A-D. Oocyte diameters are corrected for shrinkage and correspond to fresh oocyte size.

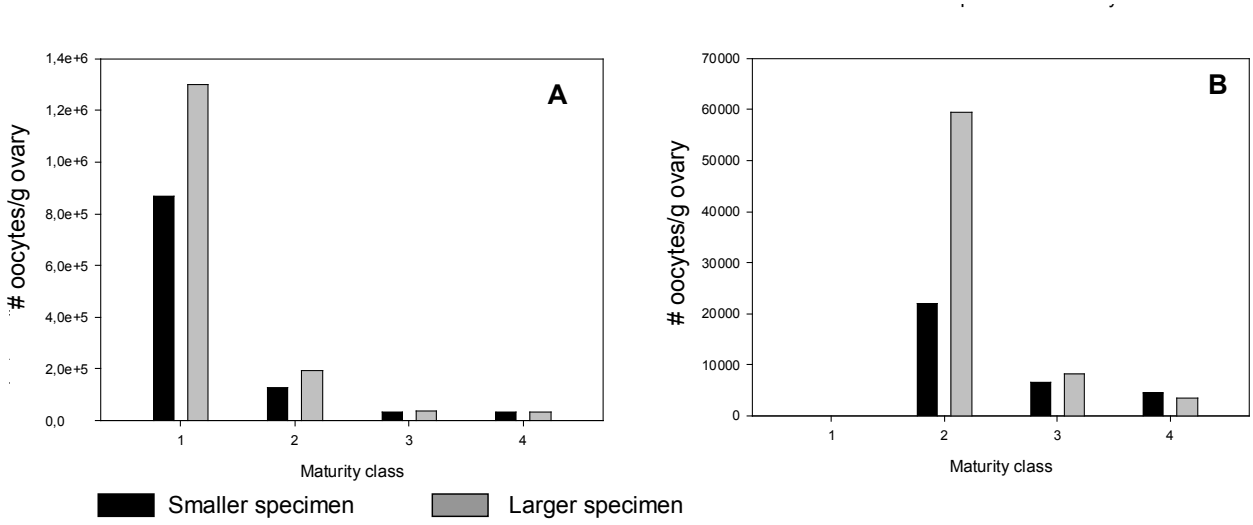

Figure 2. Number of ooytes in ovaries of herring of different size and maturity class. A. Number of primary growth oocytes per gram ovary. B. Number of developing oocytes per gram ovary. Within each maturity class, the columns illustrate an example of one relatively small and one relatively large herring.

Quantification of Ovarian Follicles in Bluefin Tuna by Two Stereological Methods

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Introduction

Estimates of fecundity are often obtained from counts of advanced (large yolked) oocytes in the ovary using the gravimetric method, stereology applied to histological sections, or automated particle counting. Data on the number of large yolked oocytes may significantly overestimate the actual fecundity (realised fecundity) because a proportion of these oocytes can degenerate and be reabsorbed before spawning (Kjesbu et al. 1991; Óskarsson et al. 2002; Kurita et al. 2003). A probably more accurate approach to determine the exact number of eggs released in a spawn is to count the number of fresh post-ovulatory follicles. The number of different types of ovarian follicles was estimated in bluefin tuna *Thunnus thynnus* (L.) using model-based and disector stereological methods. For evaluation of developing follicles, three distinct oocyte developmental stages were considered: lipid-stage, vitellogenic and migratory-nucleus. In addition, degenerating follicles in α and β stages of atresia, as well as freshly ovulated follicles (post-ovulatory follicles) were quantified by both stereological methods.

Methods

Nine mature female Atlantic bluefin tuna were sampled either during spawning migration at the Strait of Gibraltar or at the spawning area around the Balearic archipelago. The gonadosomatic index (I_G) was calculated as percentage of gonad weight relative to total body weight, $I_G = 100 \times W_G \times W_B^{-1}$. The ovarian volume ($V_G$) was estimated from the ovarian weight (W_G) according to the equation: $V_G = 0.9174 \times W_G$ (Medina et al. 2007).

Ovary samples were fixed for 24 h in 4% buffered formaldehyde, then dehydrated through increasing concentrations of ethyl alcohol, cleared in xylene, and embedded in paraffin. Serial 10-µm sections were stained with hematoxylin-VOF. Four distinct types of developing follicles were quantified depending on the respective oocyte maturation stages: lipid-stage (LSF), vitellogenic (VF), and migratory-nucleus (MNF) follicles. In addition, α-stage (αAF) and β-stage (βAF) atretic follicles and post-ovulatory follicles (POF) were counted.

Numbers of the different categories of follicles were calculated according to the formula of Weibel and Gomez (1962):

$$N_V = \frac{K}{\beta} \frac{N_A^{3/2}}{V_V^{1/2}}$$

, where N_V: numerical density (number per unit volume) of the follicle type, β: shape coefficient, K: size distribution coefficient, N_A: number of follicle transections per unit area, V_V: volume fraction. The total number of ovarian follicles (N) was obtained extrapolating N_V to the total volume of the two ovaries.

Numbers of follicles were also estimated by the physical disector method (Sterio 1984). For convenience, the oocyte nuclei of all developing follicles were counted instead of the entire follicles, whereas for quantification of POFs and atretic follicles, which lack a distinct oocyte

nucleus, the whole elements were considered. The numerical density of follicles (N_V) was estimated as follows:

$N_V = \frac{Q^-}{a \cdot P \cdot h}$, where h: separation between sections (from ¼ to ⅓ of the mean particle diameter); a: area of the counting frame, P: total number of disectors used, Q^-: total number of follicles counted in the P disectors.

Results

Excepting one individual, the estimates of LSFs, VFs and MNFs by the model-based method tended to be lower than those calculated by the disector method. In general, the counts of VFs and MNFs made with both techniques were similar (Figure 1).

Counts of αAFs by the model-based method in all specimens but two were clearly higher than those made using the disector method (Figure 1). Similarly, the model-based method overestimated the amount of βAFs in relation to the disector technique in all cases but one, both estimates differing considerably (Figure 1). In contrast, the numbers of POFs estimated by the disector method were larger than those calculated with the model-based method. The disector produced counts of POFs that were about 20% lower than those of MNFs.

Discussion

Previous estimations of fecundity of bluefin tuna have been made from stereological counts of large yolked oocytes, which define the potential fecundity. These data are thought to overestimate the actual (realised) fecundity because they do not consider follicular atresia. Therefore, a straightforward approach to determine the realised fecundity is quantification of POFs.

The estimates of numbers of developing follicles obtained with the two procedures were generally similar, the model-based method giving slightly lower values. However, high discrepancies occur in atretic follicles (especially βAFs) that may be due to the irregular shape and size of these particles. Therefore, quantitative assessments of highly irregular follicles (*e. g.*, atresia) and POFs (realised fecundity) should be carried out by assumption-free stereological procedures.

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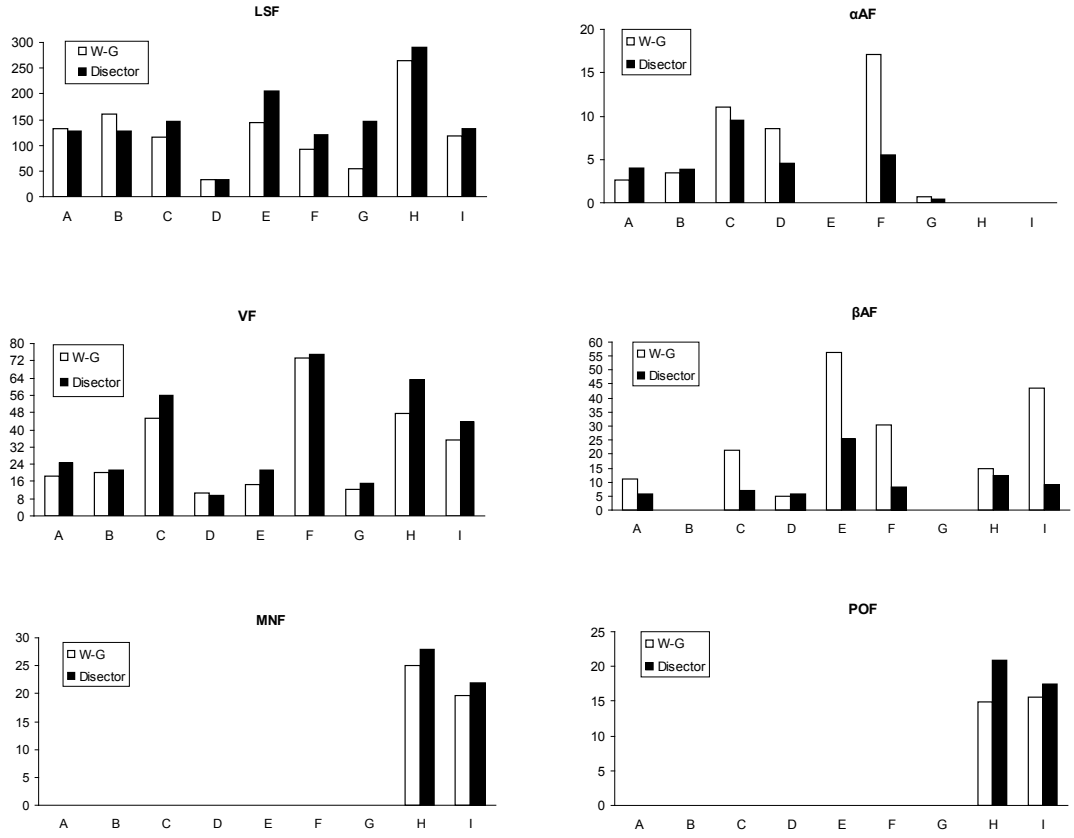

Figure 1. Counts (in millions) of the different follicle types in nine bluefin tuna obtained with the two stereological procedures: Weibel and Gomez (1962), physical dissector (Sterio 1984). The highest differences are seen in atretic follicles. LSF, lipid-stage follicles; MNF, migratory-nucleus follicles; POF, post-ovulatory follicles; VF, vitellogenic follicles; αAF, α-atretic follicles; βAF, β-atretic follicles.

Pattern of Previtellogenic Oocyte Recruitment Into Secondary Growth Phase in Indeterminate Reproductive Style Species

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The early-cycle follicular recruitment into vitellogenesis has previously been partly described and quantified in species with a determinate reproductive style. In contrast, this follicular recruitment event is even less understood in indeterminate reproductive style species due to: (i) the multiple size classes in the standing stock of oocytes that present no evidence of gap formation between vitellogenic oocytes and the pool of oocytes in the primary growth phase (Murua and Motos 2006) and thereby complicate any quantification of the vitellogenic oocytes; (ii) apparently an endless number of previtellogenic oocytes that may recruit to the secondary growth phase; (iii) the likely insignificant level of atresia during previtellogenesis but, on the other hand, a sporadic appearance of this resorption process during later phases of vitellogenesis growth; and (iv) the inefficiency of traditional methods to account for any of these three points (Witthames et al. 2009).

Each species specific reproduction model then may be somehow dependant on the dynamic organisation of the ovary, which in turn determines the methodology dealing with the quantitative measurements. By using a multidisciplinary approach that combines theoretical oocyte geometry (Kurita and Kjesbu 2009) with stereological measurements aided by image analysis, the oocyte packing density of previtellogenic, vitellogenic and final maturation oocytes have been estimated in the ovaries of European hake *Merluccius merluccius*. The corresponding maturity stages were: 1) the first follicular growth phase, 2) early secondary follicular growth phase, and 3) late secondary follicular growth phase. This approach was undertaken in order to understand the cyclical recruitment of oocytes from the pool of previtellogenic oocytes during early cycle follicle recruitment into the secondary growth phase in a species with indeterminate fecundity and to assess the ovarian dynamic model of European hake.

The explanatory variables (volume-based mean oocyte diameter (ODv), volume fraction of oocyte in the ovary, specific gravity of the ovary, oocyte shape) to estimate oocyte packing density for each developmental oocyte stage (previtellogenic, lipid vesicles, cortical alveoli, vitellogenic, advanced vitellogenic, migrating and hydrated) (Figure 1) were achieved using histological slides of ovaries in every maturity stage. ODv was deduced from the arithmetic mean oocyte volume. The volume fractions of oocytes were measured by point counting using a grid of 256 points for stereological measurements (Howard and Reed 1998) among 5 fields sampled at systematically random. Specific gravity was set to 1.072 for the ovaries below advanced vitellogenic developmental stage and 1.061 for migratory nucleus and hydrated ovary stages (Kurita and Kjesbu 2009). Oocyte shape at each developmental stage was fixed measuring the long and short axis of oocytes. Finally, the estimation of oocyte packing density was previously conducted using the auto-diametric fecundity method (Thorsen and Kjesbu 2001), and in order to relate these results with those obtained with the theoretical

estimation of oocyte packing density the shrinkage factor was determined. Data validation was also assessed.

The volume fraction of oocytes increased stage to stage from previtellogenic to hydrated ovaries in a logarithmic manner (r^2=0.76, $p<0.05$) (Figure 2A). These volume fractions were estimated with a mean error of about ± 0.05 which was deemed precise taking into account oocyte distribution in the European hake ovary was considered to be homogeneous (Witthames et al. 2009). The number-based oocyte diameter was not sufficient predictor of the oocyte packing density in the case of European hake. However, the relationship between estimated oocyte packing density and volume-based mean oocyte diameter had a coefficient of determination for the regression as high as 0.89 for oocytes ≥ previtellogenic stage (Figure 2B). The oocyte sizes determined by the two methods were interrelated with a shrinkage factor of 13.76%.

The subsequent analysis demonstrated for the first time that the European hake presents a *continuous* indeterminate reproductive model in which there is 1) always a stock of first growth phase oocytes present and 2) oocyte recruitment and subsequent developing oocyte growth follow each other closely during the reproductive season. Thus, in contrast to common believe, there is no accumulation of oocytes at any threshold size limit, i.e., the recruitment of oocytes into vitellogenesis during the follicular cycle should be considered as rather constant.

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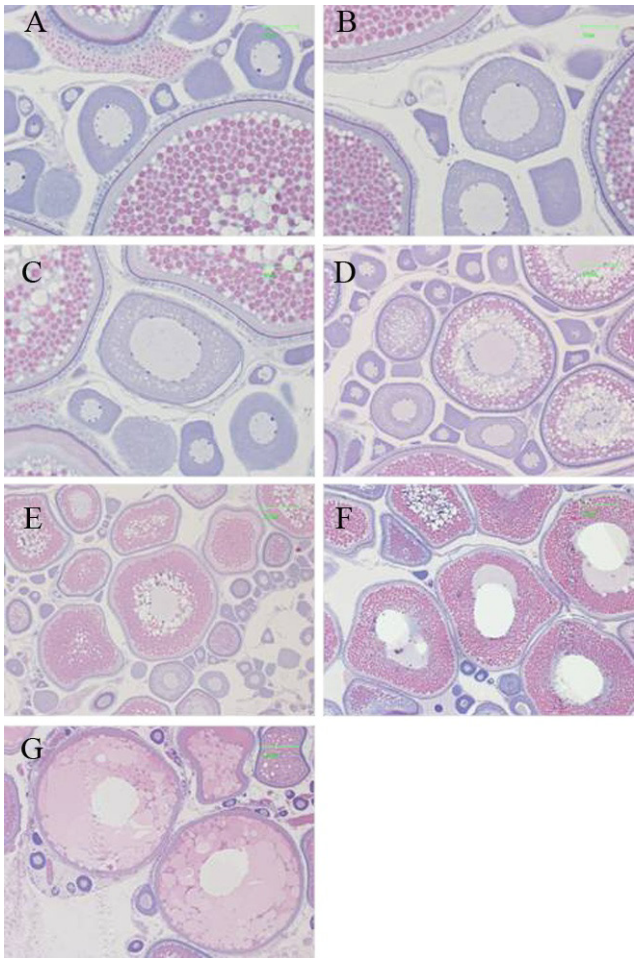

Figure 1. Each of the oocyte development stages measured in this study in order to account for recruitment. (A) primary oocyte; (B) lipid vesicles; (C) cortical alveoli stage; (D) vitellogenic; (E) advanced vitellogenic; (F) migrating and (G) hydrated .

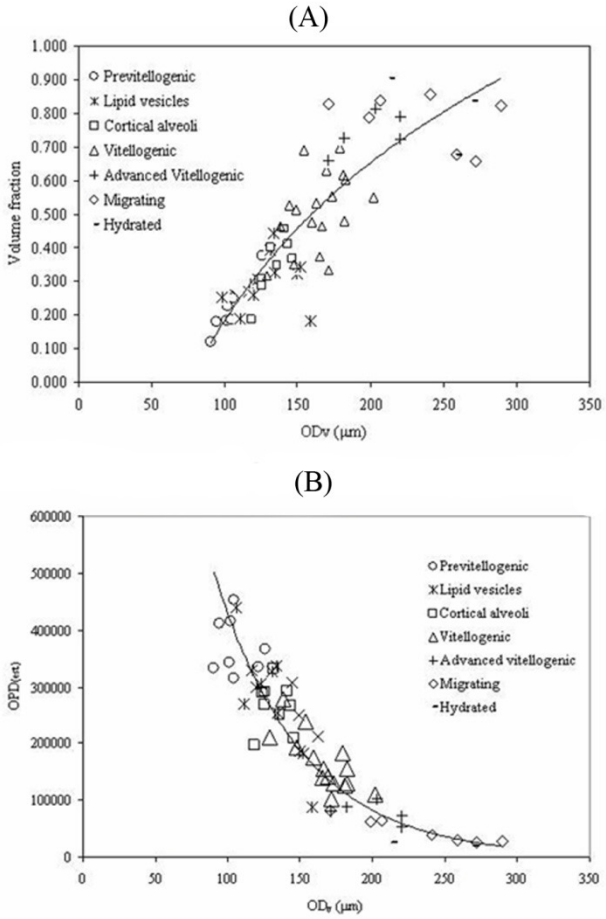

Figure 2A. Volume fractions of oocytes plotted against volume-based mean oocyte diameter (OD_v) for each maturity stage (primary oocyte, lipid vesicle, cortical alveoli, vitellogenic, advanced vitellogenic, migrating and hydrated).
Figure 2B. Theoretically calculated oocyte packing density where the number of oocytes per unit of ovary volume are plotted against volume-based mean oocyte diameter (OD_v) for each maturity stage.

Impact of Mass Atresia in Reproductive Ecology, Maturity Ogive, Spawning Migration and Population Dynamics on *S. mentella* in Icelandic Waters

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Introduction

Mass atresia or skipped spawning in fish with determinate fecundity has been documented in a number of species, particularly in freshwater and anadromous fishes and in less degree in marine species (Rideout et al. 2000). This phenomenon can occur at early stages of secondary growth oocyte development (cortical alveoli) or at any time during vitellogenesis by mass atresia of all vitellogenic oocytes (Rideout et al. 2005). In redfish, mass atresia has not been studied, but in this paper it is shown that it is a common feature in the main redfish species inhabiting Icelandic waters, *Sebastes mentella*.

Material and methods

A total of 1,086 ovaries of *S. mentella* in Icelandic waters were collected between 2000 and 2002 throughout the year. Almost a third of the analyzed fish were immature. In this study only mature fish were considered (n = 451). Included with these, however, are those fish that showed CA in any season of the year, with or without a high amount of atresia. These fish are difficult to distinguish from those showing a normal pattern of oocyte development, except in months were the presence of CA is known to be abnormal. However, it was decided to include them in the estimations of the proportion of the different stages by month. No samples were available in July and August.

Since it is not relevant to know the total number of atretic oocytes for this study, atresia was quantified in a simple manner. The number of atretic oocytes in relation to the number of oocytes in secondary growth stage was counted in 4 images from each fish. A fish was considered to be in the skipped spawning stage when more than 25% of the oocytes were atretic.

Results and Discussion

Mass atresia was observed both at the previtellogenic stage and especially during vitellogenesis. The presence of mass atresia during vitellogenesis means that fish will definitely not spawn, since there is not sufficient time to develop another clutch of oocytes.

The reproductive cycle in Iceland is similar to other cold waters species, with ovulation occurring at mid-winter (January - February), based on the proportion of females with embryos in the ovary, and spawning (parturition) occurring in April. The onset of ripening begins in spring, when females were in postspawning and recovering stages, as observed from the presence of CA oocytes and the first occurrence of oocytes in vitellogenesis.

Massive atresia was observed in February and March in oocytes at late vitellogenesis, in May and June in oocytes at the beginning of vitellogenesis and in autumn in developing oocytes. Although atresia was not fully quantified, the levels of atresia were low in fully developed oocytes (February and March), but abundant in autumn, reaching in some cases the consideration of reproductive failure (mass atresia).

During autumn in northwest Iceland, 95% of females with CA oocytes and 39% with vitellogenic oocytes showed massive atresia; in winter-spring more than 60% of the females in CA were skip spawning. In southeast Iceland during autumn, 90% of the females with CA skipped spawning, but only 8% of those in vitellogenesis did so. Interestingly, in winter-spring in this area, 81% of females with CA oocytes and 100% with vitellogenic oocytes showed mass atresia. Finally, in southwest Iceland (the spawning area), massive atresia was only relevant in females with CA oocytes, both in autumn (92%) or in winter-spring (100%).

Combining the proportion of skip spawners and the presence of spawning activity in each of the areas gives a good overview of the reproductive strategy of *S. mentella* in Iceland. Generally speaking, northwest Iceland is an area where neither spawning nor final vitellogenesis occurred, because basically immature or skip spawners are found in this area. Northwest Iceland is an area where only the onset of maturation and ripening occurs (Figure 1). Non skip spawning fish migrate in autumn towards south Iceland, but in southeast Iceland they mature (ripen) but do not spawn. This suggests an active migration in spring to southwest Iceland, where both ripening and spawning are found (Figure 1). Females that are not able to complete vitellogenesis do not migrate to the spawning area but remain in the southeast, where massive atresia is induced, possibly due to fish condition, feeding or environmental conditions.

In February and March, approximately 93% of the maturing females < 35 cm were skip spawners, while only 26 % of females > 35 cm were in such situation. In autumn, the proportion of skip spawners was lower, but still 74.3 % of the females < 35 cm were skip spawners comparing with only 18 % of the females > 35 cm. However, a proportion of the skip spawning fish may be not detected if their oocytes never progress past the primary growth stage, and thus the may be considered immature. An indication of this fact may be the unusually high proportion of immature fish between 35 and 40 cm.

These results have major implications in the estimation of the maturity ogive. Non-skipping mature fish migrate to different areas in different seasons. However, massive atresia alters this migration pattern, with skipped spawning fish remaining in the area when massive atresia occur. Thus, when and how ogives are estimated yields different results (Figure 2), with drastic differences that can completely change the perception of the SSB. When estimating size at maturity following normal procedures, i.e. considering apparently mature fish at all areas and seasons, a value of 34.8 cm is obtained. However, if we use only females at the spawning site while discounting those with massive atresia (histology is required), female size at maturity increases to 41.2 cm. This means that it is very important when and where ogives are estimated, and hence when surveys and sampling are conducted to estimate SSB.

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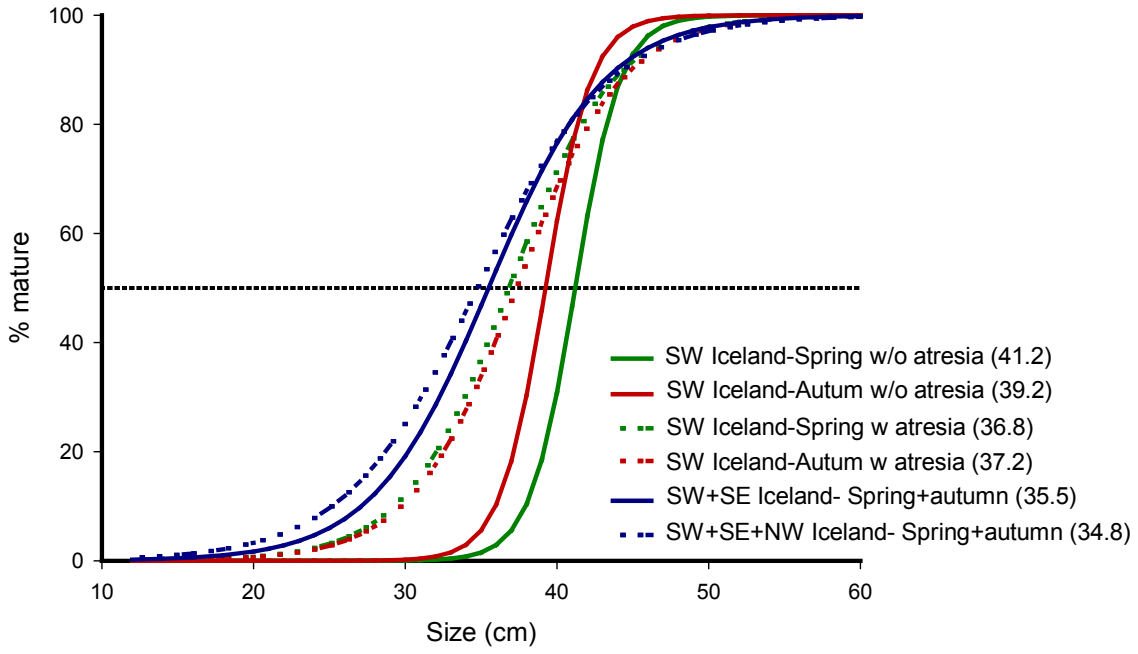

Figure 1. General pattern of female migration from ripening to spawning, and major areas where massive atresia is observed.

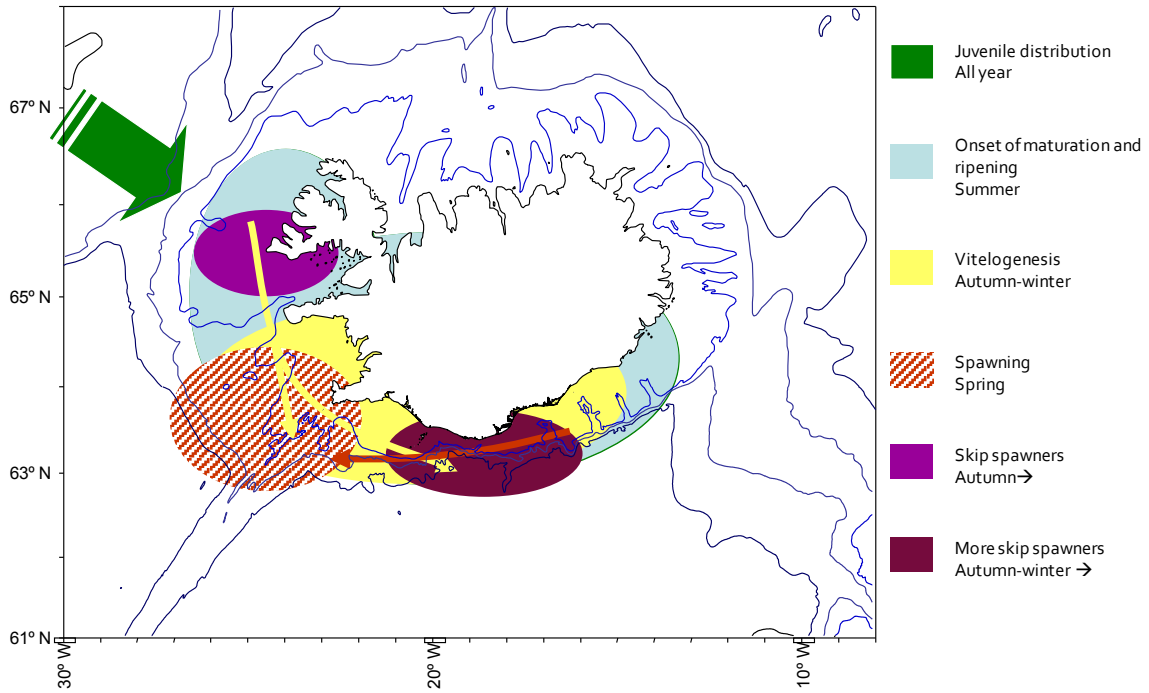

Figure 2. Maturity ogives of *S. mentella* in Icelandic waters estimated in different areas, seasons and with (w) or without (w/o) females with massive atresia. Values in brackets indicate size at maturity (in cm).

The Reproductive Biology of Female Winter Flounder (*Pseudopleuronectes americanus*): Validating Classification Schemes to Assess the Importance of 'Skip Spawning'

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Introduction

Winter flounder, like most pleuronectid flatfishes, are iteroparous. At the southern part of their range in the northwest Atlantic, winter flounder usually mature at three years of age and may live up to 16 years (Witherell and Burnett 1993). Mature female winter flounder may not, however, spawn every year after initial maturation (i.e. 'skip spawning'). Previous histological studies have demonstrated pre-vitellogenic oocytes are established 2-3 years in advance of spawning (Dunn and Tyler 1969), but some females do not advance a clutch of oocytes to spawn in some years.

Skip spawning rates by captive, laboratory winter flounder are a function of the nutritional status of the fish (Burton 1994). In the wild, winter flounder occur across a wide latitudinal range, so temperature may have a major impact on reproductive output and the prevalence of skipped spawning because of latitudinal effects on food resources and temperature effects on feeding rates. Supporting this postulation, estimates of skipped spawning in northern populations of winter flounder are higher (>20% for females in Newfoundland, Canada; Burton and Idler 1984) than observed for southern populations (~5% for females offshore of New Jersey, USA; Wuenschel et al. 2009). The latter results were, however, based solely on macroscopic criteria.

Herein, we report on our efforts to develop microscopic, histological criteria for evaluating skip spawning by female winter flounder at the southern extent of their range. We agree with Rideout et al. (2005) that intra-specific comparisons of skip spawning rates could lead to a better understanding of the environmental factors that cause it and could be used to better predict its occurrence. We are also motivated by the fact that skip spawning fundamentally means that estimates of spawning stock biomass overestimate annual reproductive potential. Undoubtedly, better estimates and predictions of skip spawning rates could be useful for stock assessment of winter flounder. Therefore, another goal of this study was to validate macroscopic maturity schemes with histological data so that skip spawning rates could be reliably measured in the field.

Materials and Methods

We collected winter flounder in the spring and fall seasons from trawl surveys by the United States' National Marine Fisheries Service (Northeast Fisheries Science Center), as well as trawl surveys operated by the State of Massachusetts and the State of Rhode Island. Fish were collected from three stocks of winter flounder (Gulf of Maine, Georges Bank, and southern New England) during the years 2006-2008. Presently the data are pooled but stock-specific data will be reported in future analyses.

Results and Discussion

We used a simple scheme (Figure 1) to assign maturity based on histological characters including the most advanced oocyte stage and the presence of postovulatory follicles. When compared to macroscopic classifications determined in the field, results indicated significant misclassifications between immature and resting fish in the spring which has implications for maturity ogives and estimates of spawning stock biomass without consideration of skip spawning. Many immature fish classified incorrectly as resting possessed oocytes in the late cortical alveoli stage indicating they were already beginning to develop for the first time, a full year in advance of spawning, but had no evidence of prior spawning. Other, more subtle differences (e.g. spent vs. resting) occurred, due in large part to the greater detail revealed through histology. These results are directing the development of training materials to reduce misclassifications of maturity class in the field.

To improve our understating of the reproductive biology of winter flounder, a more detailed classification scheme (Figure 2) is proposed as well. This scheme enabled the differentiation of first time from repeat developers, allowing us to more accurately identify individuals that were truly resting during the spawning season (i.e. about 5% were identified as skip spawners during the spring spawning season). Development from primary growth oocytes to hydration takes approximately 1 year, therefore oocytes preparing for the subsequent year enter into late cortical alveoli stage soon after spawning. Given this prolonged pattern of oocyte development it should be possible to estimate skipped spawning at intervals between spawning seasons. Therefore, we determined the most advanced oocyte stage of fish collected in the fall (a non-spawning season). By fall, most mature individuals (50-80%) had already begun vitellogenesis, with decreasing percentages of individuals in the late cortical alveolar stage. Primary growth oocytes were the most advanced oocyte stage for a small group of mature fish (~5-7%), identifying these fish as skip spawners during the fall non-spawning season.

Therefore, multiple lines of evidence (resting mature fish in spring, and most advanced oocyte stage primary growth in fall) indicate skipped spawning is low (~5-7%) for female winter flounder for these southern stocks in recent years. This may not represent a significant reduction in egg production as compared to estimates ignoring skipped spawning, however, preliminary evidence suggests skip spawners are more likely to be 2nd time spawners, and these comprise a marked proportion of mature fish for stocks which possess a truncated age structure as a result of overfishing. Future stock assessments should evaluate potential effects of various levels of skipped spawning on population abundance to acknowledge likely annual variation due to environmental conditions. Spawning and skipped spawning are potential outcomes resulting from different energy allocation strategies in fish. As such the interpretation of fish reproduction in general and gonad histology in particular can benefit from a basic understanding of seasonal cycles in energy storage and depletion.

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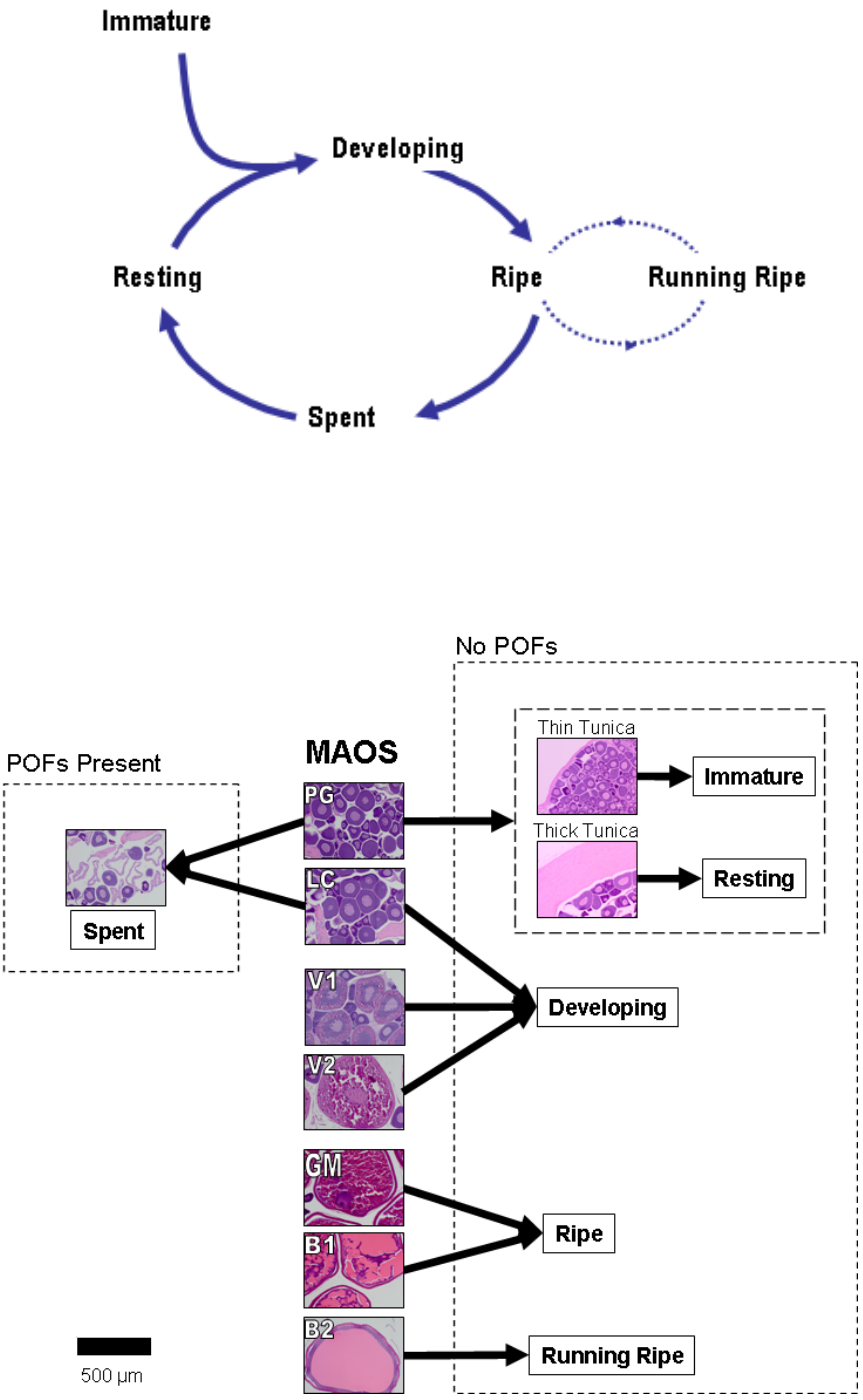

Figure 1. (Top) A general classification scheme used to describe maturity of fishes. (Bottom) The decision tree of histological characteristics used to assign those maturity classes. B1 - hydrated oocyte, still in follicle; B2 - hydrated, ovulated egg; GM - germinal vesicle migration; LC - late cortical alveoli; MAOS - most advanced oocyte stage; PG - primary growth; POFs - postovulatory follicles; V1 - early vitellogenesis; V2 - late vitellogenesis. Scale bar (lower left) applies to all images.

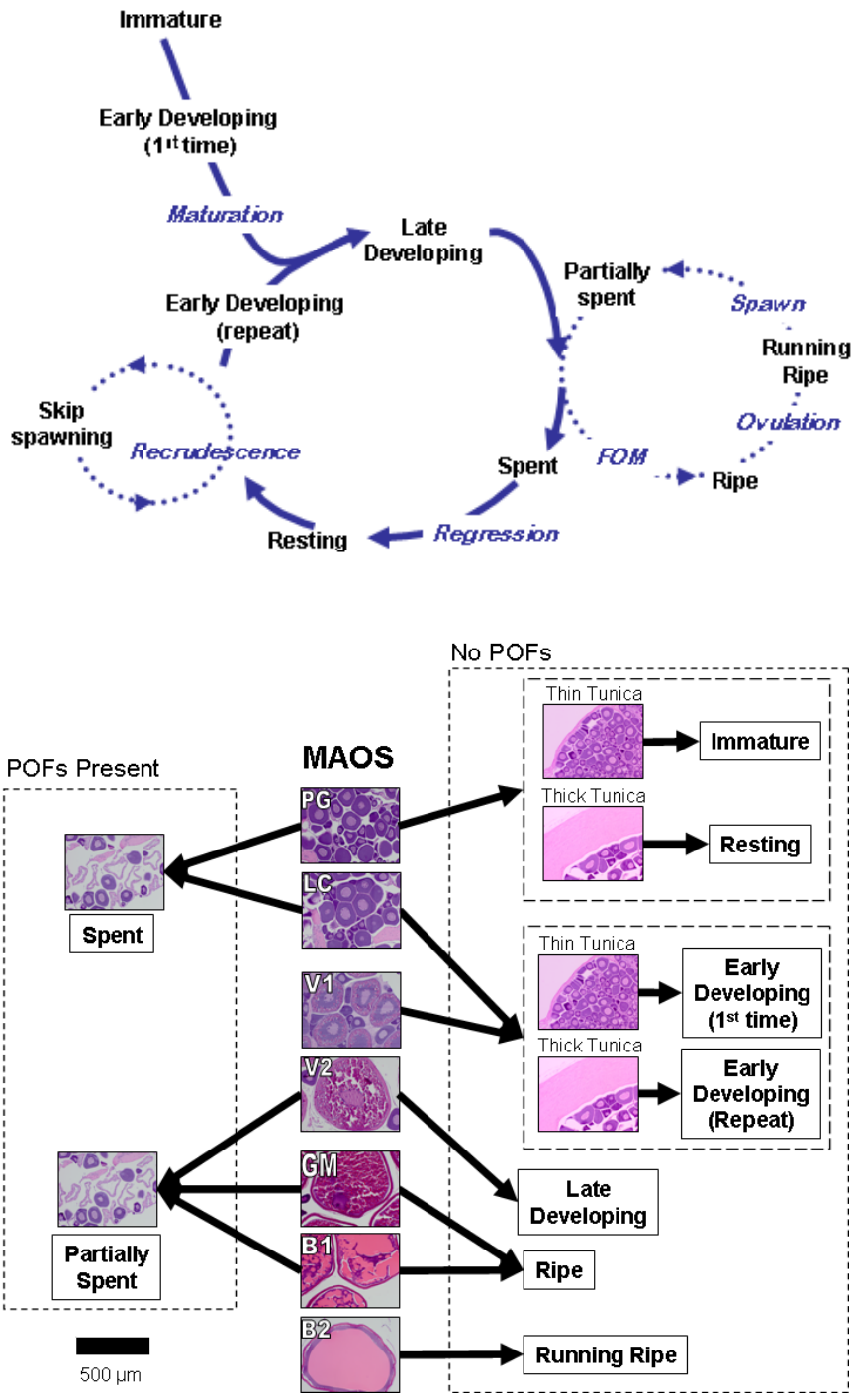

Figure 2. (Top) A more detailed classification scheme used to describe maturity of fishes that differentiates first and repeat developing fish, includes a partially spent class to reflect batch spawning, and indicates the possibility of skip spawning. (Below) The decision tree of histological characteristics used to assign those maturity classes. Labels as in Figure 1, scale bar (lower left) applies to all images.

Prevalence of Intersex in Eelpout (*Zoarces viviparus*) as an Ecosystem Status Indicator

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A histological analysis of 200 male eelpout gonads was performed as part of biological field studies carried out in coastal waters of Denmark during prespawning and spawning time in May-June. In the marine environment, the eelpout is often selected as sentinel species. Histology of the testis revealed the presence of intersexuality in specimens from all six areas investigated with a prevalence ranging from 8 to 36 % of the male population (on average 22 %). The intersex condition was defined by the simultaneous presence of primary growth oocytes within apparently normally developing testis tissue. The severity of histological alterations ranged from a single oocyte to several hundreds in a pair of testes cross sections. In severe cases, the primary oocytes and oogonia could be either clustered or evenly distributed in the tissue. Presence of oocytes in secondary growth phase was not detected in any sample. The eelpout exhibited the highest intersex prevalence at contaminated marine stations, but also occurred at sites with apparently little pollution. Severity of abnormality was not proportional to prevalence. The findings suggest that feminized male fish were exposed to endocrine disrupting substances in their environment early in life. Concurrent reference sampling of male and female eelpout in different development stages evidenced also female specimens with a high proportion of atretic oocytes in the ovaries.

Reproductive Biology of Chub Mackerel *Scomber japonicus* Houttuyn 1782 in the Middle Eastern Adriatic Sea

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Introduction

The chub mackerel *Scomber japonicus* Houttuyn (1782) is widely distributed in moderate and warm waters of the Atlantic, Pacific and Indian Oceans. It is also rather widely distributed thought the Mediterranean and Adriatic Sea. This middle-sized pelagic fish species has been located in the deeper waters during the colder part of the year whereas during warmer parts of the year its schools were found in the coastal waters where they spawn and feed. Subsequently, the spawning season is depended on water temperature (15 to 20 °C) (Collete and Nauen 1983). The reproductive biology of chub mackerel, in particular the sexual pattern, was studied in order to determinate the time and duration of the annual spawning activity as well as the size at first sexual maturation and fecundity of this species.

Materials and methods

Chub mackerel specimens (n = 4,157) were collected monthly from January 1998 to December 2007, in the area of the Middle Eastern Adriatic Sea. Their total lengths were measured (nearest 0.01 cm), weighed (body and gonad weight; nearest 0.01 g) and sexed. Overall gonads were dissected immediately after capture and the assessment of gonad maturity stages was determined macroscopically using an empiric scale described by Sinovčić (1978). A sub-sample of 100 gonads was processed for histological analysis, for which the oocytes were classified according to their morphology and to the presence and position of lipid droplets, yolk vesicles and granules (Yamamoto 1956), while the spermatogenic cells were classified according to Grier (1981). Additional information of sexual cycle was obtained from the ratio of gonad weight and total weight of each individual, using the gonosomatic index (GSI). Specimens with the most progressive stages of gonads (ripe, spawning, spent - with hydrated and semi hydrated oocytes) were used for the calculation of the maturity curve and size at first maturity. For the estimation of minimal length at 50% and 100% maturity of the chub mackerel population, the logistic function was applied. The gravimetric (Laevastu 1965) and volumetric methods (Sinovčić 1983-1984) were used to estimate fecundity. Namely, ovaries were put in Gilson's solution for 4 weeks, and then the number of oocytes was counted. The fecundity was the result of the mean of the count of three subsamples. A power model was fitted for the relationships of fecundity versus fork body length, total body weight and gonad weight. Diameters of hydrated oocytes were measured with the computer program Olympus DP-Soft.

Results

A total of 4,157 chub mackerel were collected during the study including 1,073 males (25.8 %) and 1,579 females (37.9 %). The fork length (LF) of all observed specimens ranged from 10.1-39.0 cm, the mean value was 23.79 ± 4.68 cm. The males ranged from 17.9-38.8 cm (mean 26.00 ± 3.83 cm) and females from 14.9-39.0 cm (mean 25.28 ± 3.46 cm). Overall sex ratio was 0.68, which significantly deviated from the hypothetical distribution of 1:1 (χ^2 =

102.62; $p < 0.001$); a significant preponderance of females over males was observed throughout the investigated period.

The sexual cycle of chub mackerel was analysed by the maturity states of gonads as well as by gonad weight, gonadosomatic ratio and the gonad tissue histology. According to the monthly percentage composition of gonad maturity stages, the most advanced stages (V, VI, VII) were dominant during the spring - summer season for both sexes (Figure 1). The mean values of GSI ranged from 0.01 % (February) to 14.84 % (May) in males and 0.02 % (February) to 13.77 % (June) in females, showing distinct seasonal changes. Namely, for both sexes GSI was low from February to April, then increased until the peak in May for males and in June for females. The GSI values were elevated until September and remained low in winter time. The seasonal variation of GSI is consistent with the percentage composition of each maturity stage in gonads, suggesting that the spawning season of chub mackerel is from April to August. Histological examination of gonadal tissue confirmed the spawning pattern of chub mackerel; all stages of oocyte development were present in mature ovaries, but during the peak of spawning migratory nucleus and hydrated oocytes were the largest group of oocytes present in the ovary while in testis all four types of spermatogonic cells were present fo most of the year (Figure 2).

Both ripe males and females (corresponding to stages V, VI and VII from the maturity key of Sinovčić 1978) were collected during the May - July period. Fifty percent of the chub mackerel population becomes mature at 18.3 cm of fork length ($r^2 = 0.789$), with males acheiving maturity at a smaller size (16.8 cm; $r^2 = 0.695$) than females (20.4 cm; $r^2 = 0.852$).

The fecundity ranged from 99,166 to 394,120 eggs per individual, with amean value of 181,277 ± 62,090 oocytes per ovary. The total number of eggs per ripe ovary increased with fork length (LF) in accordance with the equation: $F=534.7\ LF^{1.8}$. Fecundity also incerased with total body weight ($F=10274.3\ Wuk^{0.55}$) and gonad weight ($F=107609\ Wg^{0.289}$). The diameter of hydrated eggs (n = 1,000) ranged from 0.372 to 0.806 mm; mean value was 0.595 ± 0.069 mm.

Conclusions

The number of *Scomber japonicus* females is significantly greater than that of males (♂/♀ = 0.68). They first attain sexual maturity at 18.3 cm LF, and males begin maturing earlier than females. Temporal evolution of gonad masses, gonadosomatic index, stage of gonads as well as the gonad histological analysis showed that the spawning season of chub mackerel was from April to August. Average fecundity was 181,277 ± 62,090 and could be described as a power function of fork length, total body weight and gonad weight.

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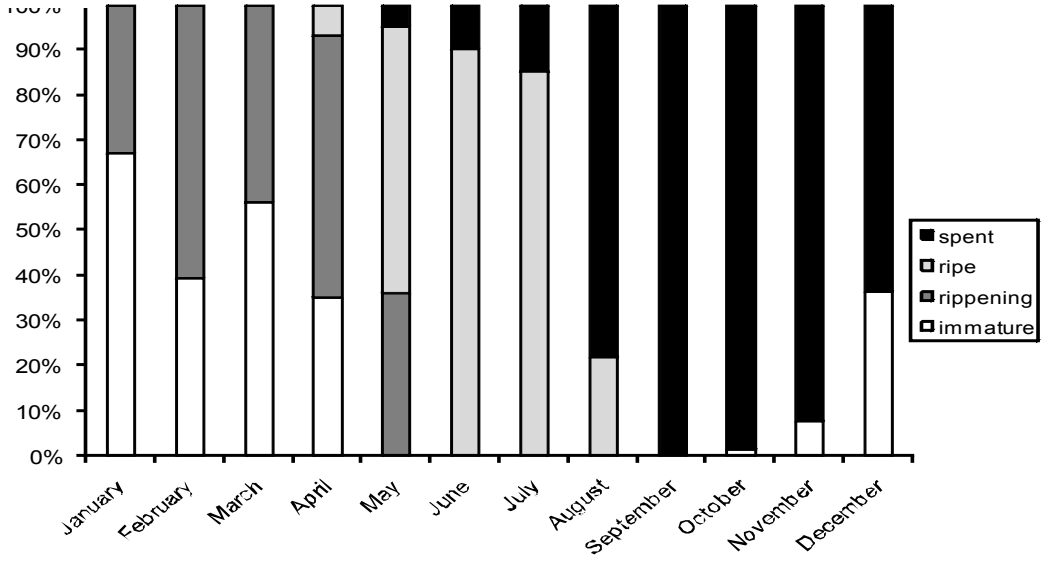
a)



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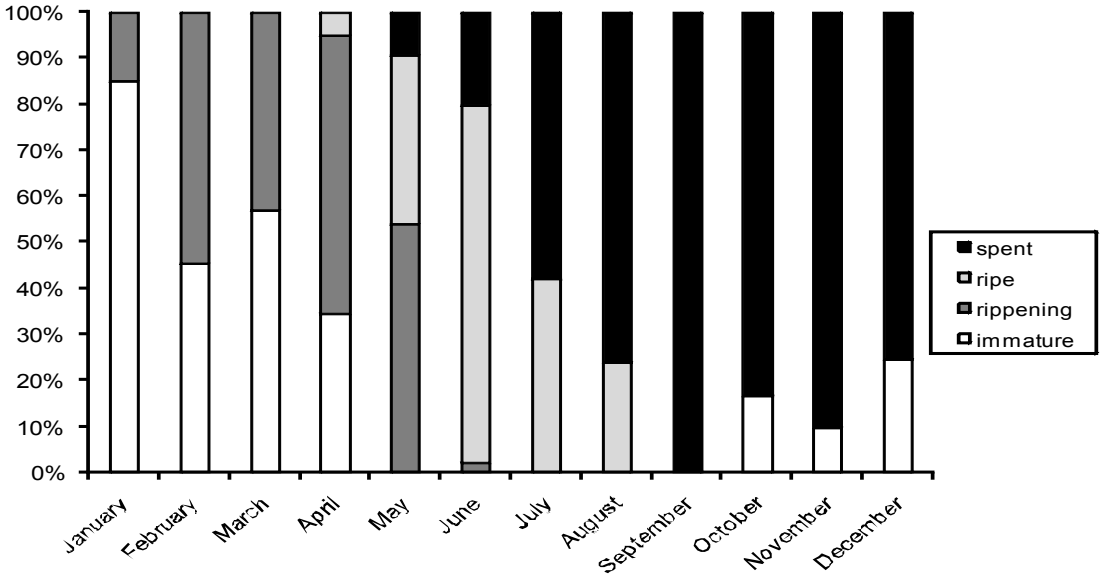

Figure 1. Monthly variations of maturity stages for male (a) and female (b) specimens of chub mackerel collected in the middle eastern Adriatic Sea, 1998 – 2007

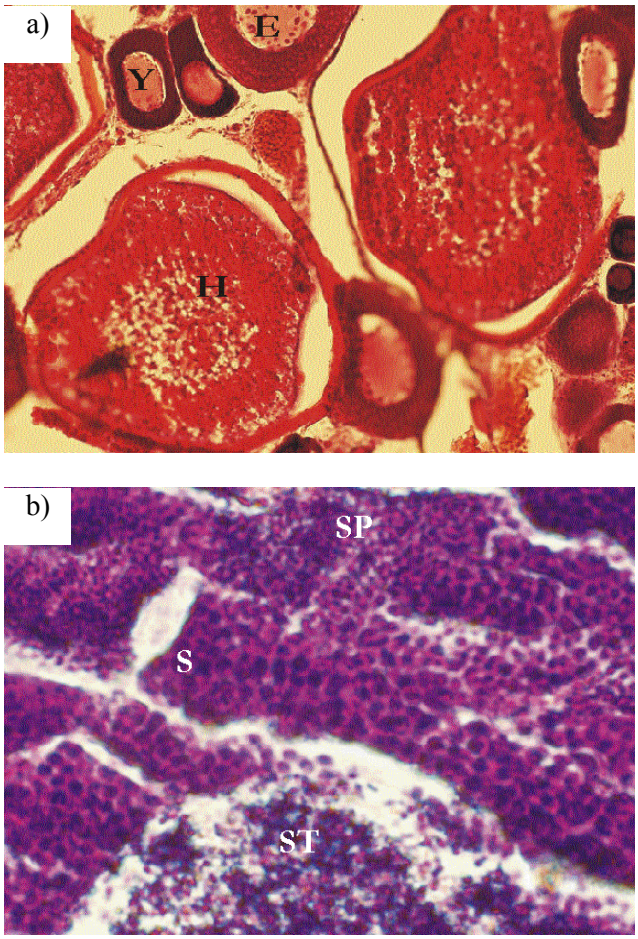

Figure 2. *Scomber japonicus*. a) Ovarian tissue with oocytes in different development stages (Y - yolk-vesicle formation, E- early vitellogenesis, H – late vitellogenesis) (100x); b) Testicular tissue with stages of spermatogenesis (S - spermatogonia, SP - spermatocytes, ST - spermatids) (100x)

Reproductive Strategy of the Common Wolffish *Anarhichas lupus* L. in North East Atlantic Waters

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The common wolffish *Anarhichas lupus* produce large eggs (5-6 mm), apparently pair before spawning, exhibit internal fertilization and the spawned eggs stick together in a ball-like mass made by the female and guarded during a long incubation period by the male (Jónsson 1982; Keats et al. 1995;Falk-Petersen and Hansen 1991; Pavlov and Novikov 1993).

Seasonal gonad analyses of apparently sexually mature common wolffish, *Anarhichas lupus*, along the coast of northern Norway, including fish from 5-22 years of age and lengths between 36 and 112 cm, showed that main spawning took place during autumn (Falk-Petersen and Hansen 1991; Falk-Petersen et al. 2009). Mean ovarian indices (GI's) varied between 1-12% during the year; they increased slightly during late spring and significantly during summer followed by a marked decrease in November (Figure 1). The seasonal fluctuations in testes weights were very moderate (mean GI´s between 0.1-0.2%; Figure 1). Histomorphological studies of the latter showed that spermatids and spermatozoa were most numerous during summer and autumn, but maturing and mature stages of gametes were present during most of the year.

Ovaries with early secondary growth stage oocytes of the cortical alveolus stage (200-1100 µm) in addition to primary growth oocytes dominated during winter and early spring. The cortical alveolus oocytes were present at all seasons in ovaries of all females sampled in this investigation, while vitellogenic oocytes with clearly visible yolk granules (800-1600µm) were most commonly found from April or May and dominated in maturing fish caught during late summer and early autumn (2000-4000 µm; Figure 2A). The fecundity of females increased with fish size, at least up to 80 cm length. A few ovaries contained vitellogenic oocytes in February/March. The annual maturing population of oocytes recruit from an existing batch of cortical alveolus oocytes in wolffish. Ovaries of *A. lupus* may remain at the cortical alveolus stage for several years, however (see also Gunnarsson et al. 2006). Some (30-40%) of the smaller and medium sized females in our investigation apparently skipped spawning, based on the observation that only early secondary growth phase oocytes (cortical alveolus oocytes/early vitellogenic oocytes) were present in August/September, but the thickness of the ovarian wall indicated that many of these individuals were not first-time spawners. Furthermore, the largest cortical alveolus oocytes/early vitellogenic oocytes showed atresia during summer and autumn in presumably non-spawning females (Figure 2B).

Liver indices varied annually and appeared to reflect energy storage during the most intensive feeding period of the year (late winter/spring and summer). Females with atretic oocytes during summer/autumn generally had lower liver indices than maturing females, suggesting that lack of energy stores may be the reason for skipped spawning.

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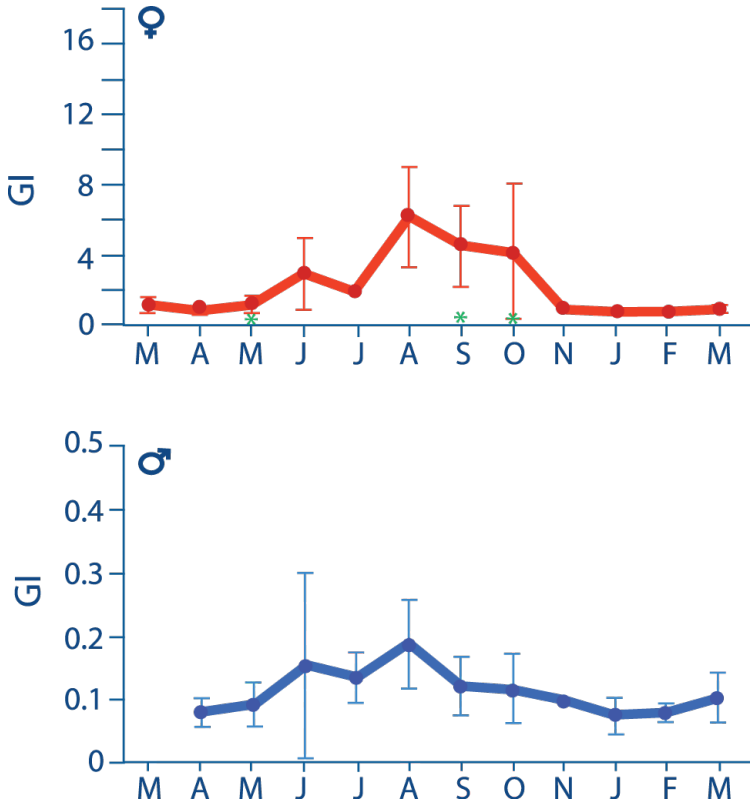

Figure 1. Annual gonad indices (GI) of female and male *A. lupus* from north-Norwegian waters.

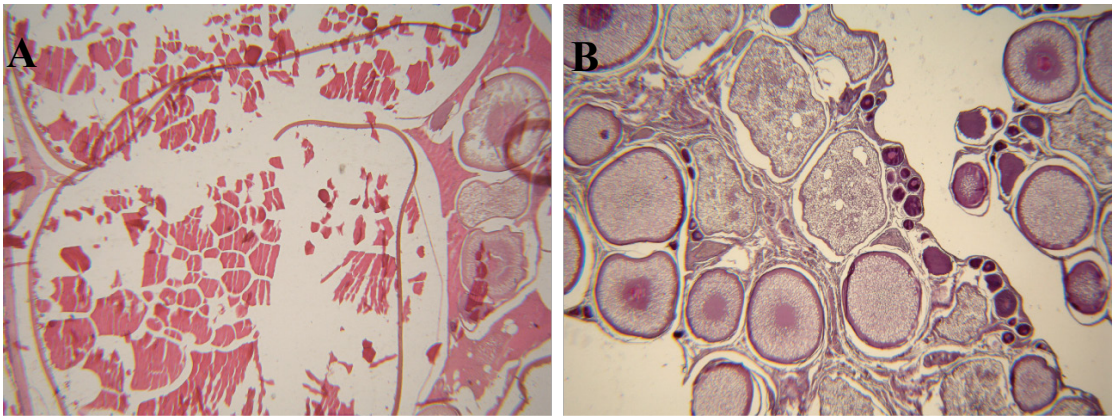

Figure 2. Ovarian histology of female wolffish captured in October. A. Large, maturing oocytes in ovary close to spawning. B. Numerous atretic oocytes among cortical alveolus oocytes and primary oocytes in ovary from a non-spawning, sexually mature female.

Features of Oogenesis in Two Species of the Family Mullidae from the Nha Trang Bay, South China Sea, Vietnam

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Introduction

Owing to increased anthropogenic pressure to marine ecosystems, there is an urgent need to study coastal organisms to evaluate their potential use as indicators and/or key species for coastal ecosystem monitoring and management. Fishes of the family Mullidae are regarded as good indicators of the environmental conditions of bottom biotopes because of their strong reaction to certain natural or anthropogenic changes. In addition, these fishes can be referred to as key species in the ecosystem due to specificity of their foraging behavior with barbels and mouth, which leads to the looping back of nutrients from bottom sediments into the open water and surrounding areas by resuspension and formation of multi-species foraging associations (Uiblein, 2007). The goal of this study is to evaluate the features of reproductive biology, and in particular oogenesis, in *Parupeneus multifasciatus* and *Upeneus tragula*, two of the most abundant representatives of the family Mullidae from the Nha Trang Bay of the South China Sea (Vietnam).

Methods

The material was collected from July to December 2008 and from February to April 2008 and 2009. The fishes were collected with nets, mesh size up to 1.5 cm. To study the morphology of the gonad, widely used histological methods and light microscopy were applied. The biological parameters and gonad structure of *P. multifasciatus* were studied more extensively.

Results and Discussion

During the period of investigation, ovaries of the majority of adult individuals of both species were in IV or IV-V maturity stages (based on visual observations). The size of such fish varies substantially. The minimal fork length (FL) of sexually mature female *P. multifasciatus* and *U. tragula* was similar, reaching 110 mm (about 20 g). The maximal size of the females (206 mm FL and 139 g) is larger in *U. tragula*, than in *P.multifasciatus* (179 mm FL and 97.36 g).

The dynamics of GSI of *P. multifasciatus* shows a comparatively small variability that suggests an occurrence of a protracted season of reproduction. The increase of GSI is observed in March, as well as in December (Figure 1) and represents peaks of spawning.

Anatomical differentiation of the gonads of *P. multifasciatus* is observed by 50 mm FL and 1.5 g, and cytological differentiation in the females is registered by 58 mm FL and 2.6 g. The ovaries possess the entovarian cavity (Figures 2a, 2b). The fish reach first sexual maturation at approximately 110 mm FL.

The oocyte diameter distribution in sexually mature females of both species collected during the spawning season shows that oocytes of the smallest size groups prevail, and the oocytes of intermediate size (between previtellogenic and the largest vitellogenic) are present. Thus, the

species are characterized by continuous oogenesis and indeterminate fecundity (Götting, 1961). Actual fecundity determined in nine individuals of *P. multifasciatus* ranged from 1527 to 26,423 eggs, on the average 10,343 eggs.

In histological sections of ovaries of mature females from both species, oocytes of different sizes from various phases and periods of development are seen (Figures. 2c, 2d). In many females *P. multifasciatus*, abnormalities in morphology of oocytes during maturation, vitellogenesis and (less often) previtellogenesis are seen (Figures 2e, 2f). These abnormalities are as follows: uneven distribution of nucleoli, partial separation of the cytoplasm and its lysis, destruction of zona radiata, hypertrophy of the cells of follicular epithelium, appearance of cell detritus, vascularization of the ovarian stroma, etc. Based on these observations, unfavorable environmental conditions in the Nha Trang Bay induced by anthropogenic factors can be suggested. Similar destructions are registered in other fish species under the influence of anthropogenic factors of different nature (Savvaitova et al. 1995; Belova et al. 2007).

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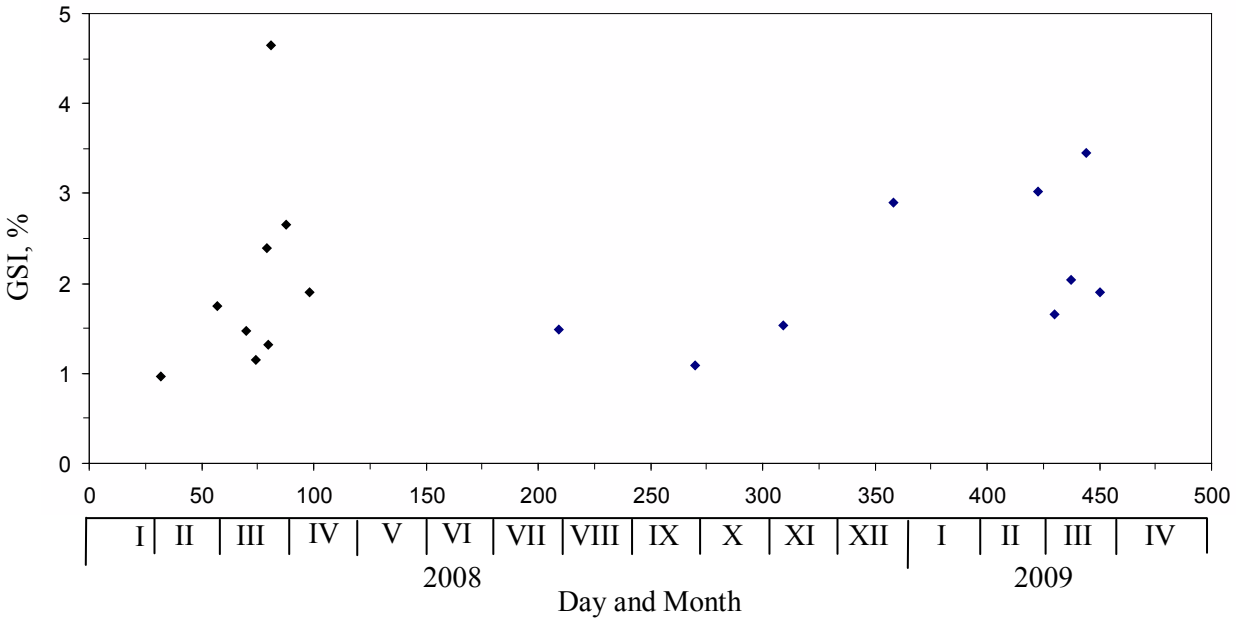

Figure 1. Gonadosomatic index (GSI) of female *Parupeneus multifasciatus* during the period of investigation. Each point is the mean of 3–19 (on the average, 7) values. Day 1 = 1 January 2008.

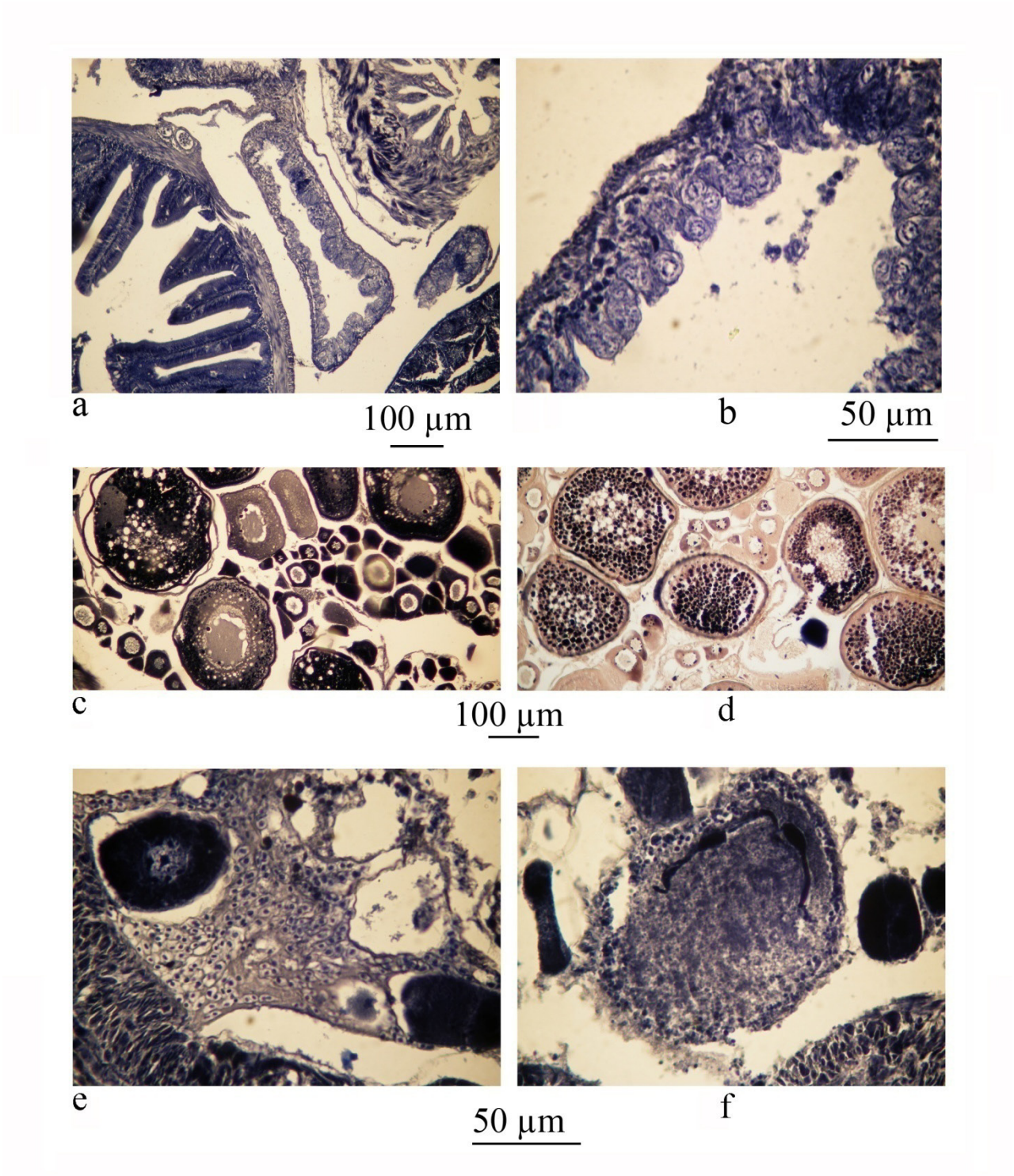

Figure 2. Ovaries of *Parupeneus multifasciatus* and *Upeneus tragula*. (a) general view of the ovary of *P. multifasciatus* with the entovarian cavity; (b) fragment of the ovary of *P. multifasciatus* with a low number of oocytes; normal condition of oocytes in sexually mature individuals of (c) *P. multifasciatus* and (d) *U. tragula*; resorption of oocytes in *P. multifasciatus* during the periods of (e) previtellogenesis and (f) maturation.

Effect of Radiation on the Reproductive System of Freshwater Teleost Fishes – Consequences of the Chernobyl Nuclear Power Plant Accident

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Light and electron microscopy were used for a 20 year study of the effects of radionuclides on the reproductive systems and capabilities of freshwater fishes. Twelve species of freshwater fishes from various water bodies of Ukraine, Russia, and Belarus that were impacted by the Chernobyl Nuclear Power Plant (NPP) Accident were examined in this study.

A wide spectrum of morphological and functional abnormalities of sex glands with high frequencies of occurrence is revealed, including asymmetry and abnormal gonadal morphology, mass destruction of follicular and sex cells at various developmental stages (Figure 1A, B), displacement of sex cells by connective tissue (sterilization) (Figure 1C, D), hermaphroditism (Figure 1E, F), and development of unusual structures.

The high intensity radiation, resulting in heavily polluted water bodies, led to the appearance of a large number of serious gonadal abnormalities in *Esox lucius* (pike) and *Abramis bram* (rudd). A decreasing population abundance of these species is revealed. A species specificity of the resistance of the reproductive system to radiation is clearly demonstrated (Table 1). The lowest destructions are seen in the gonads of *Perca fluviatilis* (perch), *Tinca tinca* (roach), and *Carassius auratus* (goldfish, triploid form). The differences in the resistance are determined by ecological, morphofunctional, sexual, and genetic (polyploidy) features of the species. In general, the frequencies and degrees of abnormalities in the reproductive system are positively correlated with ^{137}Cs content in the body of the fish and degree of pollution of the water body by radionuclides.

An assessment of the spectrum of morphofunctional abnormalities and frequency of their occurrence in the reproductive system of various fish species in several post-accidental generations shows the maximal values in offspring from the second to fourth (F_2 – F_4) generations. We suggest that the gonadal abnormalities revealed in the fish of these generations from the weakly polluted water bodies (the Kiev Water Reservoir and Teterev River) are connected with the effect of "prolonged" mutagenesis. In heavily polluted water bodies, a negative effect of radiation on the fish reproductive system can be followed for subsequent decades.

Acknowledgments
The study was supported in part by project RFBR no. 07-04-00002 and part CR-6 INTAS project 556 "Respond".

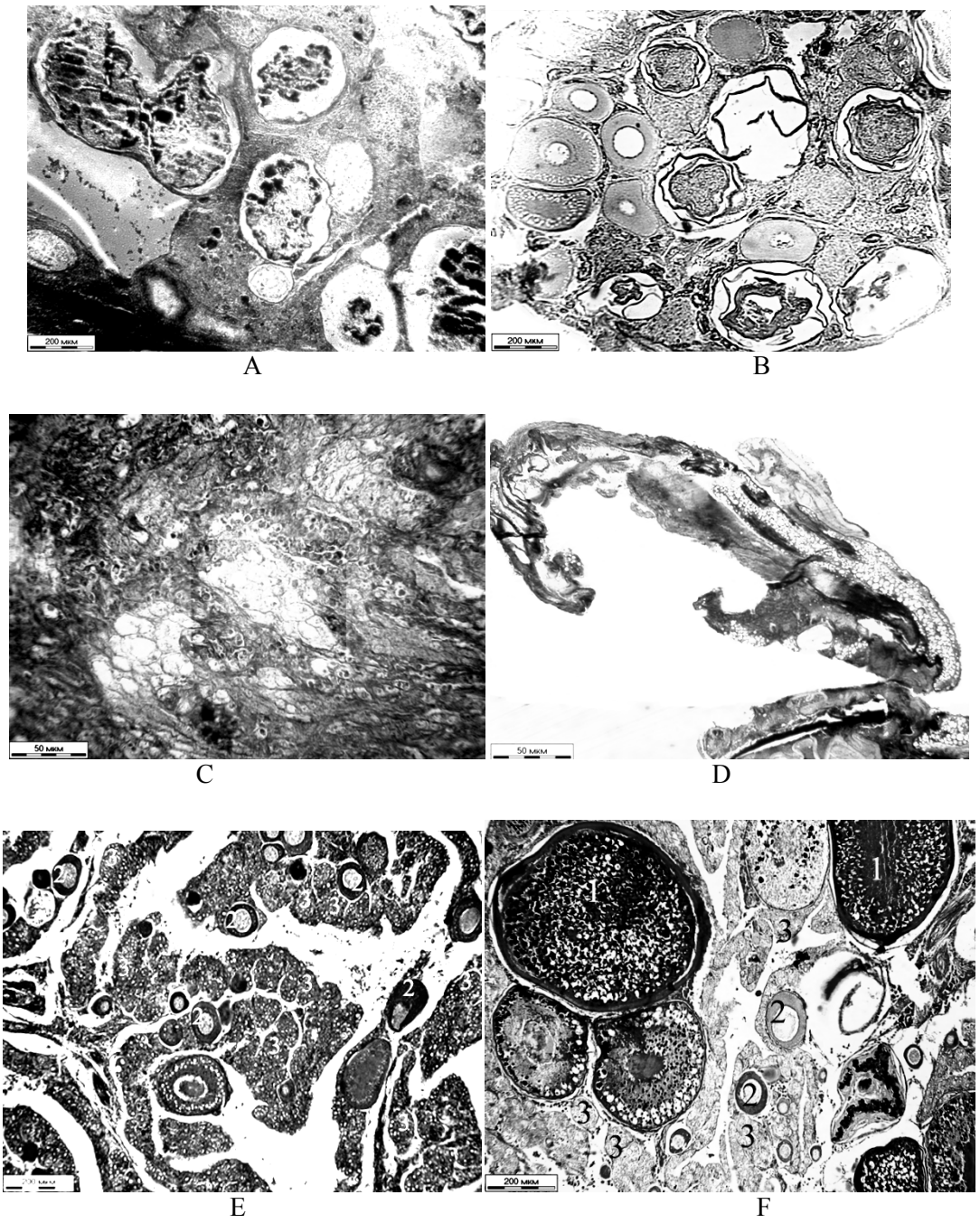

Figure 1. Morphological and functional abnormalities of sex glands and cells in several species of freshwater fishes from water bodies polluted by radionuclides. A, B. Mass destruction of sex cells at various developmental stages; C, D. Displacement of sex cells (sterilization); E, F. Hermaphroditism in normally gonochoristic species.

Table 1. The frequencies of occurrence (%) of abnormalities in the reproductive system in several species of freshwater fishes from water bodies polluted by radionuclides.

Year	Silver bream	Roach	Goldfish	Crucian carp	Bream	Rudd	Tench	Perch	Pike
	Cooling pond of ChNPP								
1992	37.5	37.5	50.0	-	100	-	-	-	-
2002	0	50,0	-	-	-	66.7	-	0	-
2003	80.0	40.0	0	-	-	0	-	30.0	-
2004	100	100	100	-	100	100	-	90.0	-
Total	48.0	50.0	66.7	-	100	70.0	-	36.7	-
	Glubokoe Lake								
2003	-	-	50.0	-	-	66.6	0	-	100
2004	-	50.0	50.0	-	-	100	-	50.0	100
Total	-	50.0	50.0	-	-	75.0	0	50.0	100
	Kojanovskoye Lake								
1993	-	0	9.1	-	-	-	-	-	0
1997-1999	-	-	45.8	50.0	50.0	-	-	-	66.7
	Svyatoe Lake								
1997-1999	-	25.0	28.5	-	-	-	72.7	11.2	100
	Kiev Reservoir								
1997, 1999-2002	0	10.0	50.0	0	46.9	75.0	11.1	19.4	59.1
2003-2005	0	25.0	50.0	100	51.3	33.3	36.4	36.4	25.0
Total	0	15.6	50.0	75.0	50.0	57.1	20.7	23.4	53.8
	Teterev River								
1997, 1999-2002	16.7	16.7	12.5	0	22.2	-	5.3	11.1	23.1
2003-2005	50.0	20.0	0	0	58.3	0	10.0	0	42.8
Total	33.3	17.4	6.7	0	42.9	0	6.9	8.0	27.3

Size, Age, and Reproductive Status of Atlantic Bluefin Tuna (*Thunnus thynnus*) from the Gulf of Mexico Spawning Grounds

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Introduction

Atlantic bluefin tuna (*Thunnus thynnus*; ABFT) is a long lived species known to spawn in two widely separated locations: the Gulf of Mexico and Mediterranean Sea. ABFT are annual spawners and spawning site fidelity has been suggested. Differences have been suggested in maturity schedules for the two stocks. The eastern stock is believed to mature at the age of 3–5 years and the western at 8–12 years (Medina et al. 2002; Baglin 1982), although the latter assumption might represent a sampling bias.

For Mediterranean spawning fish, size and age at 50% maturity is reported as 103.6 cm CFL and 3 years (Corriero et al. 2005), respectively. Over the past decade there has been extensive documentation of reproductive status of ABFT in this region. There are no studies recording size at 50% maturity for ABFT in the Gulf of Mexico, and the most recent study examining size/age at maturity for Western ABFT was conducted in the 1970's (Baglin 1982). Baglin (1982) reported maturity beginning at age 6 and 100% maturity being reached at age 8 and 190 cm CFL. Since then, there have been dynamic changes in ABFT size classes and availability. Additionally, decadal shifts in growth rates have occurred in Southern bluefin tuna, *T. macoyii* (Hearn and Polachek 2003), and ABFT could experience similar shifts resulting in changes in size and age at maturity. Analyses of reproductive patterns and fecundity of western ABFT are needed for accurate assessment of life history parameters as well as stock productivity. Our study objectives are to understand the reproductive dynamics of fish sampled in the Gulf of Mexico, and to compare our results with fish sampled in the Mediterranean using similar analytical methods.

Methods

Gonads were obtained from 124 ABFT sampled by NMFS Pelagic Observer Program observers onboard Gulf of Mexico longliners during March - July, 2007–2008. Fish length was measured on board, to the nearest cm, and is reported as curved fork length (CFL). Total body weight (BW) was calculated from dressed weight (DW) using the standard conversion equation (DW*1.35). Whole gonads were weighed to the nearest gram, and subsamples were taken from each gonad and preserved in formalin. Gonadosomatic Index (GSI) was determined (100·GW/BW) and we assessed level of maturity of females using histology. Female fish were assigned reproductive stage based on the classification scheme outlined by Schaefer (1998). Male fish were not classified, as evidence of spawning is only present for 12 hrs. post-spawning (Schaefer 1998).

Results and Discussion

Size and reproductive data were obtained from the 54 female and 50 male ABFT sampled during the 2007–2008 spawning season (Table 1). Using current growth equations, fish were aged to 8–10+ yrs. Mean GSI was 2.6 ± 0.91. The lengths, weights and GSI values support previously recorded values for Gulf of Mexico spawners (Baglin 1982). Histological

examination indicates most fish in the active spawning phase (Figure 1) with only one inactive fish measuring 245 cm. These results are in agreement with recent results showing rare presence of non-spawners on the spawning grounds during the reproduction season. The actively spawning fish we sampled include fish smaller than the currently accepted size at maturity (185 cm). While our sample includes only giant ABFT, this may not be indicative of all size classes spawning in the Gulf of Mexico as previous studies indicate longline fishing vessels catch significantly larger fish than other methods (Medina et al. 2007). Pacific bluefin tuna of different sizes spawn in different locations, indicating the possibility that smaller fish could be spawning in a location yet to be discovered.

Future Work

Gonadotropin gene expression levels will be measured from pituitary glands of actively spawning fish, and results will be compared with Mediterranean spawners. We will determine the fecundity of all female fish sampled. A major objective of this project is to compare the reproductive status of the western and eastern stocks. Therefore, upon the conclusion of analyses, combined with data from the eastern spawning stock, a life history model of ABFT reproductive strategies will be constructed.

Acknowledgements

We thank Dr. Steve Turner, Larry Beerkircher and the National Marine Fisheries Service Pelagic Observer Program for sample collection, the University of New Hampshire Veterinary Diagnostic Lab and Gilad Heinisch for assistance with sample processing. This work was funded by NOAA grant NA04NMF4550391 to M. Lutcavage

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Table 1. Length, weight and gonadal data for Atlantic bluefin tuna (ABFT) collected during the 2007–2008 spawning season in the Gulf of Mexico.

	Fork Length (cm; n=68)			Dressed Weight (kg; n=18)			Gonad Weight (kg; n=39)			Gonadosomatic Index (n=18)		
Sex	μ	SD	Range	μ	SD	Range	μ	SD	Range	μ	SD	Range
Male	257.3	21.2	200–284	237.4	55.8	185–302	8.6	3.2	0.8–13.2	2.33	0.90	1.61–4.00
Female	252.7	20.4	180–274	228.5	48.3	194–326	6.8	3.3	2.7–15.2	3.12	0.80	1.15–3.45

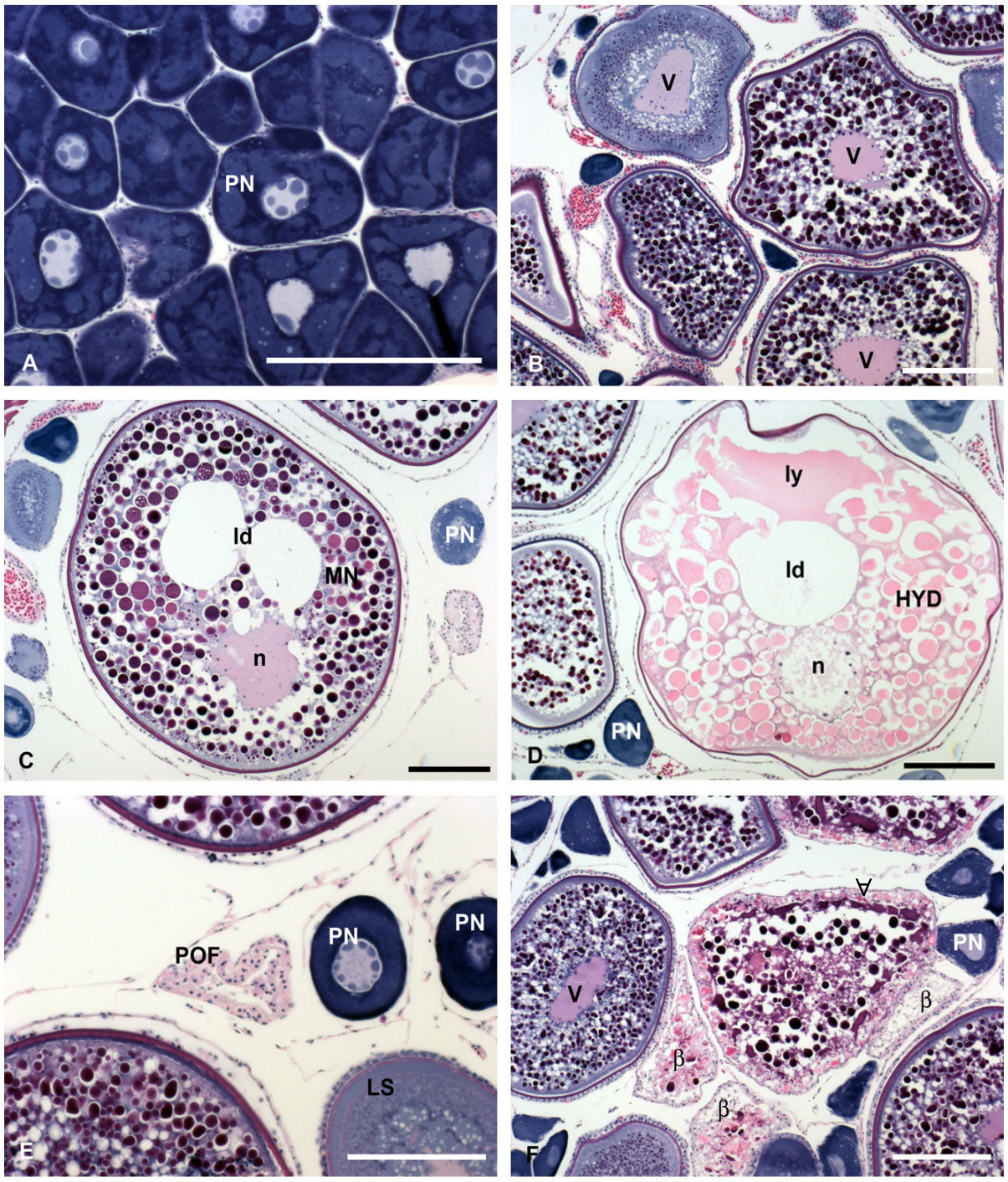

Figure 1. *Thunnus thynnus*. Micrographs of oocyte development stages. A. immature tuna, B. active nonspawning, C-E. active spawning, F. inactive mature. PN: perinucleolar oocytes, V: vitellogenic oocytes, MN: migratory nucleus oocytes, POF: post-ovulatory follicles, LS: lipid-stage oocytes, HYD: hydrating oocytes, α: α-atretic follicles, β: β-atretic follicles, ld: lipid droplet, n: nucleus, ly: liquid yolk, yg: yolk granules. Scale bars = 100 µm.

Immature/Resting Macroscopic Misclassification: What Happens with the European Hake Males (Southern Stock Case)?

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Introduction

The European hake *Merluccius merluccius* (L.) is a demersal gadoid species widely distributed throughout the north-east Atlantic. Within its geographical area, International Council for the Exploration of the Sea (ICES) recognizes the existence of two stocks, the northern and the southern stock. The southern stock is distributed along the Atlantic coast of the Iberian Peninsula (ICES Div. VIIIc and IXa). Assessment and management of fish populations currently rely on correct estimation of the spawning stock biomass (SSB) (Hilborn and Walters 1992 in Dominguez et al. 2008), which is based on accurate maturity ogives of the population. In European hake it is not possible to macroscopically distinguish between immature and resting females, which may lead to an under or overestimation of the proportion of these maturity stages (Murua et al. 2003; Vitale et al. 2006 in ICES 2007) and consequently of the SSB. The majority of the histological studies have been focused on females, and only a few histology studies on males have been conducted (ICES 2007). As recommended by the WKMSHM (ICES 2007), histology studies on males should be undertaken since the two hake stocks use sex combined maturity ogives. Taking this into account, the aim of this work was to analyze the histological slides of male hake gonads in order to explore the hypothesis of misclassification occurrence between immature and resting stages as has been done for females.

Methods

Samples of male hake gonads in macroscopic maturity stage I – Immature (maturity stage scale key resulting from the WKMSHM, ICES 2007) were collected during three cruises carried out in March 2007, January 2008 and April 2009 along the Portuguese coast. Forty-two histological slides were analyzed in order to distinguish between the Immature and Resting/Recovering stages. The different cell types – spermatogonium (9.9 – 16.6 µm), primary spermatocyte (4.7 – 6.9 µm), secondary spermatocyte (1.8 – 5.7 µm) and spermatid (1.1 – 2.1 µm) – were identified, measured and the maturity stage was defined by the proportion of each one of this cells in the gonad.

Results and Discussion

A large percentage of small males macroscopically classified as immature were microscopically classified as resting (Figure 1). The distinction between immature and resting males is only possible by the means of histology (Figure 2), as has been shown previously for females. This misclassification of immature/resting could lead to an underestimation of the spawning stock biomass and therefore result in inadequate management options. For the southern hake stock, the use of histology maturity keys appears necessary to achieve an accurate SSB estimation.

Acknowledgements
The authors are grateful to all the technicians and research vessel crew that performed the biological sampling that made this work possible. We thank Leonel Gordo for his valuable help in the identification of microscopic maturity stages. The sample collection is supported by the PNAB/EU DCR-Data Collection Regulation. PG was also supported through a grant from that program.

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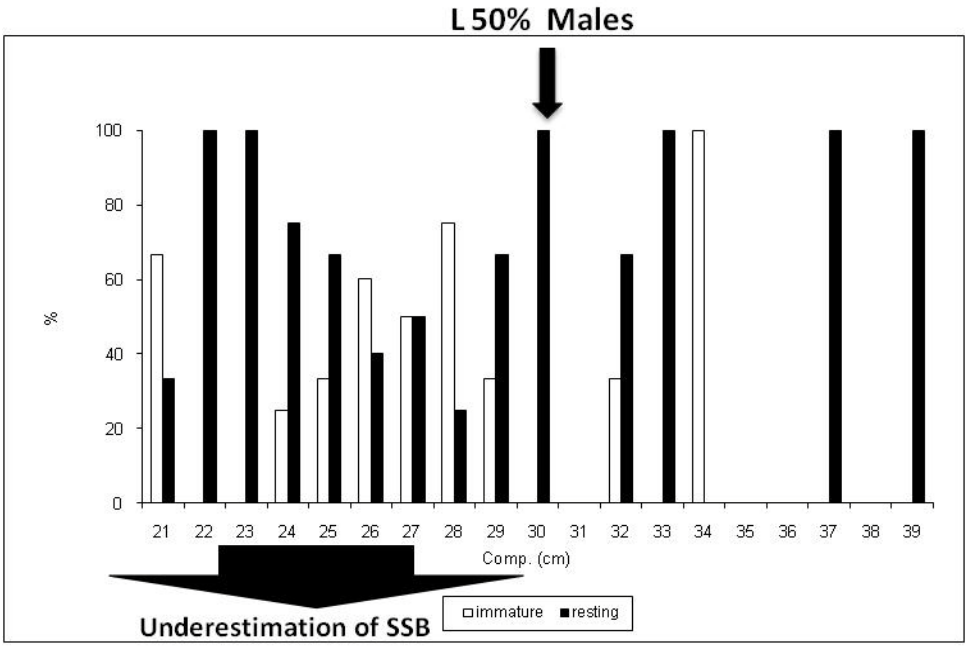

Figure 1 - Number of male hake microscopically classified as immature and resting per length class (cm) (n = 42).

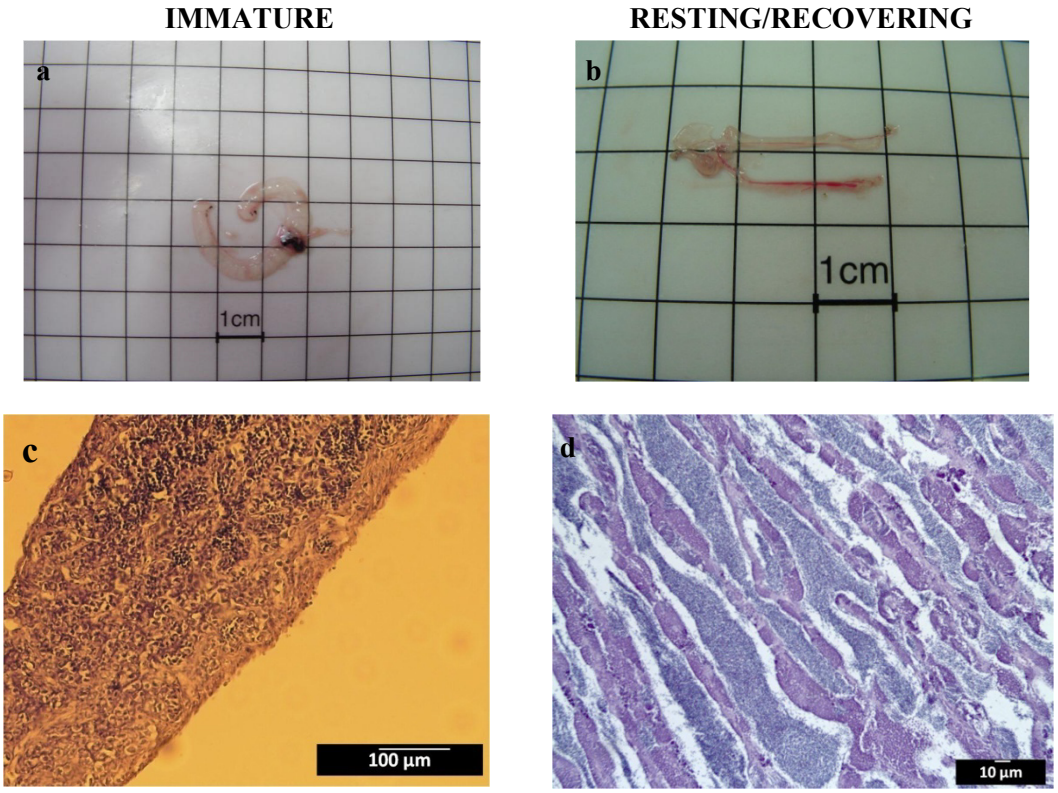

Figure 2 – Immature (a, c) and resting/recovering (b, d) macroscopic (a, b) and microscopic (c, d) identification of male hake.

Automatic Software Tool to Determine Fish Fecundity by means of a Computing Aided Image Analysis and Stereology on Histological Images

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Introduction

Presented here is a new software suite, still under development, that is intended for an easy estimation of fish fecundity from histological images. This preliminary tool aims to automate the process of counting, classifying and measuring different types of oocytes from histological images using the image analysis techniques. Based on the principles of stereology (Delesse principle) and combined with newly designed algorithms which can detect oocyte profiles, the major goal of this tool will be to unify in a single program all measures and calculations needed for fish fecundity estimations in a user-friendly interface.

Results and Discussion

The tool allows using any image format, although in the latest version calibration values need to be introduced. Several images from the same specimen can be added and analyzed together, yielding final results by specimen and not by image. Another advantage of this tool is the flexibility in its use, providing a number of drawing tools for editing the layer with preliminary results.

The process starts with the automatic detection of oocytes edges, based on new algorithms still being developed. Although preliminary results are very promising (Figure 1A), this facility is not yet implemented in the tool, but will constitute the main advance relative to any other commercial software used in fecundity estimations. The automatic detection discriminates between oocytes with and without a nucleus in order to obtain the correct oocyte diameter and the parameters K and β for Emerson's formula (see below), which are needed for a proper fecundity estimation.

The rationale of the automated detection is the algorithms used for image analysis based on texture and/or edge segmentation which can automatically detect oocytes in the image of the histological section; this process that can be supervised by the user to improve results (Figure 1B). Oocytes can be drawn easily using the drawing facilities incorporated into the software. If this is time consuming and if it is not possible to use the automated oocyte detection, a traditional Weibel Grid can be superimposed (Figure 2A). The size of the grid and the number of points can be modified by the user. The results of the Weibel method will feed into the system automatically to yield the same results as with automated detection.

The next step is to assign each oocyte to a defined development stage. The user can define as many stages (cortical alveoli, vitellogenic, etc.) as necessary according to the characteristics of the species or the estimation that wish to obtain (potential fecundity, batch fecundity,

atresia intensity, etc.). The assignment of oocytes to stages has to be made manually by the user, but the software provides tools to make this step easy and quick.

Stereological estimation of fecundity requires some input parameters based on Emerson's formula:

$$F = O_v \times \frac{k}{\beta} \times \frac{\sqrt{N_a^3}}{\sqrt{v_i}}$$

Shape coefficient (β) and size distribution coefficient (K) are automatically estimated from the measures of diameters of each individual as explained above. However, if preferred, the user can introduce these parameters directly. Number of profiles (N_a) and volume fraction (v_i) are calculated directly by the system based in the number of pixels occupied by each oocyte, from which the partial area is computed. Ovary volume (O_v) and Scherle factor (fresh ovary weight) should be added by the user. Finally, the software will return the number of oocytes by class in the whole ovary, from where different fecundity values or atresia intensity are estimated and exported to different formats (Figure 2B).

Current situation

We are currently working on two parallel processes: i) the oocyte automatic detection software is not yet implemented into the tool, because results are not fully satisfactory, but the combination in the selection criteria of different algorithms is producing reliable results that will allow the incorporation of this important element in the near future; ii) the rest of the elements are almost finished and we are working on a beta version to be released soon. This will be a freeware program.

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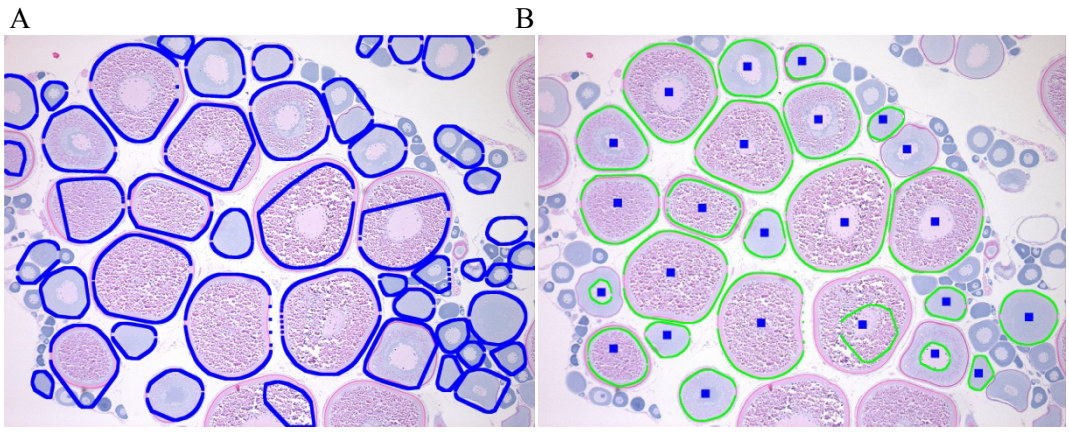
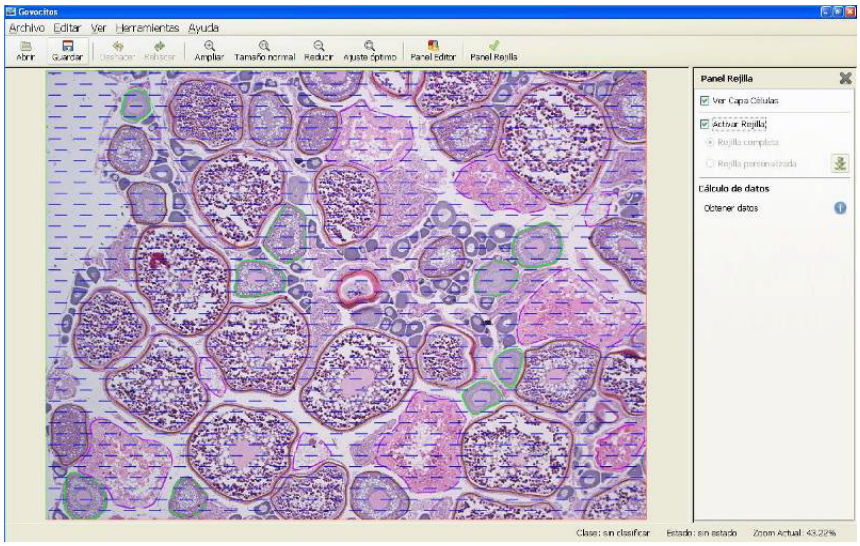


Figure 1. Automatic oocyte detection (A) without supervision and (B) supervised by user.

A



B

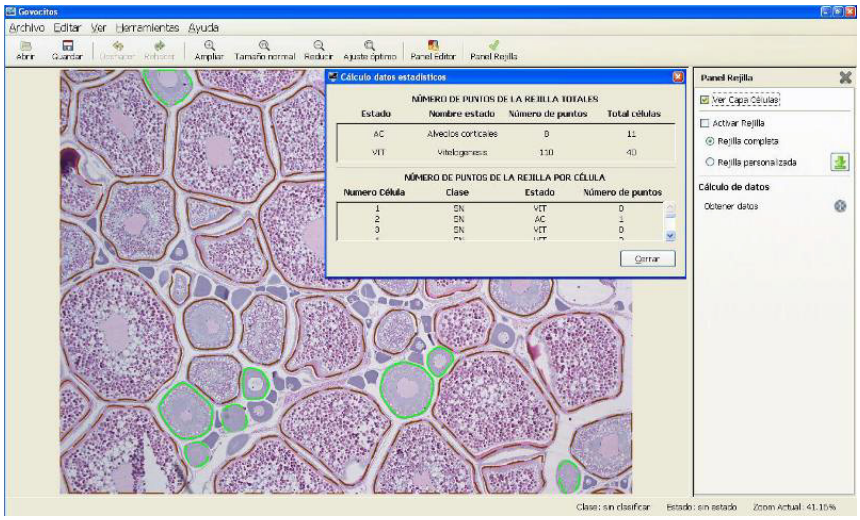


Figure 2. Examples of software. A. Weibel grid. B..Results panel. Shows the results per oocyte (developing stage, number of points) and per developing stage (total number of cells, points).

Counts of Postovulatory Follicles with the Stereological Disector Method as a Proxy of the Realised Fecundity in Bluefin Tuna *Thunnus thynnus* (L.)

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Abstract
Estimations of fecundity are often obtained from counts of advanced vitellogenic (large yolked) oocytes in the ovary (potential fecundity), but a probably more accurate approach to determine the actual number of eggs released in a spawn (realised fecundity) is to determine the number of fresh postovulatory follicles (POFs). For quantification of recently ovulated follicles (POFs) we applied the physical disector method to ovaries of seven bluefin tuna spawners, including experimental captive fish treated with GnRHa (gonadotropin-releasing hormone agonist) and wild breeders sampled from spawning grounds. The estimated realised batch fecundity ranged between 0.5 and 19 million eggs. These data are in agreement with the average potential batch fecundity of about 9 million eggs calculated from stereometric counts of final maturation oocytes from histological ovary samples of wild spawners.

Introduction
Fecundity is a biological parameter of paramount importance to fishery scientists both as a critical parameter for stock assessments and as a basic aspect of fish biology and population dynamics (García-Díaz et al. 2003). A number of stereological techniques applied to histological samples can estimate the number of advanced vitellogenic oocytes within an ovary (potential fecundity) (Emerson et al. 1990; Murua et al. 2003), but only the disector method (Sterio 1984) can provide unbiased information about the presence and number of postovulatory follicles (POF), and hence estimations of realised fecundity. In the present study we applied the physical disector method for obtaining unbiased counts of POFs in ovaries of seven bluefin tuna spawners, including experimental captive fish treated with GnRHa (gonadotropin-releasing hormone agonist) and wild breeders sampled from spawning grounds.

Methods
Sample collection
Five female spawners were caught by purse seine at the spawning ground around the Balearic Islands. Additionally, two farmed specimens were treated with GnRHa**.** Following evisceration, the ovaries were weighed to the nearest 1 g, and their volume measured to the nearest 10 ml. The gonadosomatic index (GSI) was calculated as percentage of gonad weight relative to total body weight.

Histology
Tissue samples were removed from the broadest part of one the ovaries of each fish, and comprised the whole ovarian wall. Following fixation for 48-96 h in buffered 4% formaldehyde (10% formalin), they were dehydrated and embedded in paraffin. Serial 10 µm

sections were stained with hematoxylin-VOF (Gutiérrez 1967), and mounted with Eukitt for observation on the light microscope.

Physical disector

The disector is the only objective method for quantification of particles of variable size in a given volume. It requires two adjacent sections (disector pair) a known distance (h) apart; h should lie between ¼ and⅓ of the particle's mean small diameter. In practice, the chosen separation between consecutive sections was 50 µm. Two digital micrographs were taken from each disector pair. A calibration scale was photographed at the same magnification for calculation of the real dimensions of the structures. A counting frame was placed randomly onto the images from the reference section and then (thoroughly aligned) onto the look-up section. Only those POFs that appeared in the counting frame on the reference section but not on the look-up section were counted. In order to increase the efficiency of the counting, the disector pairs were worked in both directions. If the follicle touched the forbidden lines of the frame in the reference section (red lines in Figure 1), they were not counted. The number density of follicles (number per gonad unit volume, Nv) was estimated as: $N_V(\text{fol},\text{ref}) = \frac{Q^-(\text{fol})}{a \cdot P(\text{ref}) \cdot h}$ where h is the disector thickness; a is the area of the rectangular reference frame of each disector; P(ref) is the total number of disectors used for a gonad; Q^-(fol) is the total number of POFs counted in the P(ref) disectors in a gonad, namely the added number of follicles present in the reference frames but not in the look up sections.

Results and Discussion

The physical disector method was used in the current study to asses the numbers of POFs in ovaries of seven bluefin tuna (Table 1). Total POF counts per ovary were computed as $N(fol) = V_G \cdot N_V (fol,ref)$, and these ranged between 0.5 and 19 million eggs. These data are in agreement with the average potential batch fecundity of about 9 million eggs calculated from stereometric counts of final maturation oocytes from histological ovary samples of wild spawners (Medina et al. 2007).

Conclusion

The disector method is a powerful stereological procedure for quantifying oocytes from bi-dimensional histological samples of ovaries. Although the implementation of the disector is time-consuming (i.e, serial sectioning of ovary fragments and thorough alignment of the sections), the increased work is rewarded by the high accuracy of the method and the possibility of counting any particle, regardless of its shape and size. Estimates of the numbers of POFs may be considered a good indicator of the actual number of eggs released in a spawn (realised fecundity), albeit these structures are only occasionally present in bluefin tuna ovaries.

Acknowledgements

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Table 1. Biometric and stereological data from seven females of bluefin tuna, *Thunnus thynnus* (L.).

Body Weight (Kg)	Ovarian volume (ml)	GSI	Nv (fol,ref) (POFs/mm^3)	Total POFs (millions)
130	2789	2.3	6.56	18.3
112	3454	3.3	5.44	18.8
90	2959	3.5	4.04	12.0
185	4067	2.4	6.9	28.1
98	2312	2.5	5.68	13.1
32	1202	4.1	3.92	4.7
17	554	3.4	0.99	0.5

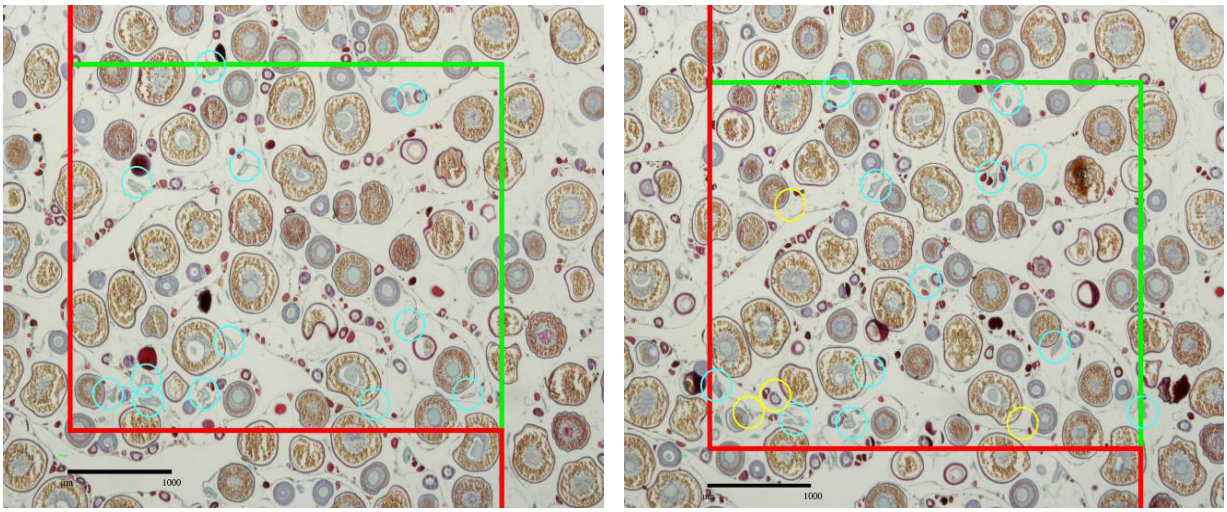

Figure 1. Example of the disector counting method. The left-hand section contains 2D counting frame and is known as the “reference” section. The right-hand section is known as the “look-up” section. POFs that appear in the reference section (blue circles) but not in the look-up section (yellow circles) were counted. Follicles placed in the forbidden lines (red lines) were not counted. (a= 14.231mm2, h= 50µm, P(ref)= 24). Scale bars: 1mm.

Aspects of the Histological Analysis of the Batch Fecundity on an Example of the Rockall Bank Haddock (*Melanogrammus aeglefinus*).

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Histological examination showed that asynchronous vitellogenesis of oocytes and batch spawning are typical of the Rockall haddock. Recruitment of vitellogenic follicles from the precursory population of previtellogenic follicles appears to occur synchronously. Later in maturation the vitellogenic follicles show more asynchronous development. Batch production from the vitellogenic group follows and the size of the vitellogenic population reduces as spawning progresses. The remnants of postovulatory follicles on histological preparations indicate that some quantity of eggs have already ovulated in batches spawned previously.

Histological analysis would have to be used to select prespawning females and the criteria to identify eggs comprising the fecundity. The criteria to identify eggs comprising the fecundity was based on size of separation of the developing oocytes from the previtellogenic ones. According to most researchers, generally the mean diameter of the advanced stock of oocytes is the accurate measure of ovarian maturity. The vitellogenic oocytes of haddock had the mean diameter of 663 μm (±S.D.=112) and hydrated oocytes 1417 μm (±S.D.=102).

Absolute potential fecundity (the mean value of individual potential fecundities per age) was $78x10^3$eggs in first spawning fish aged 2 years, $170x10^3$eggs in three -year-olds, $340x10^3$eggs in four-year-olds, $555x10^3$eggs and $947x10^3$eggs in eight-year-olds. The actual and potential fecundity may differ depending upon the numbers of oocytes in the potential that are resorbed during the spawning season. In investigated ovaries of Rockall haddock it was not revealed resorption of vitellogenic oocytes and other abnormalities of the gametogenesis that usually result in reduction of the fecundity and the miss of the spawning. However, the resorption of the unshed mature oocytes were found.

The percent size of an egg batch did not change greatly with age and varied from 5.3 to 9.5% of the total number of eggs found per female. The Rockall haddock produces 11–19 batches over the course of a spawning season.

Philometrid Parasites are Present in Wahoo, *Acanthocybium solandri*, Ovaries but do not Preclude Spawning by the Host

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Introduction

More life history information about wahoo, *Acanthocybium solandri*, has become published recently using collections from Florida's Atlantic coast and the northern Bahamas during 1997-2006 (McBride et al. 2008; Maki Jenkins and McBride 2009). Ages from sectioned otoliths show wahoo are short-lived (< 10 years) but grow about 1 m in size in their first year and reach a maximum size of approximately 2 m. Gonad histology shows that wahoo are summer spawners, spawning on average every five days during June-August. The size and age at 50% female maturity are 925 mm fork length and 0.64 years, respectively. Batch fecundity was positively correlated with fish size, varying between 0.44 and 1.67 million eggs, and annual fecundity estimates are on the order of 10-100 million eggs. Much of these data confirm earlier reports in other geographic regions or expand on preliminary data available in the gray literature.

Results

Unexpectedly, a nematode parasite was observed in 18 of 147 (12%) histological preparations of ovarian tissue (Figure 1). Whole worms were not noted during gross evaluation of ovaries, and there are no reports of this parasite occurring previously in this host. Infection site and morphology of the parasites in histology sections were consistent with members of the family Philometridae. Although collections occurred year round, all fish with ovarian parasites were collected during May-September. One host was immature, 15 were mature-active, and two were mature-inactive. They ranged in size from 982-1522 mm fork length. Most hosts were in their second summer of growth (age 1, n = 9) but several were older (age 2, 3, or 4, n = 5, leaving four fish with no age). Most parasitized fish had vitellogenic oocytes, and several had oocytes with migrating nuclei or post-ovulatory follicles as illustrated in Maki Jenkins and McBride (2009), so these parasitized fish were capable of spawning.

Discussion

Philometrid parasites are widespread in this geographic region (Moravec et al. 2008b;) as well as globally (e.g., Moravec et al. 2008a). The prevalence and occurrence of this parasite in wahoo is similar to that reported for the spotted seatrout, *Cynoscion nebulosus* (13% of ovaries, found in mature females during the spawning season; Perez et al. 2009). They are reported to cause significant pathological effects in ovarian tissue in other host species (Clarke et al. 2006).

Although we did not find evidence that this parasite negatively affected its host, we recommend that fisheries biologists examining gonad histology slides should note the occurrence of this parasite, especially if it is not destructive, to develop a better understanding

of how this type of parasite may or may not affect the population dynamics of marine fisheries.

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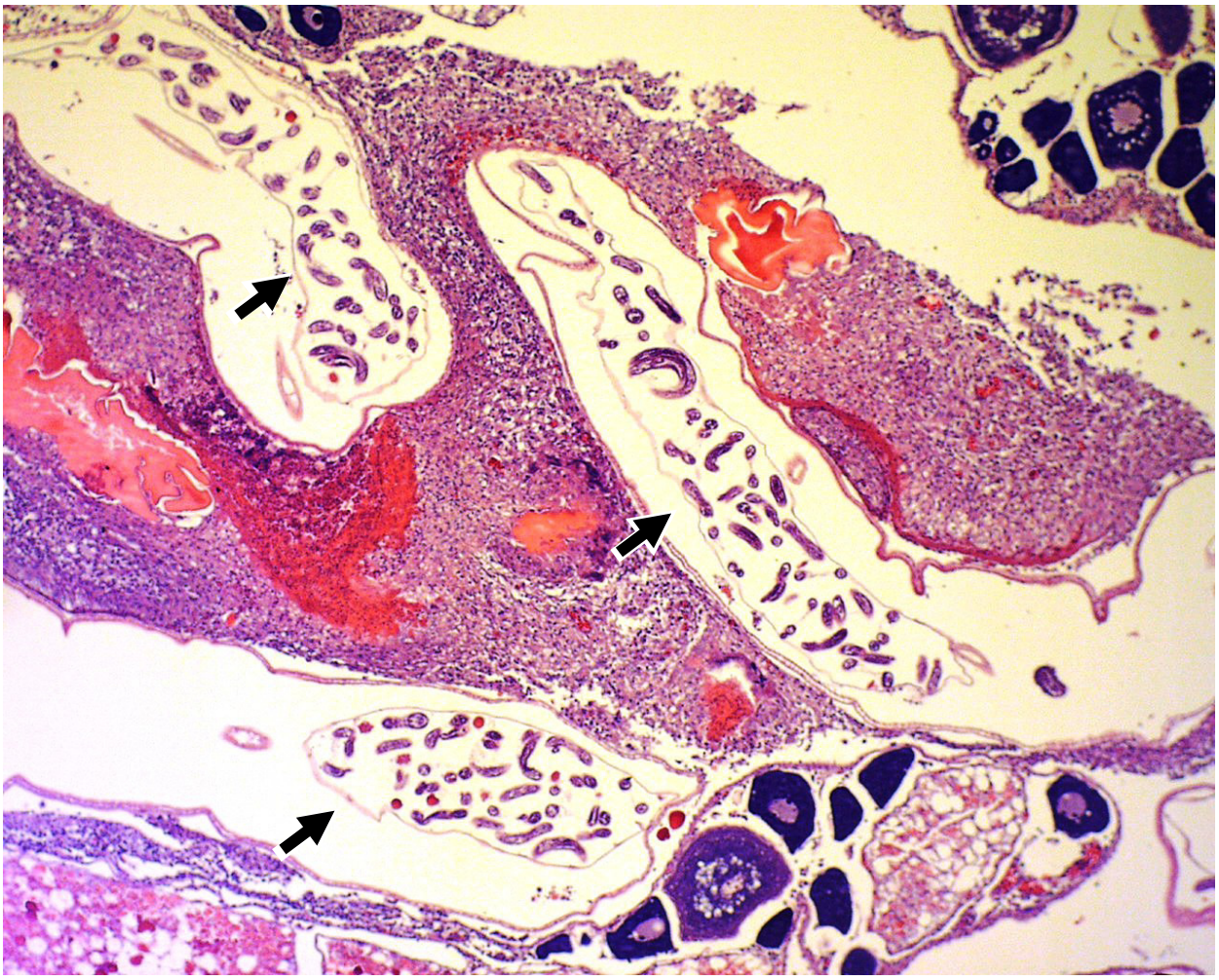

Figure 1. Histological (hematoxylin and eosin staining) section of wahoo ovarian tissue showing three sections of parasite (arrows). The host was a mature-active female (with vitellogenic oocytes). It was 1337 mm fork length, 14.9 kg total body weight, 3.4 years old, and caught in August, 2003.

Intersex and Other Gonadal Abnormalities in Sturgeons from the Missouri River, USA

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Sturgeons (Acipenseridae) are normally gonochoristic; however, pseudohermaphrodites with intersex gonads collected from the impounded and highly altered Missouri River, USA have been reported sporadically in the literature since the 1960's. Over a twelve-month sampling period (2001-2002), we attempted to collect 15 male and 15 female shovelnose sturgeon (*Scaphirhynchus platorynchus*) from the Missouri River, Missouri to document seasonal changes in the reproductive physiology. During the course of that study, we observed intersex rates in the shovelnose sturgeon of 0 to 23% of total fish caught monthly. Fifty percent of the intersex fish were captured in January and February. Intersex over this time averaged 12.5% of males, about six times greater than observed rates in this species in the Missouri River 40 years ago (Moos 1978). This increased occurrence of intersex over historic levels has remained relatively stable since 2002. Moreover, we have since documented intersex in a second species, the endangered pallid sturgeon (*S. albus*).

The pattern and severity of intersex varies widely among individual fish. Most intersex gonads were predominantly testicular with grossly visible patches of black or yellowish oocytes (Figures 1A and 1B). However we occasionally observed, microscopically, small patches of testicular tissue among ovarian follicles deep within an ovarian lobe (Figure 1C) or single primary oocytes in the center of a testicular lobe (1D). In most intersex fish, oocytes were distributed more-or-less randomly across the exterior surface of a testis (Figure 1B). In a few individuals, a mass of oocytes was located anteriorly, whereas the posterior portion of the gonad lobe was testicular (Figure 1A). Also, sturgeon with one normal-appearing testis lobe and one normal-appearing ovary lobe were encountered.

Gametes in gonads with mixed-sex tissue may be at any stage of development and may or may not be associated with lamellar folds (Figure 1E); in some individuals we observed only lamellar folds embedded in testicular tissue. Primary oocytes have been seen within a fat matrix (Figure 1F), whereas others are found just under the tunica albuginea. In addition, maturing black eggs are common and may be normal or atretic; no follicular cells are apparent around the oocytes; and the testicular parenchyma appears normal. Other gonadal abnormalities were regularly observed. These included: deformed or severely shortened testes; teratomas associated with the gonad or free in the body cavity; and post-spawning period ovaries either completely spent and containing only atretic oocyte follicles or partly spent with many atretic follicles.

Reports of intersex and reproductive abnormalities in sturgeons from other river systems with declining sturgeon populations have suggested chemical contaminants as a potential cause (Feist et al. 2005). Missouri River sturgeons contain elevated concentrations of chemicals classified as endocrine disruptors, including polychlorinated biphenyls (PCBs), chlordane,

and mercury (Gale et al. 2008). Fish consumption advisories have been issued since 1997 due to these chemicals (Missouri Department of Health and Senior Services 2008). However, in our intersex fish, assays for biomarkers of exposure show low ethoxyresorufin O-deethylase (EROD) activity (mean = 2.8 pmol/min*mg) and vitellogenin protein levels in males (an indicator of exposure to estrogens) are not elevated (Figure 2).

The causative agent(s) or factor(s) contributing to the high incidence of reproductive pathologies observed in Missouri River sturgeons have not been determined. Wild fishes with aberrant mixed-sex gonads collected from fresh and marine waters from a number of countries have been reported with increasing frequency over the past decade (Blazer et al. 2007). However, in contrast to sturgeon, intersex in these fishes has only been observed microscopically. Research has shown that testicular oocytes (primary oocytes scattered among testicular tissue) and even sex-reversal can be induced in fish by exposure to natural and synthetic steroids and some chemicals (Ankley et al. 1998). It is also known that testicular oocytes can result from hybridization, thermal stress, radiation exposure, and other causes. Regardless of the cause, the exact mechanism(s) of how a primary oocyte is produced within the testicular environment of a genetically male (XY or ZZ) fish is unknown. Given the importance of some sturgeons as endangered relic species and others for their caviar and meat, our research will continue to focus on causative factors leading to intersex and the mechanisms involved. The normally low rate of intersex in sturgeons generally as well as the extreme and grossly visible nature of intersex in Missouri River sturgeons make them uniquely suited as indictor organisms for environmental stress.

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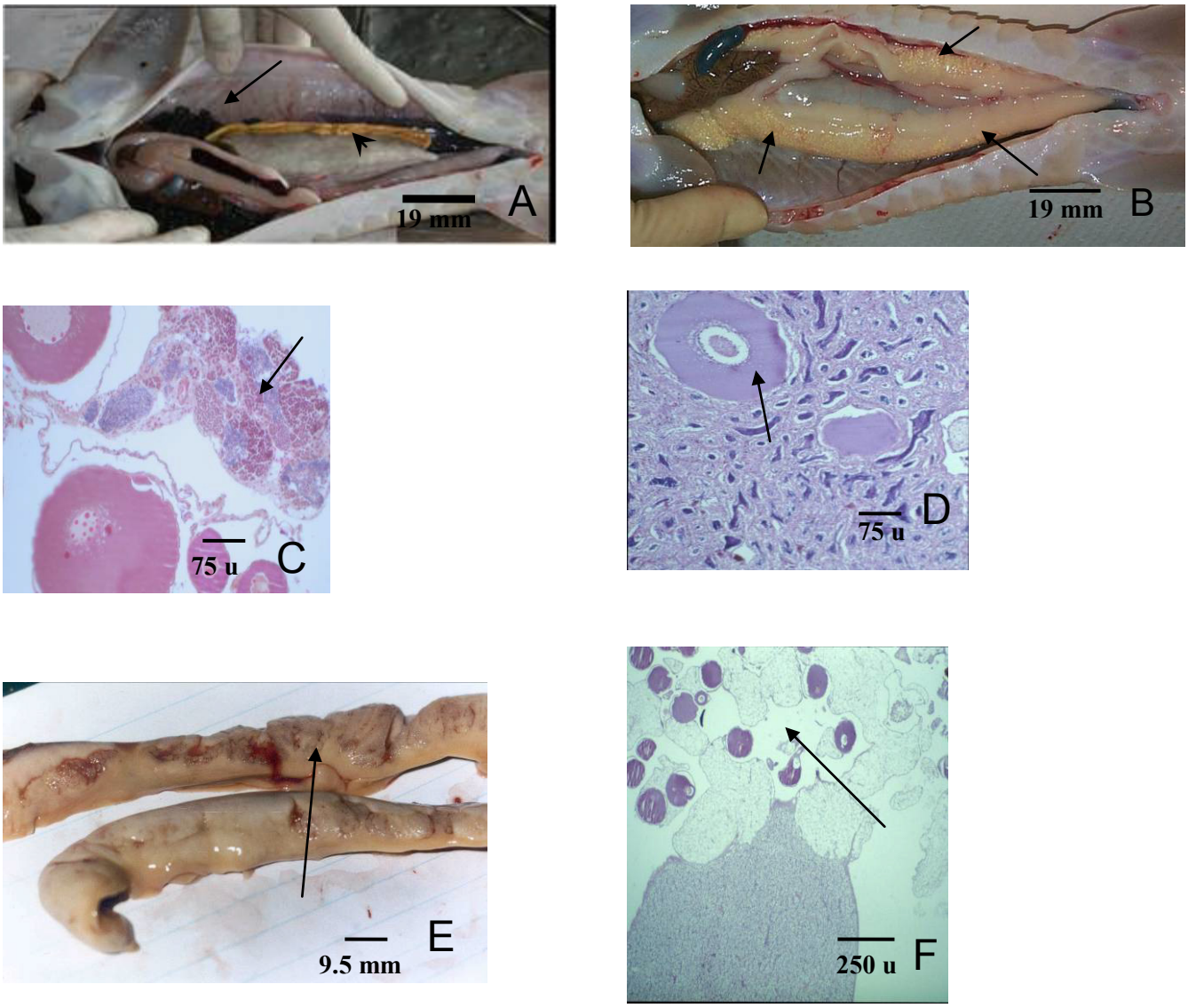

Figure 1. Morphology of intersex gonads of shovelnose sturgeon from the Missouri River, USA. A. Mature black oocytes (arrow) at the anterior end of a testis lobe. B. Vitellogenic oocytes (arrows) distributed along the length of the testes. C. H&E section of ovary with a focal area of testicular tissue (arrow). D. H&E section of testis with testicular oocytes (arrow). E. Intersex gonad with lamellar folds (arrow). F. Pre-vitellogenic oocytes within fat (arrow) on surface of testis.

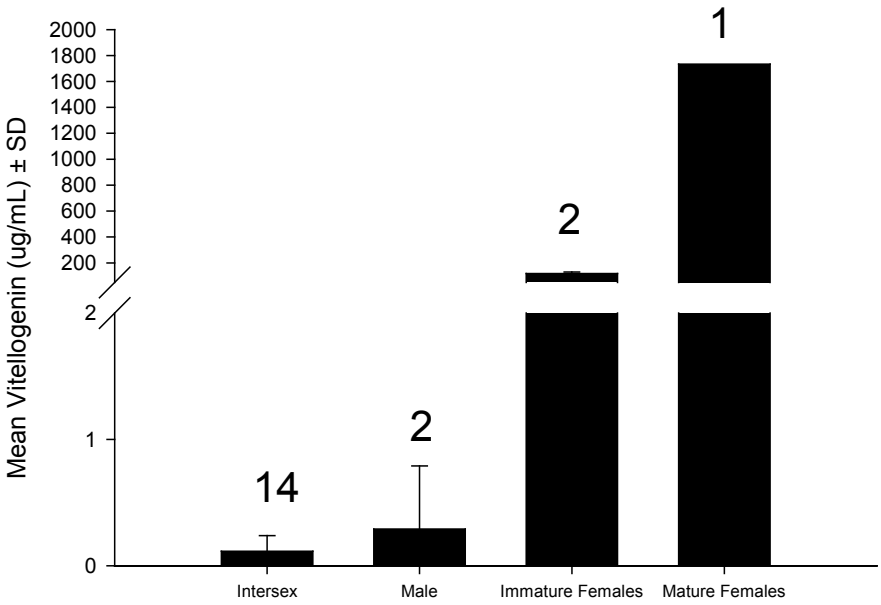

Figure 2. Measured plasma vitellogenin in shovelnose sturgeon from the Missouri River, USA with intersex gonads compared to normal males and females. Numbers above bars indicate number of individuals in sample.

Understanding Temporal Reproductive Patterns in Marine Fish

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Introduction

We define "temporal reproductive patterns" as those parameters associated with reproductive timing, as well as the gonadal development necessary to reproduce. Temporal reproductive patterns, like all aspects of a species' reproductive strategy, are driven by energetic and mortality rates. A number of environmental cues act as triggers for gonadal development and spawning, the most common being: temperature, photoperiod, and lunar and tidal cycles. Spatial factors, such as distance from home range to spawning site and distance from spawning site to nursery habitat will also affect temporal reproductive patterns.

These reproductive patterns occur over four temporal scales: lifetime, annual, intraseasonal and diel (Lowerre-Barbieri et al. 2009). Although all fish go through similar reproductive patterns, timing differs depending on the species' reproductive strategy (Figures 1 & 2). At the lifetime scale, fish mature, go through one or more reproductive cycles, reach maximum age and die. Semelparous species, such as Pacific Salmon (*Oncorhynchus* spp.), go through the reproductive cycle only once. However, iteroparous species, which are more common, go through the reproductive cycle multiple times within a lifetime. The reproductive cycle reflects spawning seasonality, which for most species, occurs on an annual basis. Two reproductive strategies occur at this scale: batch spawners, which spawn multiple times within a season and total spawners, which spawn only once in a season. The intraseasonal scale refers to events within the spawning season. Two events commonly studied are oocyte recruitment from cortical alveoli to yolked and the frequency of spawning. If yolked oocytes are recruited once in a season, a species has determinate fecundity. Species with indeterminate fecundity recruit oocytes to the yolked stage multiple times within a season. The diel scale reflects the time of spawning within the diurnal period and is indicated by oocyte maturation, ovulation and postovulatory follicles (POFs). Many marine fishes exhibit diel periodicity, and spawn in the evening.

Lifetime Reproductive Patterns

Reproductive parameters estimated at the lifetime scale include maturity, reproductive lifespan, and the interval between reproductive cycles. These reproductive parameters are used to estimate the reproductive potential of the population, commonly expressed as the spawning stock biomass (SSB). Although this is the scale at which most stock assessment models incorporate reproduction, it is becoming increasingly clear that there is a need for

better estimates of reproductive parameters at this scale, as well as an improved understanding of the relationship between SSB and reproductive potential (Morgan et al. 2009). Maturation is the most-used reproductive parameter in traditional stock-assessment models and the trait expected to have the greatest impact on fitness (Lowerre-Barbieri 2009). Yet there is no standardized methodology to estimate this parameter. We suggest adopting the seasonal filter method of Hunter and Macewicz (2003) to improve maturity estimates. With this method, the proportion of immature and regenerating females is evaluated over the spawning season and data from months with few or no regenerating females is used to estimate size and age at maturity. Improved and standardized maturity estimation methods are necessary to effectively assess changes in maturity over space and time and their potential cause. There is also a need to evaluate whether all mature fish spawn each year. Skipped spawning appears to be especially common in cold-water fish that exhibit spawning migrations, with individuals in poor condition skipping entire spawning seasons, especially younger fish (Rideout et al. 2005; Jorgensen 2006).

Reproductive Timing

Annual, intraseasonal, and diel reproductive patterns interact to determine reproductive timing. Knowledge of reproductive timing is important to understand: birth date dynamics, the number of breeding opportunities, fecundity in indeterminate species, the spatial distribution of spawning and factors affecting egg survival. It is also a prerequisite for applying the daily egg production method. We define birth date dynamics as those aspects of recruitment which are directly linked to where and when eggs are spawned. Understanding where fish spawn is important to assess effective populations, population connectivity and habitat needs, as well as for developing marine protected areas. However, where fish spawn cannot be determined unless it is known when fish spawn, both at the annual and diel scale. How these parameters are estimated will differ by species as fish go through their reproductive cycles at different rates depending on reproductive strategy, food availability, metabolic rate and water temperature. Most cold water species show slower rates of gonadal development than warm-water species. For example, cold water species commonly show a lag between vitellogenesis and spawning whereas warm-water species do not. Similarly, POFs in cold water species can persist for months (Rideout et al. 2005) but in warm-water species they are usually resorbed in 48 h. These differences will affect how histological indicators can be used. The spatial distribution of spawning will also affect our ability to estimate temporal parameters such as spawning frequency, as the assumption that fish do not move in or out of the sampling areas is often violated by species which demonstrate aggregate spawning.

Birth date dynamics are also greatly impacted by spawning seasonality and demographic trends in spawning periodicity (i.e., individual spawning seasons). At the population level, spawning seasonality can be either extended (more than three months) or restricted. Restricted spawning seasons are associated with specific environmental conditions and populations with this pattern are expected to be more highly impacted by climate change and exhibit greater recruitment variability. Cold water species are more apt to demonstrate restricted spawning seasons in comparison to warm-water species. Batch spawners with extended spawning seasons produce year classes with a wide range of sizes-at-age. This in turn will affect time at maturation, recruitment to the fishery and when individuals begin to spawn. Spawning periodicities can be quite variable. Older, larger individuals of many

species appear to begin spawning earlier and often spawn longer and more frequently than younger, smaller fish. Cod have been shown to double their spawning seasonality over the course of their lifetimes. This, combined with size-related trends in fecundity, has the potential to greatly increase reproductive success of older, larger fish.

A manuscript based on this presentation has been submitted to the journal Marine and Coastal Fisheries as part of the special module "Emerging Issues and Methodological Advances in Fisheries Reproductive Biology". Publication is anticipated in fall 2010. Web page: www.fisheries.org/mcf.

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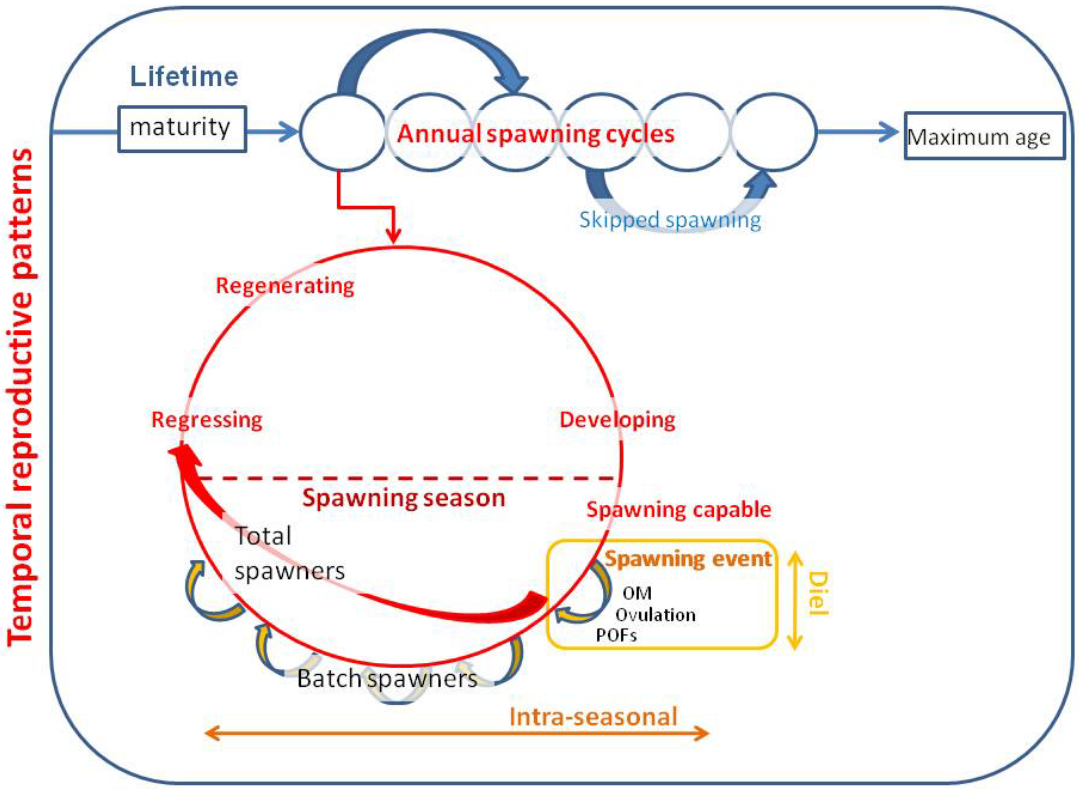

Figure 1. A conceptual model of temporal reproductive patterns occurring over four temporal scales: lifetime, annual, intra-seasonal and diel scale.

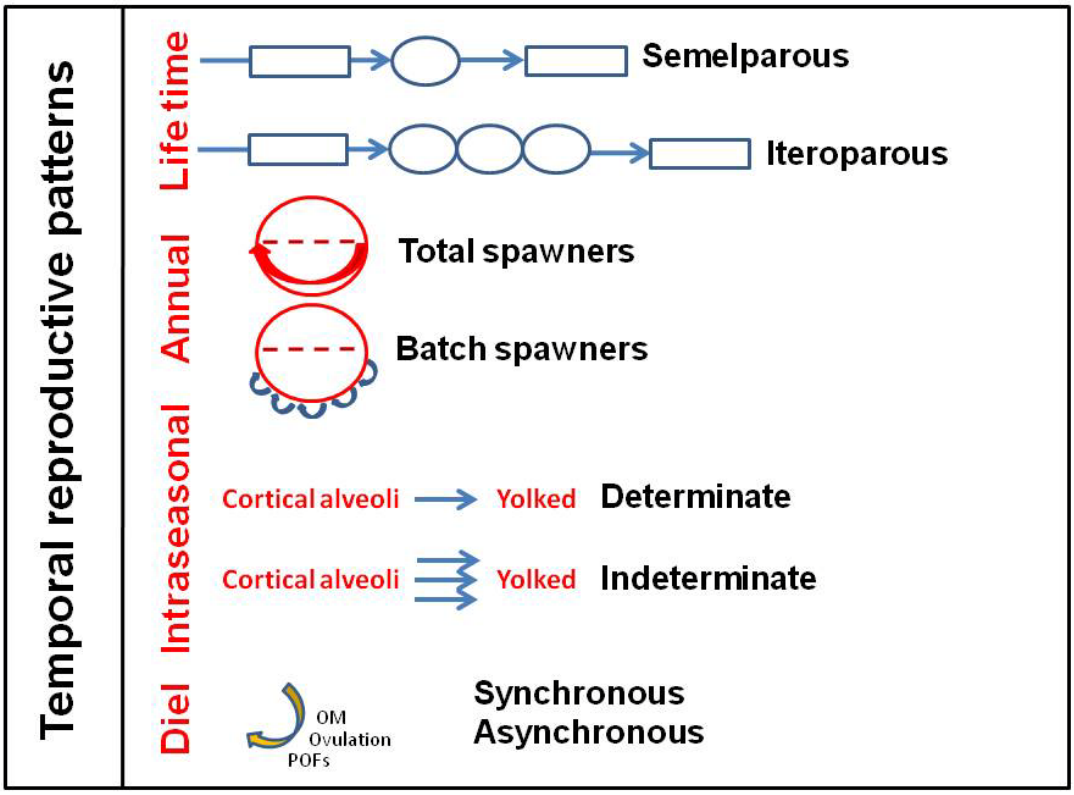

Figure 2. The role temporal patterns play in fish reproductive strategies.

Importance of Histological Information in the Assessment of Southern Stock of European Hake

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Introduction

Maturity stage is a classification of fish gonadal development. Maturity stage is used to differentiate between mature and immature individuals, and is therefore useful for spawning stock biomass (SSB) calculations, spawning season definition, monitoring long-term changes in the spawning cycle, and many other research needs regarding reproductive fish biology. SSB is one of the main criteria for judging the status of an exploited fish population. Usually maturity ogives are estimated based on macroscopic observations of gonads. However, macroscopically, virgin ovaries (immature) and resting ovaries (mature) are similar. Distinguishing between these stages is only possible using histological maturity staging identification. The present work presents quick and easy measures to minimize the effect of macroscopic maturity misclassification on the Southern hake stock (ICES Div. VIIIc and IXa). The work developed is based on conclusions from the Workshop on Sexual Maturity Staging of Hake and Monk (ICES 2007). Based on histological results, some recommendations are made to improve maturity data for stock assessment proposes.

Material and Methods

Samples were caught in 2 different geographic locations of the Southern hake distribution area in Spain: Galicia (north Atlantic coast) and Gulf of Cadiz (southern Atlantic coast). The peak of spawning activity was determined in each area based on the presence of hydrated oocytes and recent POFs in the ovary. Misclassification of the proportion between immature and mature fish by length class (n = 81 ovaries) was estimated. A correction factor was also estimated based on the proportion of regenerating and immature females within maturity group I (regenerating or immature).. Maturity ogives based on (i) macroscopic data ("macroscopic ogive"), (ii) validated data by histology ("microscopic ogive"), and (iii) macroscopic data corrected by the correction factor ("corrected ogive"), were compared. Data used for ogive estimations include only female gonads from the peak of spawning.

Results and Discussion

In Vigo, peak spawning was observed in February-March while in the Gulf of Cadiz the peak was detected in August (Figure 1). When distribution of maturity misclassification was analyzed by length, it was observed that the percentage of classification errors changed depending on female size. Misclassification percentage was higher in females between 31 and 35 cm.

Length at first maturity (L_{50}) estimated in Vigo was 43.4 cm (macroscopic), 41.2 cm (microscopic) and 42.1 cm (corrected), whereas in Cadiz it was 42.4 cm (macroscopic) and 41.3 cm (corrected). In Vigo, the "macroscopic ogive" was significantly different from the

"microscopic ogive", and the "corrected ogive". However, the "corrected ogive" did not differ from the "microscopic ogive" (Figure 2a). In Cadiz, the "corrected ogive" was significantly lower than the "macroscopic ogive" (Figure 2b). Although the "microscopic ogive" has not been estimated in Cadiz we can assume that results would be similar to those observed in Vigo, so the "corrected ogive" should not be significantly different from the "microscopic ogive". A comparison between these results (with data from the peak of spawning) and previous studies in Vigo (Domínguez-Petit 2007) using year around data shows that even without applying the correction factor, differences between "macroscopic" and "microscopic ogive" were small. This fact is related to the low proportion of regenerating females during the peak of the spawning season, and consequently the low number of immature misclassification. Additionally, the application of the correction factor based on histology significantly improves L50 estimations, minimizing differences from the "microscopic ogive". Overestimation of L50 leads to underestimation of SSB, as happens when L50 is estimated based on macroscopic maturity classification. The estimation of a correction factor based on histological validated data improves SSB estimations. Given the low number of microscopic data in this study, it is recommended to extend this study with a larger sample size covering the whole Southern European hake stock distribution area.

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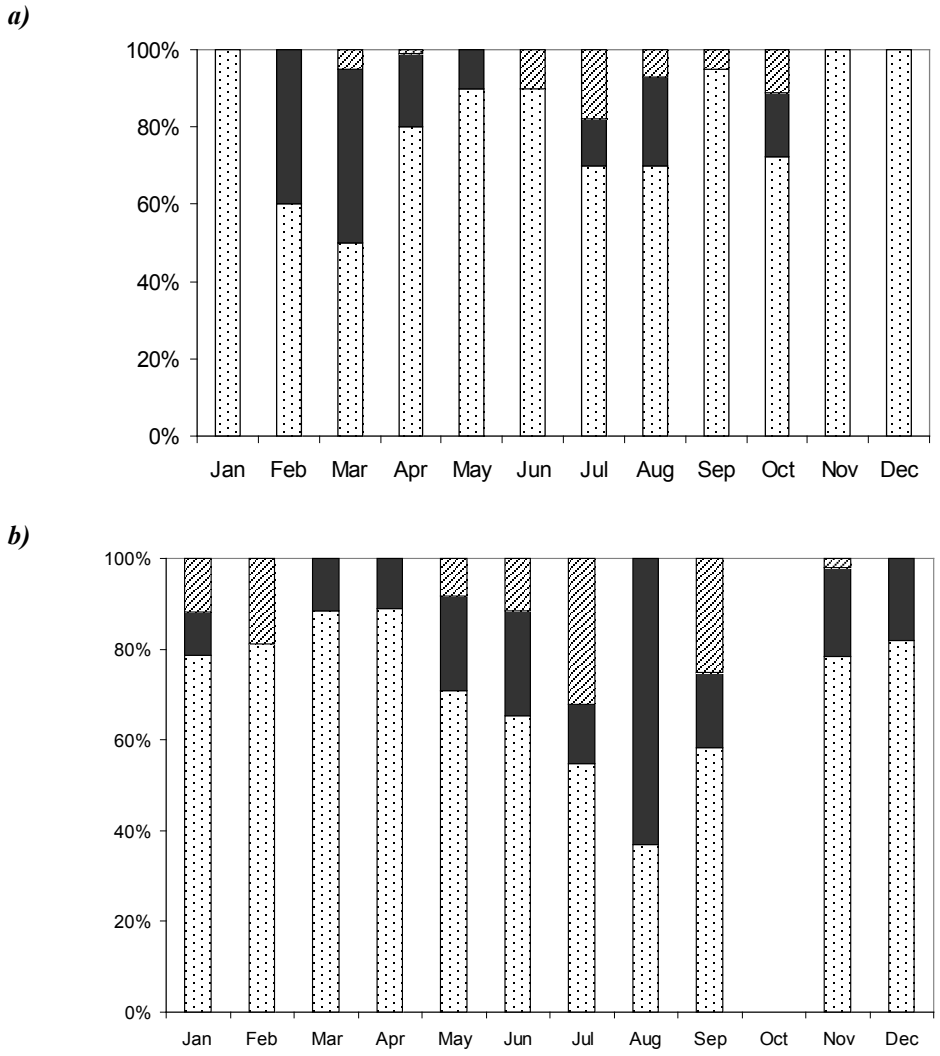

Figure 1. Monthly variation of female maturity stage proportion a) in Vigo and b) in the Gulf of Cadiz. Dotted bar = pre-spawning females; solid bar = spawning females; striped bar = post-spawning females.

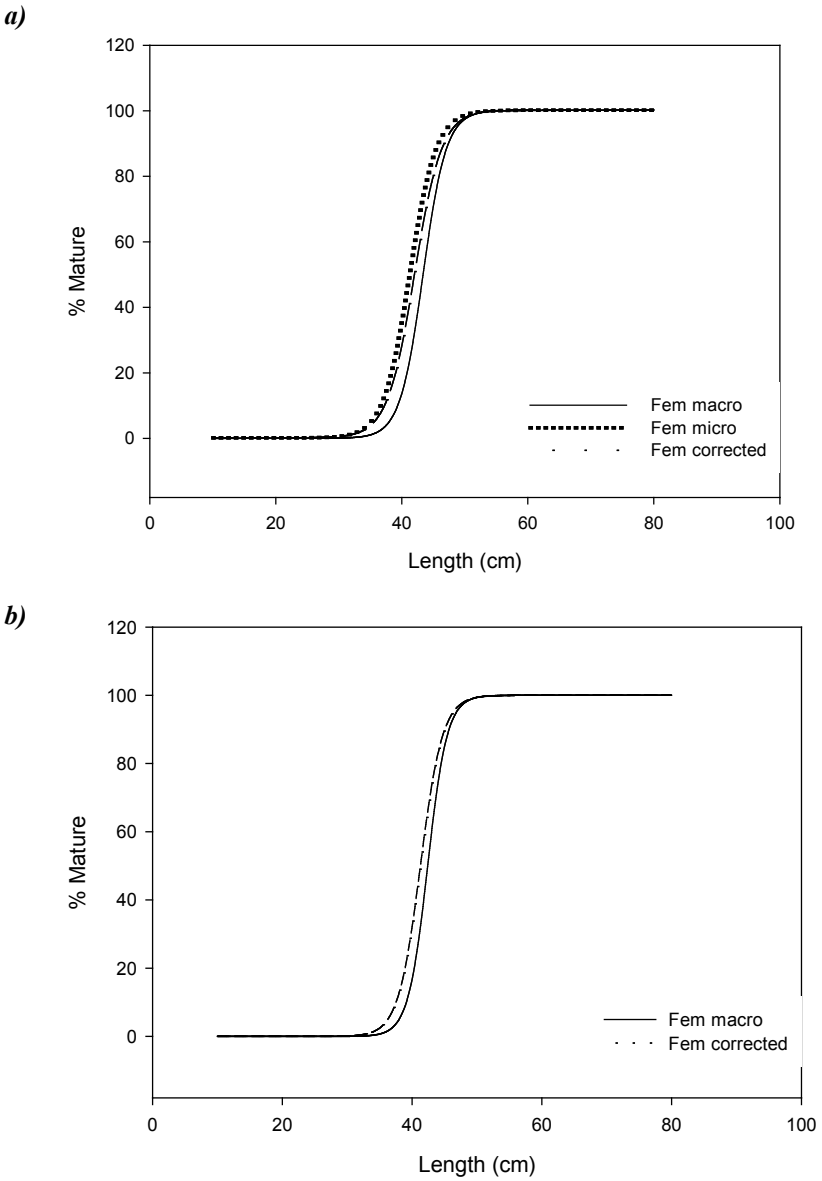

Figure 2. Maturity ogives estimated with different maturity data in a) Vigo and b) Gulf of Cadiz.

Gonad Histology of Vendace *Coregonus albula* in Lake Paravani, South Georgia

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Introduction

Vendace *Coregonus albula* Linnaeus is an introduced species in Georgia. It was introduced during 30th of the 20th century, from Ladoga Lake in Russia to Lake Paravani, which is the biggest lake by its surface area (37.5 km ²) in Georgia. Histological structures of gonads of vendace from Lake Paravani have not been studied before our research. The aim of the present research was to study the structure of sexual organs and cyclic changes occurring in the gonads of vendace, in addition to examining the development of gametes and possible factors related to the disturbance of these processes.

Methods

Fish were collected on a seasonally basis from Lake Paravani during 1999-2007. The following measurements were made for each sampled fish: total body length (TL), wet body weight and wet gonad weight. Gonadosomatic index (GSI) was also calculated. Fish age was assessed by counting annual rings. The gonad analysis included visual examination of the condition of gonads, weight of gonads and their histology. Variation of maturity index during the sexual cycle was investigated and maturity stage of each specimen was examined according to size and weight of gonads. For histological studies, gonads from females and male were dissected, fixed in Bouin's solution and embedded in paraffin. Sections at 6 µm thickness were stained according to Mallory's method and with Heidenhain's hematoxylin. Development stages were defined according to the scale by Sakun and Butskaya (1968).

Results and Discussion

We have described 5 sexual stages for female and males vendace. Ovaries of vendace in maturity stage I have thin, transparent, or some times yellowish or pinkish string-like form of 1.3 ±0.86 cm in length. The size of immature oogonium reaches 9.7 ± 0.2 µm. The nucleus of oogonium were often roundish, with a size of 6.5 ± 0.1µm. Two groups of cells were distinguished in the period of previtellogenesis. At the early step of this stage, size of sexual cells was 10.5 ± 0.1 µm, growing in the next phase to 19.2 ± 0.4 µm. At the second stage of maturation the majority of sexual cells in the ovary are represented by oocytes in the protoplasmic growth phase. We distinguished 3 groups of oocytes of different size in the second stage of maturation. Size of the oocytes of the first group equaled 33.2 ± 0.5 µm (range 29.6-42.2 µm), size of the oocytes of the second group equaeds 78.7 ± 1.9 µm, and size of the oocytes of the third group equaled 115 ± 0.7 µm (range 98.4–116 µm). At the third development stage, the size of ovary greatly increased because of the intensive growth of oocytes. Oocytes could be observed clearly even with the naked eye. Their size reached 167 ± 1.8 µm (range 138.3-197.4 µm) (Figure 1a). At the fourth stage of maturation trophoplasmic growth is ended or almost ended in the oocytes, and the oocytes reach the final size typical of vendace species. Ovaries occupy the largest part of the body and blood vessels

are prominent. The micropyle is formed, and in some it is already formed and filled up with follicular cells. Ovulation takes place at the V stage of maturation. At this stage, vendace can release oocytes with a slight touch on the sides. The size of matur oocytes varies from 1,035 to 1,250 µm (Figure 1b). After spawning, ovaries looked like red wrinkled plates, and contained many small oocytes although some large oocytes were observed as well. This stage begins at the end of November and lasts until December-January in Lake Paravani. Only follicles and germ cells are left in the ovaries: oogonia and oocytes from protoplasmic growth. In August, oocytes of vendace are wider, and GSI varies between 2.47-7.1%. The GSI values reach their maximum before spawning (Figure 2b).

Testes in the immature stage contained spermatogonia and small cysts of spermatocytes. Mature testes of vendace occupied most of the abdomen, and spermatozoa, spermatocyts and spermatids are present. Spermatogenesis was completed by the fourth stage of maturity (Figure 1c), and spermatozoa became more abundant and filled the lumen of the lobules. The GSI increased from 0.88 to 4.57% (Figure 2a). In November, the sperm ducts testes are full with spermtazoa that came from the cysts (Figure 1d). The processes of termination of spermatogenesis began slightly before mating, with a decrease in GSI to 2.63 ± 0.25%. The testes before spawning are at IV-V stage. After spawning, GSI of vendace males varied from 0.32 to 0.65%.

Our research has shown that maturation stages progress normally only in certain ecological conditions. Development of vendace gonads depends on photoperiod and temperature. The II stage of development of gonads in Paravani Lake requires water temperature of 0.8º-9ºC, stage III requires 15ºC, and optimal temperature for IV stage is 20-24ºC. Gonads turn to stage V stage in Paravani Lake at 7-10 ºC. We have observed that early stages of gametogenesis require low temperatures, while stages III-IV of development require an increase in temperature and light.

Disorders in the process of gametogenesis could be caused by different factors, especially ecological changes (photoperiod, water temperature). Anthropogenic factors, such as human mismanagement of fish habitat, intensive poaching and fishing, climate changes and global warming are leading factors altering the sexual cycle of vendace in Lake Paravani. From 1958 to 1995 massive migrations tospawning sites was observed until 18-20 November (Demetrashvili 1960, Japoshvili 2002). Our investigation has shown, that recently water in lake Paravani has become warmer due to global warming, and the spawning period of *Coregonus albula* has shifted to December (Japoshvili 2002), which coincides with the V stage of ovary development.

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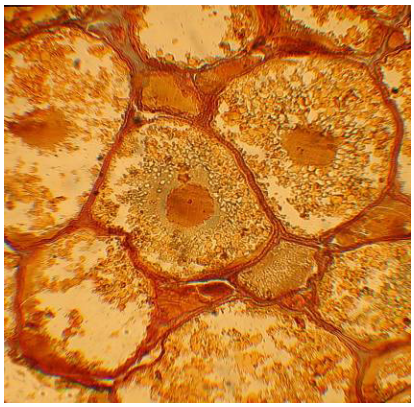

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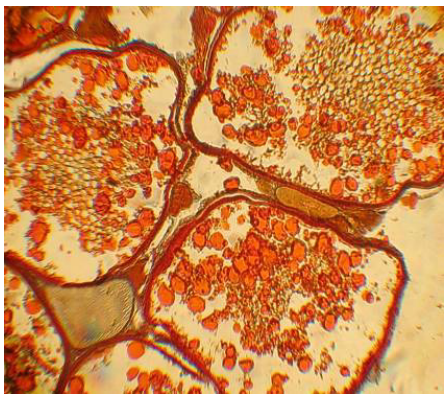

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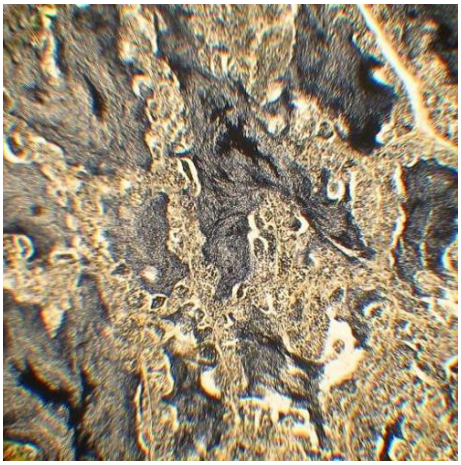

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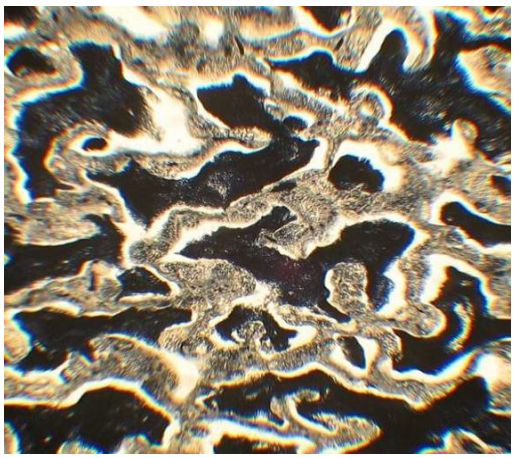

d

Figure 1. Histological sections of ovaries and testes of *Coregonus albula*, at different stages of maturation. a) ovary in maturity stage II; b) mature oocytes; c) completed spermatogenesis, stage IV, July; d) section of testes ready for spawn, November.

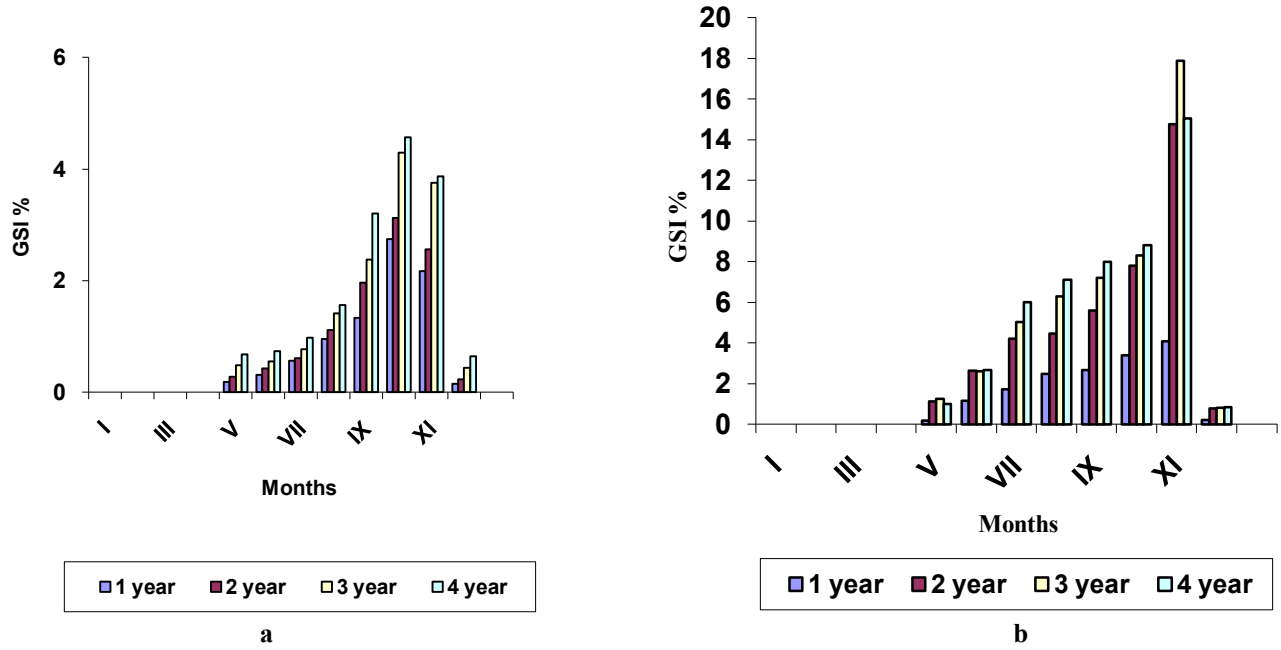

Figure 2. Monthly changes in GSI of vendace. a) Males b) Females

Quantification of Seasonal Follicular Cycle of an Indeterminate Fish: the European Hake Model

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To clarify the seasonal follicular dynamics, and hence the regulation of fecundity, in indeterminate reproductive style fish species with asynchronous oocyte development, a considerable amount of effort has been directed towards quantifying temporal relative changes between a heterogeneous population of first growth phase follicles and clutches of larger growing follicles.

Traditional methods may be considered inefficient to estimate the number of oocytes because they are based on assumptions about the size, shape, and orientation of the oocytes and an arbitrary sampling over the ovary, and thus, they influence the estimation of the individual reproductive investment through the season. In this study, stereological design-based methods based upon Cavalieri estimator (Cavalieri 1635) and the case-adapted disector technique, the "double disector" (Marcussen 1992), have been used, under a uniform random probability sampling regime (SURS) (Gundersen and Jensen 1987), to obtain unbiased and accurate estimation of the total number of chromatin nucleolar, perinucleolar, pre-cortical alveoli, cortical alveoli, early vitellogenic and advanced vitellogenic oocytes (Figure 1A) within known volume ovaries throughout the reproductive season. Besides, these methods have allowed assessing the contribution of biological variability to the variation in the sample estimates in order to evaluate the sampling efficiency and precision (coefficient of error) of the estimates.

One ovary lobe from each of 13 individuals was embedded in agar (7%) and sectioned exhaustively, in parallel slabs of 2.5 mm in width, along the longitudinal axis. Five slabs were selected using SURS and from their areas of the sectional profiles the lobe volume was estimated. Each slab was embedded in methacrylate and seven 4-□m-thick parallel slides, with a known distance between them, were sectioned from each of the embedded slabs. Ten fields were selected, spread as evenly as possible through the 5 slabs in order to place an unbiased counting frame, and the counting rules were applied (Köning et al. 1991) through the ribbon of collected section pairs: 1st-6th, 1st-11th, 1st-6th, 1st-21st, 1st-26th and 1st-36th (Figure 1B). The number of oocytes at each developmental stage was estimated from the sum of nucleus count within the counting frame divided by the disector volume (which was estimated multiplying the area of the counting frame by the disector height or the distance between the pair of sections that form the disector) and multiplied by the ovary volume (2 times the estimated lobe volume).

The results of this study provee that this method, which has been used for the first time in our knowledge with an asynchronous oocyte development fish, can be considered a valid tool to estimate the number of oocytes from different stages, i.e. from very small chromatin nucleolar to advanced vitellogenic oocytes. In that sense, the biological variance contributed around 90% at each oocyte stage to the observed variance in total oocyte number among the 13

individuals. This was satisfactory because the sampling variance introduced by the stereological estimation procedure was a minor fraction of the observed variance; therefore, the results confirmed that this method is an accurate method to quantify the total number of oocytes of a wide range of diameters.

The estimation of the number of oocytes at these stages for species showing an asynchronous ovary development provides valuable information of oocyte recruitment and the dynamics of oocyte growth; this, in turn, provides an opportunity to investigate the fecundity dynamics that seem to be regulated by a complex mechanism**.** Quantitative results (Table 1) revealed that the number of previtellogenic oocyte stages was several times greater than the populations of growing oocytes all over the spawning season. The number of chromatin nucleolar and perinucleolar oocyte stages represented almost 70% of the total number of oocytes in the ovary, whereas other stages remain at a level below 20% (pre-cortical alveoli stage) or below 10% (cortical alveoli, early vitellogenic, and advanced vitellogenic stages).

The results obtained also suggested that morphometric variables affect individual reproductive investment, as there was a significant correlation between fish length and the number of oocytes at the pre-cortical alveoli stage (r^2=0.67, $p<0.05$), which was not observed in any of the other oocyte stages. Thus, body size may indicate when a fish can dedicate effort to reproduction and how much it can invest. Chromatin nucleolar stage oocytes increased in number after March and up to July when the gonadosomatic index was lowest, showing that the recruitment of this stage may be determined during these months.

A manuscript based on this presentation has been submitted to the journal Marine and Coastal Fisheries as part of the special module "Emerging Issues and Methodological Advances in Fisheries Reproductive Biology". Publication is anticipated in fall 2010. Web page: www.fisheries.org/mcf.

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(A)

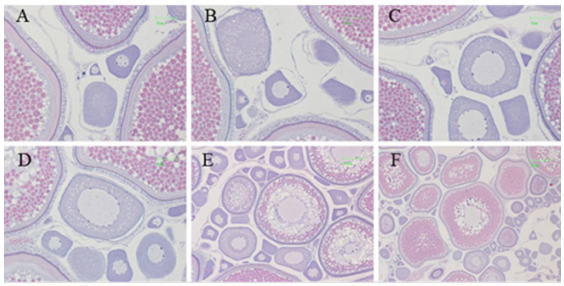

Figure 1A. Histological examples of each of the oocyte development stages chosen for this study. A) chromatin nucleolar stage (>0.045 mm), B) Perinucleolar stage (0.045-0.100 mm), C) Pre-cortical alveoli stage (0.060-0.150 mm), D) Cortical alveoli stage (0.150-0.250 mm), E) Early vitellogenic stage (0.250-0.450 mm), F) Advanced vitellogenic stage (0.450-0.600 mm).

(B)

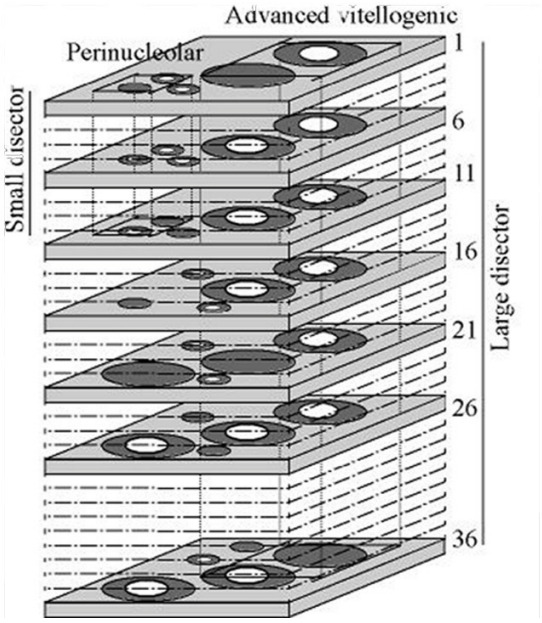

Figure 1B. An illustration of the "double disector" or "multiple double disector" as applied in this pilot study. A ribbon of 4 µm-thick histological sections is generated by the microtome. Oocytes are in dark grey with white nucleus (not in scale). In "small disector", the first and 11th sections are used to estimate the numerical density of perinucleolar oocytes. In "large disector" the first and last sections (36th) are used to estimate the numerical density of the advanced vitellogenic oocytes.

Table 1. Data from 6 oocyte populations of 13 ovaries showing mean values, standard deviations, and coefficients of variation. S.D.=observed standard deviation; CV=observed coefficient of variation, S.D./mean; ∑Q=The total number of oocyte nuclei counted on all slabs with double disector; meanQ=The mean number of oocyte nuclei per disector; Nv=number of oocyte nuclei per mm3; N= total number of oocytes in the lobe (N=V x Nv).

Ovary No.	ΣQ	No. Disectors	*MeanQ*	N_V ($10^3 mm^3$)	N (10^6)	N ovary (10^6)
0·550 mm						
Mean	71	70	1.02	0.007	0.188	0.375
SD	11.75	5.80	0.14	0.001	0.071	
CV	0.165	0.083	0.137	0.137	0.378	
0·350 mm						
Mean	83	72	1.13	0.010	0.251	0.502
SD	28.52	5.99	0.31	0.003	0.078	
CV	0.345	0.083	0.273	0.336	0.309	
0·175 mm						
Mean	88	72	1.22	0.015	0.367	0.734
SD	39.67	2.92	0.52	0.006	0.121	
CV	0.449	0.041	0.423	0.423	0.330	
0·100 mm						
Mean	102	89	1.26	0.025	0.605	1.210
SD	49.09	19.06	0.75	0.012	0.279	
CV	0.482	0.214	0.595	0.465	0.461	
0·075 mm						
Mean	193	108	1.77	0.089	2.158	4.316
SD	100.74	5.24	0.92	0.046	0.981	
CV	0.523	0.048	0.517	0.517	0.454	
0·035 mm						
Mean	90	108	0.83	0.083	2.104	4.208
SD	27.76	5.42	0.25	0.025	0.921	
CV	0.310	0.050	0.298	0.298	0.438	

Seasonality of the Reproductive Activity and Resource Allocation in Sardine from the Iberian Waters

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Sardine *Sardina pilchardus* (Walbaum, 1792) is a small pelagic clupeid, with indeterminate fecundity and multiple spawning, releasing batches of pelagic eggs repeatedly during a protracted spawning season (Stratoudakis et al. 2007). Producing eggs during such an extended reproductive season requires a considerable amount of energy resources. These resources could be supplied either by energy reserves accumulated prior to spawning and/or directly from food input during the spawning season, as has been reported for another clupeid, the northern anchovy *Engraulis mordax* (Hunter and Leong 1981). Sardine off the Portuguese coast feed all year round, with a peak of intensity in winter-spring (Garrido et al. 2008), but accumulate fat mainly during summer (Bandarra et al. 1997). Understanding the way energy resources are traded inter-seasonally between reproduction and fat deposition/somatic growth is important not only to characterize the reproductive strategy, but also because the energy available to produce eggs could affect the reproductive output of the species. The present study thus aimed to 1) describe the reproductive activity of sardine off the central coast of Portugal over a year, and 2) make inferences on the allocation of energy resources among the different body compartments.

Sardine samples were obtained from commercial landings at Peniche twice a month during a year, the individual biological information (length; total, gutted, gonad and liver weights, age, fat stage) was recorded for both sexes (n= 1133 females + 901 males), and the monthly evolution of the following biological indices was obtained: the gonado-somatic index (GSI), the hepato-somatic index (HSI), the relative weight factor (Wr), and the mean fat stage (Fat_m). Additionally, female gonads (n= 416) were preserved and processed histologically to obtain the following histological indices: the stage of the most advanced batch of oocytes (to determine the maturity stage), the presence and age of postovulatory follicles (POF; to estimate the spawning fraction), and the prevalence and intensity of atresia in pre-vitellogenic (all stages of atresia) and vitellogenic (alpha-atresia) oocytes.

The results for the GSI showed a similar pattern for both sexes and indicated that sardine were reproductively active mainly from October to March, with a small “pulse” of activity in May (Figure 1A). For females, the histological analysis of the ovaries confirmed this temporal pattern (Figures 1E and 1F). Oocytes with cortical alveoli appeared in the ovaries in September, vitellogenesis started in October and lasted mainly until March, while during summer ovaries were mainly composed of primary oocytes and atretic follicles at different stages of resorption (Figures 1E and 2D). Spawning fraction increased in October, peaked in December (14%) and decreased sharply from February onwards (Figure 1E). During the main reproductive season, all indices of atresia were very low, but a significant increase of both prevalence and intensity of alpha-atresia in the vitellogenic oocytes was observed in March

and May. The prevalence of atresia in pre-vitellogenic stages of oocytes also increased in March and remained high until August (Figure 1F).

The HSI showed a similar pattern for both sexes outside the reproductive season, whereas it always presented higher values for females during the spawning period: it increased in spring for both sexes, increased again for females in October and decreased for males during most of the autumn (Figure 1B). On the contrary, the two condition indices (Wr, Fat_m) showed an identical seasonal evolution for the two sexes: they decreased from October to March and increased from April to September (Figures 1C and 1D).

The observed seasonal patterns in these biological parameters suggest a seasonal transition of resource allocation from reproduction in autumn and winter to fat deposition and improvement of condition in spring and summer, as has also been reported by Ganias et al. (2007) for sardine in the Mediterranean Sea. Taking into account that somatic growth in sardine off the Portuguese west coast takes place mainly in spring (Silva et al. 2008), it could be hypothesized that a physiological shift allocation from reproduction to growth/condition would occur at this time, a shift regulated by endogenous rhythms and/or determined by external factors. For example, sea water temperature seems to play an important role in determining sardine spawning periodicity in European waters (Stratoudakis et al. 2007). On the other hand, all condition indices reached maximum values just before the beginning of the reproductive season and minimum levels when the reproductive activity was significantly reduced, suggesting that body energy reserves could also influence the duration of the fish reproductive period, and in particular that the end of the main reproductive activity in March could be caused by the synergetic effect of physical condition and depletion of energy reserves. The role that energy supplied directly from food could have in the reproductive output (not considered in this preliminary study) should be evaluated, especially at the end of the reproductive season when most energy reserves are exhausted. For example, could direct input from feeding partly explain the small “pulse” of reproductive activity observed in May? The results also showed that all condition indices were significantly related to each other, and in particular they suggested that fat/energy storage around the gut (mesenteral fat) and in the muscle (somatic condition) seem to take place in parallel. Furthermore, if it is recognized that liver in fish has a major function in nutrient metabolism and storage, the significantly different patterns observed for HSI between males and females during the reproductively active season likely illustrate the dual function of the liver in females during that period, i.e. the synthesis of the yolk precursors. Our preliminary examination of female liver histological slides showed different histomorphological features in the spawning and the resting periods.

A manuscript based on this presentation has been submitted to the journal Marine and Coastal Fisheries as part of the special module "Emerging Issues and Methodological Advances in Fisheries Reproductive Biology". Publication is anticipated in fall 2010. Web page: www.fisheries.org/mcf.

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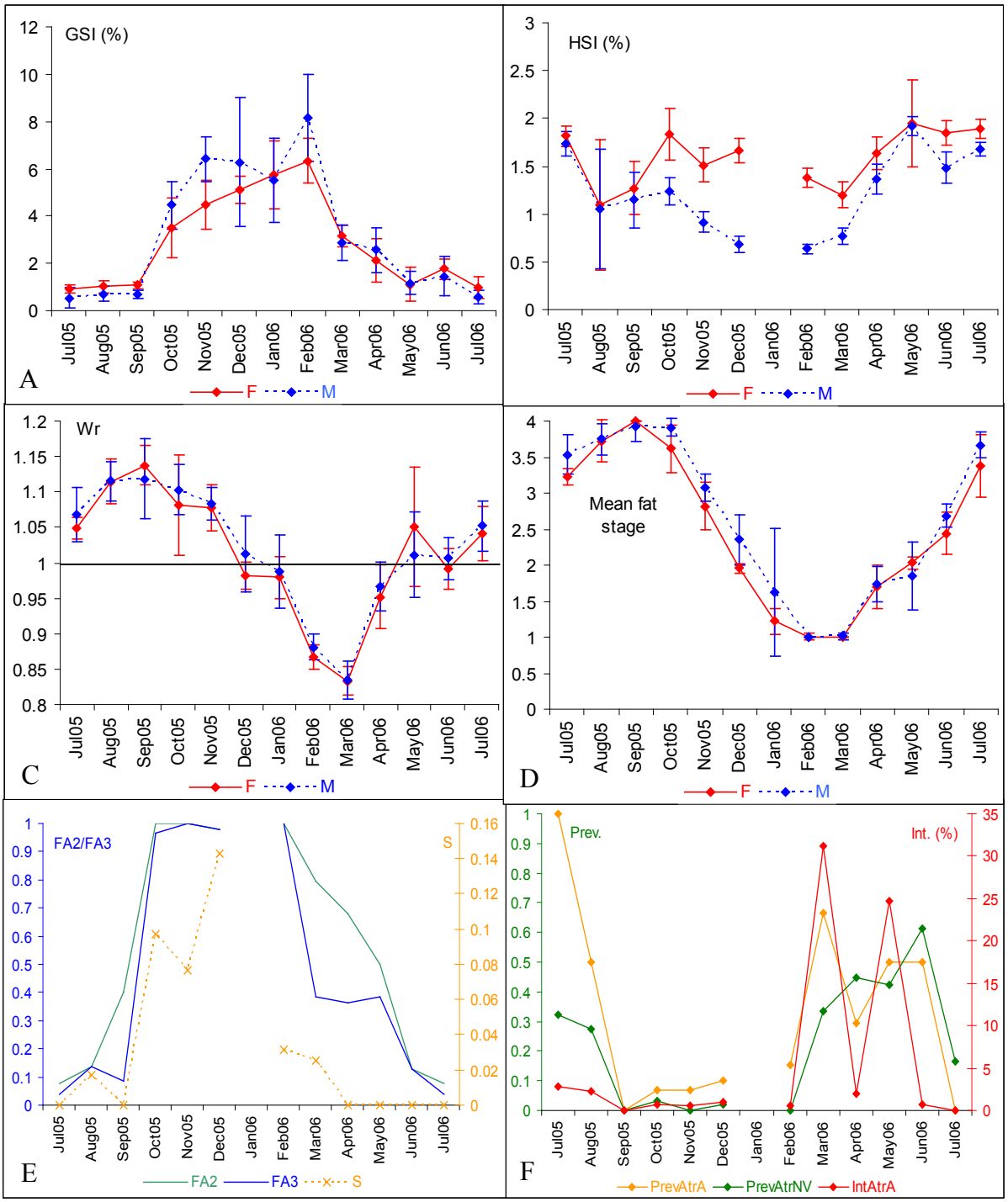

Figure 1. *Sardina pilchardus*. Monthly evolution of A) the gonado-somatic index (GSI), B) the hepato-somatic index (HSI), C) the relative weight factor (Wr), D) the mean fat stage, E) the fractions of females having oocytes with cortical alveoli (FA2) and vitellogenic oocytes (FA3), and the spawning fraction (S), and of F) the prevalence of atresia in vitellogenic (alpha-atresia: PrevAtrA) and in pre-vitellogenic oocytes (all stages of atresia: PrevAtrNV) and the intensity of alpha-atresia in vitellogenic oocytes (IntAtrA). For the biological parameters in A) to D), mean ± standard deviation is represented (F: female, M: male).

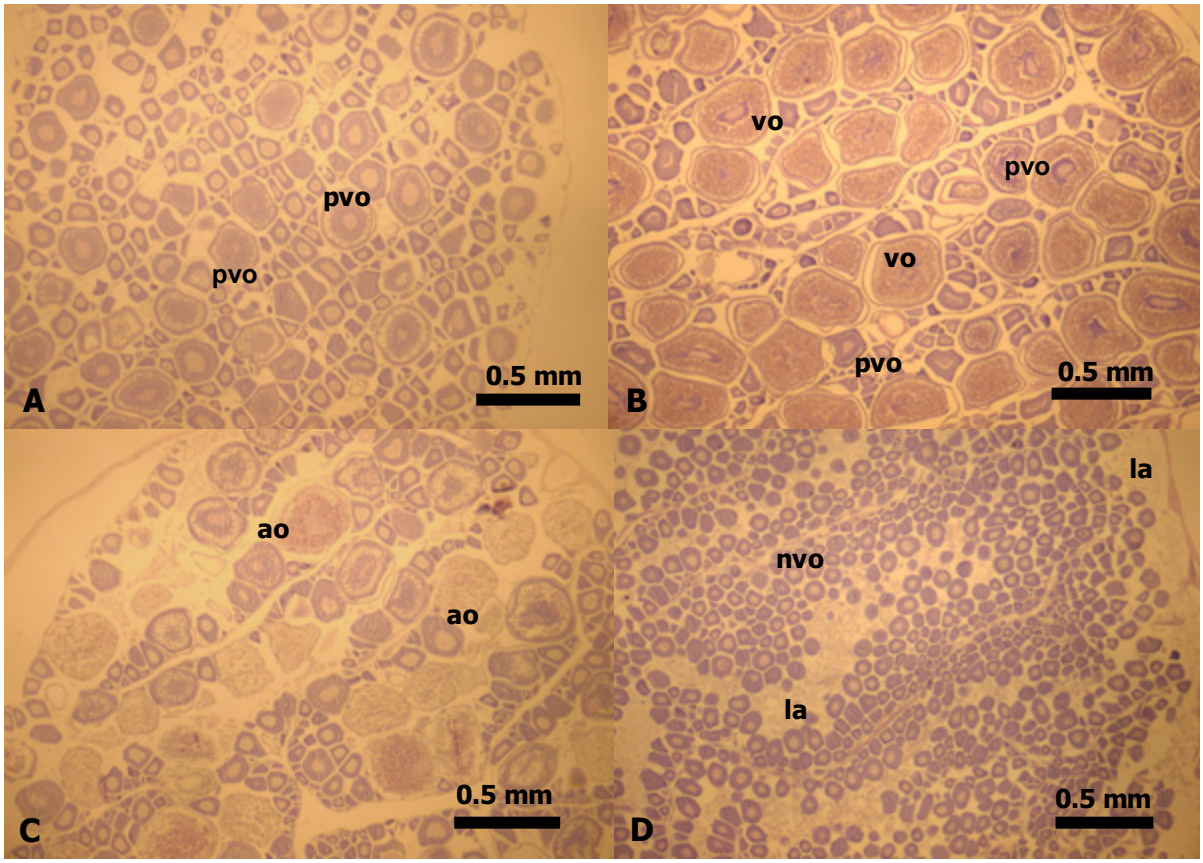

Figure 2. *Sardina pilchardus*. Histological sections from different periods of the reproductive cycle of sardine (paraffin embedding, 5 µm sections, hematoxilin-eosin staining). A: ovary from September 2005, whose most advanced batch of oocytes have cortical alveoli (pre-vitellogenic oocytes: pvo); B: ovary from December 2005, with pvo and vitellogenic oocytes (vo); C: ovary from March 2006 with most of the oocytes atretic (ao) at different stages of alpha-atresia; D: ovary from August 2005, with primary (non-vitellogenic) oocytes (nvo) and late (delta) atresia (la).

Contribution to the Reproductive Biology of Atlantic Albacore (*Thunnus alalunga*, Bonaterre 1788)

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A research cruise by the Japanese research vessel "Shoyo-Maru" took place from June to October 2002 in the Central North Atlantic and in the tropical North and South Atlantic. Sampling of albacore was organized to provide better knowledge of its reproductive biology. A total of 39 adult albacore (*Thunnus alalunga*, Bonnaterre 1788) with a fork length ranging from 91 cm to 119 cm were caught. From the histological analysis of gonads, information on spawning pattern based on development stages of oocytes, estimates of ovary development and fecundity were obtained by areas and time. All albacore studied were sexually mature. Male albacore caught in the North Central Atlantic areas were in an active pre-spawning stage and the specimens from the tropical Atlantic were spawning. A single female caught in the Central North Atlantic was in an active pre-spawning ripening stage, while the four females caught in the south-western tropical Atlantic were fully ripe or spawning. The estimated batch fecundity (1.16 million oocytes) and relative fecundity (47 oocytes per gram of body mass) were calculated for the single fully mature prespawning female albacore caught in September in the South western tropical Atlantic ocean. These results constitute the first approach to estimate the albacore fecundity in the Atlantic Ocean.

Histological Examination of Ovarian Development of Anchovy (*Engraulis encrasicolus)* from the Agadir Bay (Morocco Atlantic Coast)

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Introduction

The anchovy *Engraulis encrasicolus* is a pelagic fish species and is the only species of the Engraulidae family inhabiting the margin of the Moroccan Atlantic coast. The anchovy stock supports large commercial fisheries in Morocco and is exploited intensively and specially by the Moroccan purse-seine fleets. Spatial and temporal patterns of anchovy eggs and larvae along the Atlantic coast of Morocco demonstrated that sheltered zones and bays, like the Agadir bay, are preferential spawning grounds of this species (Ettahiri 1996).

There have been a number of investigations to determine some systematic and biological characteristics of anchovy in the vicinity of Moroccan waters (Garcia and Palomera 1996).
We aim to describe the oogenesis process of anchovy on the basis of histological observations. Different stages of oocyte development are identified and measured. In parallel, other key reproductive parameters are calculated, such as Gonadosomatic Index and sex ratio.

Material and Methods

The Agadir bay extends from Cape Ghir (30°37'N, 9°54'W) to Cape Aglou (29°40'N; 10°W). Samples of *Engraulis encrasicolus* were collected between June 2004 and July 2005 from commercial catches throughout the study area.

The gonads of 92 adult female specimens collected monthly were used for the description of oocyte stages. Gonads were weighed and fixed in 4% neural-buffered formalin. Samples were dehydrated, and submitted to classical histological processing using different staining methods, trichrome, hematoxylin-eosin and Periodic acid- shiff (PAS) solution.

Nuclear evolution and cytoplasm modifications were used to classify stages of oocyte development. The classification used based on our observations divide this development into five stages: previtellogenic, cytoplasmic growth, vitellogenic growth, yolk oocyte and mature oocyte. The incidence of oocyte atresia in anchovy ovaries was checked. Morphometric and statistic treatments were done using the image processing software ImageJ.

Results

The ovaries of Anchovy are paired, and lamellar, and are spread along the length of the body cavity.

Previtellogenic oocyte stage (PV)
Oogonia have an irregular shape and can be recognised by the large, lightly stained nucleus and the deeply stained cytoplasm (Figure 1a), and have a mean diameter of 0.066 ± 09.003 µm. Nucleoli are situated under the membrane all around the periphery of the nucleus. A maximum of 16 nucleoli are observed in this phase. They were observed, at high frequency, in all gonads and at all stages of the sexual cycle. Previtellogenic oocytes were seen during the whole year with the maximum frequency in December and January (Figure 2).

Cytoplasmic growth stage (CG)
Oocytes are characterised by the appearance of fat vacuoles in the cytoplasm and mean oocyte diameter is 0.192 ± 0.006 µm. The first small vacuoles appear in oocytes with a diameter as small as 90 µm. The nucleoli are arranged in the periphery of the nucleus (Figure1b). The vitelline membrane begins to develop. At the end of this stage, following the appearance of large vacuoles, yolk accumulation begins. A maximum of 26 nucleoli were counted. The CG stage was most predominant from February to June (Figure 2).

Vitellogenic growth stage (VG)
The cytoplasm loses affinity for haematoxylin (Fig. 1c). In this stage, the formation of the yolk vesicles and yolk globules are evident. A great volumetric increase of the oocyte is observed, with a mean ooocyte diameter of 0.36 ± 0.013 µm. The nucleoli number at the periphery of nucleus stabilizes between 12 and 26. Oocytes in the VG stage were found most commonly from June through November (Figure 2).

Yolk oocyte stage (YO)
The intensive and complete accumulation of yolk was observed in this stage with oocyte diameters ranging from 330 - 630 µm (mean 0.52 ± 0.016µm; Figure1d). The thickness of the vitelline membrane is 4-5 µm. The YO stage was found year-round with the exception of September, December and January (Figure 2).

Maturation oocyte stage (MO)
The oocyte size and morphology characteristics differ slightly from those at the final phase of yolk deposition. The wavy characters of the nuclear membranes become smooth in some sections. At the beginning of this stage, lipid droplets combine in the near nuclear zone forming 1 to 4 large lipid drops (Figure1e). Thee nucleus migrates toward the animal pole where the nuclear membrane dissolves. The MO stage was seen in April, June and July (Figure 2) and corresponds to actively spawning females.

After ovulation, the spent ovaries were composed of atretic follicles, immature oocytes and postovulatory follicles (Figure 1f). Atretic oocytes were most commonly found during the main reproductive season from April through July (Figure 2).

The GonadoSomatic Index (GSI) shows a gentle and progressive increase from December to April, remains at a high level until September (4.65 – 5.8), and reaches its minimum at the end of the year (1.1). The overall proportion of females is higher than males; the sex ratio fluctuated from 0.7 to 2.5, with the ratio most skewed in July.

Discussion

According to the classification of Wallace and Selman (1981), the Moroccan anchovy has a 'group asynchronous ovary' because all stages of oocyte development were observed year-round, except in December and January. In almost all studies, hematoxylin-eosin is used as the standard stain for clupeid species. However, in our study shifts between early stages seem to be more highlighted by the PAS stain.

The anchovy maintains an inactive oocyte stock from September to January, and starts a new maturation process from February onwards. In spite of the high GSI in September, the histological results confirm the inactive status of the ovary with a high percentage of immature oocytes during that month.

The final maturation appears to start in late March and ovulation occurred mainly between April and August. This clearly places spawning mainly between April and August. However, no hydrated oocytes were found in our study. The time of spawning of the anchovy seems to occur many hours prior to capture. This observation is in agreement with other anchovy populations who spawn at dusk and during the night (Blaxter and Hunter 1982). The catches in our study area are mainly concentrated at dawn between 06:00 and 09:00.

The maximum frequency of atretic oocytes was observed at the same time as the maturation period. According to Hunter et Macewicz (1985), atresia is a key histological marker for the cessation of spawning. Unusual environmental stress may have been the cause of the increased atresia during the spawning season (West 1990). This case may suggest a special strategy of the females which sacrifice some oocytes to recover enough energy to ensure the maturation of the rest.

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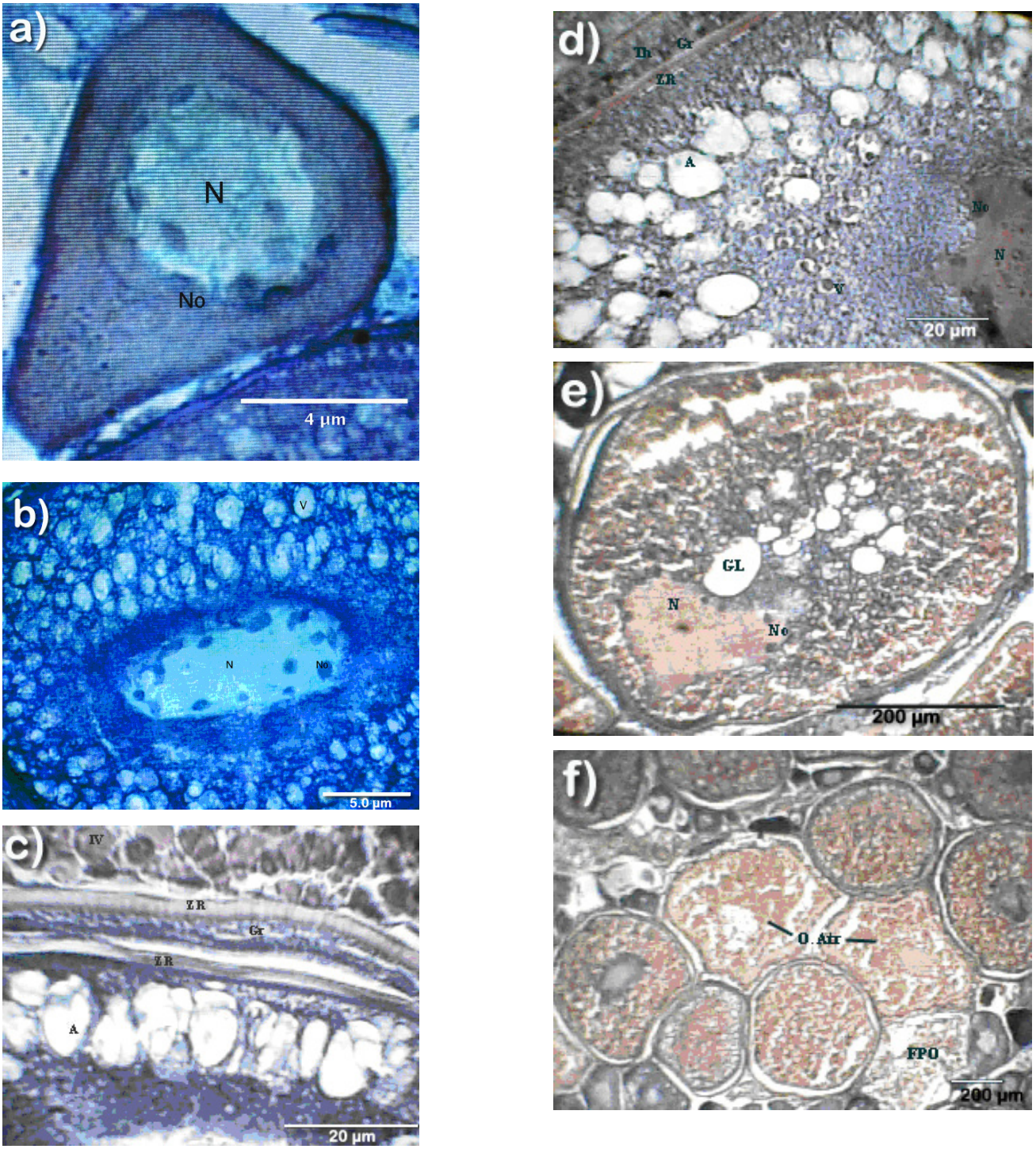

Figure 1 : Oogenesis of *E. encrasicolus* **a)** Previtellogenic stage; (Trichrom x 20), **b)** Cytoplasmic growth stage (Hematoxylin Eosin x 20). **c) V**itellogenic growth stage (Hematoxylin- Eosin x 40), **d)** Yolk oocyte stage (Hematoxylin-Eosin x 40). **e)** Mature oocyte stage (Hematoxylin-Eosin x 20), **f)** Atretic oocytes and postovulatory follicles (FPO) (Hematoxylin Eosin x 20). KEY: A - Alveol; C - cytoplasm; FPO - postovulatory follicle; Gr - Granulosa; N - Nucleus; No - Nucleolus; O.A - Atretic oocyte; Th - Thecal layer; V - vitelline granule; ZR - Zona Radiata; ZR : Zona Radiata .

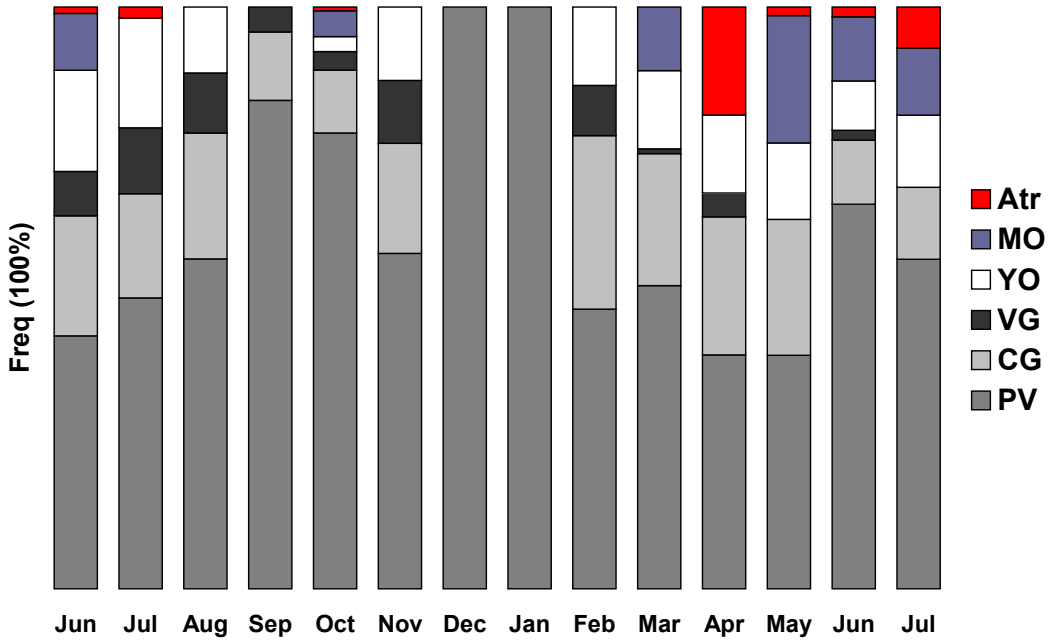

Figure 2. Monthly evolution of oocyte stage frequency (%) (PV, PreVitellogenic; CG, Cytplasmic growth; VG, Vitellogenic Growth; YO, Yolk Oocyte; MO, Mature Oocyte; Atr, Atretic Stage).

Estimation of Spawning Frequency for the Bay of Biscay Anchovy (*Engraulis encrasicolus*, L.)

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In the application of the Daily Egg Production Method to the Bay of Biscay anchovy, spawning fraction (S) was initially derived from the average incidence of Day 1 spawners (although since 1994 Day 2 spawners were also included) (Motos 1996). In order to obtain unbiased estimates of the spawning frequency, the effect of over-sampling of Day 0 spawners was usually corrected by replacing the observed Day 0 females by the expected (not over-sampled) values of spawning females (Picquelle and Stauffer, 1985; Motos 1996).

Recently, a new approach for the independent identification of oocytes and postovulatory follicle -POF- stages in the ovary was developed for the Bay of Biscay anchovy (Alday et al., 2008). A Key of 8 oocytes maturation stages and 7 POF degeneration stages was applied. The novelty of this procedure is that separates the classification of the oocytes and the staging of POFs from their ageing process. The paper corroborated the findings of Motos (1996) about the times of incidence of the phases of nucleus migration and hydration, as well as in placing the spawning peak around midnight. Additionally, the age of the proposed POF stages was validated, suggesting that ageing of POFs younger than 24 hours was more secure than ageing older POFs, and favoured the use of Day 1 for the estimation of S. However, the implications of the proposed analysis criteria in the complete identification of the pre- and post- spawning cohorts and estimation of spawning frequency were not fully worked out by previous authors.

Based on the results of previous studies, we proposed and assessed a matrix system to allocate oocyte and POF stages to daily (pre & post) spawning cohorts in relation to the time of capture of the sample. This system was subsequently applied to assign each individual female to pre and post spawning cohorts. And finally, the incidence of each spawning cohort in the total set of samples collected from 1990 to 2005 was studied, along with the over-sampling effect of the hydrated females, to get the best estimator of spawning frequency.

A total of 11,948 females from 471 samples collected by purse seiners from the commercial fleet and by chartered pelagic trawlers during 1990 to 2005 were examined. Five daily spawning cohorts going from 0600 hours (GMT) to 0600 hours of the next day could be identified: Day 1, Day 2 and Day 3 post-spawning cohorts corresponded to different stages of POFs,. whereas Day -1 and Day 0 pre-spawning cohorts corresponded to advanced stages of oocyte development (from early nuclear migration to hydrated stages). In addition, Day 0 females caught between spawning time (2300 hours) and 0600 hours were identified by the early POF stages (I to IV if occurring at those times). Many of the individuals included in the pre-spawning cohorts according to their most mature oocytes were also independently allocated to Day 1, Day 2 or Day 3 post spawning cohorts on the basis of their POFs. Once

the females were allocated into daily cohorts, the incidence of each cohort was represented throughout five consecutive days.

Figure 1 shows the incidence of spawning cohorts crossed with the occurrence of maturing oocytes. The most remarkable result was the overall coherence of S for the Day 0 and Day 1 cohorts at around 40%. On the other hand, the Day -1 cohort could only be partly identified, as the early nuclear migration (indicative of that cohort) does not start before the 1600 hours, *i.e.,* during the second half of the day. It is also remarkable that most females of Day 2 post-spawning cohort have already advanced oocyte stages (and therefore they will be spawning during that day), thus giving an indication of high spawning fraction. As such, the drop in the incidence of Day 2 cohort after midnight must be due to the recruiting process of that cohort to the new spawning cohort , with the subsequent appearance of young POFs corresponding to the new Day 0 cohort.

The incidence of the cohorts were analysed in order to propose unbiased estimators of the spawning fraction. Four estimators were analysed: The first two were those directly based on the incidence of Day 0 or Day 1 spawning cohorts (without any correction for oversampling of Day 0 cohorts); the third one was the corrected incidence of the Day 1 cohort (Day 1 / (Total mature females – Females allocated to Day 0 + Females allocated to Day 1)); and the fourth one was the joint incidence of the Day 0 +Day 1 divided by 2 (without any correction).

Figure 2 shows the incidence of Day 0 (left) and Day 1 (right) cohorts over 2 days for the whole set of available samples. The average spawning fraction was again around 40%, but some oversampling effect (by about 10%) can be observed on Day 0 around spawning time (2300 hours), with a parallel under-sampling of Day 1. Therefore, estimators of spawning fraction based on Day 0 or Day 1 can only be used outside of those time intervals, otherwise a correction for the sampling bias is required.

The joint incidence of Day 0 and Day 1 females was a more precise estimator of S than the traditional Day 1 estimator corrected for over-sampling. The high spawning frequencies observed in this population result in the over-sampling of hydrated and recent spawned females (Day 0, around spawning time) and the under-sampling of the Day 1 cohort. As a consequence, the joint incidence of Day 0 and Day 1 spawning cohorts could be used as a rough estimator of two times the spawning fraction S, without introducing any other correction.

New estimates of S are around 41% (with a SD of 7.3%) and invariant throughout the study period. These estimates are 64 % higher than the average estimates of S reported in previous studies (Motos 1996, Somarakis et al. 2004).

A manuscript based on this presentation has been submitted to the journal Marine and Coastal Fisheries as part of the special module "Emerging Issues and Methodological Advances in Fisheries Reproductive Biology". Publication is anticipated in fall 2010. Web page: www.fisheries.org/mcf.

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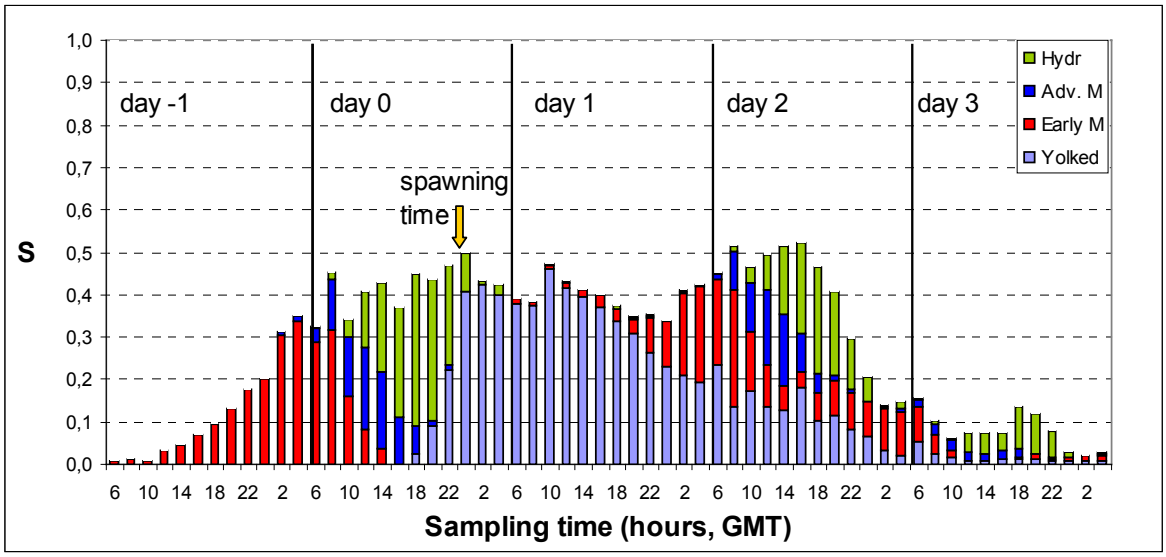

Figure 1. Incidence of oocyte development stages in 2 hour intervals for five spawning cohorts. GMT = Greenwich mean time; S = spawning fraction. Legend: Hydr = hydrated oocytes, Adv. M = advanced nuclear migration, Early M = early nuclear migration, and Yolked = yolked oocytes.

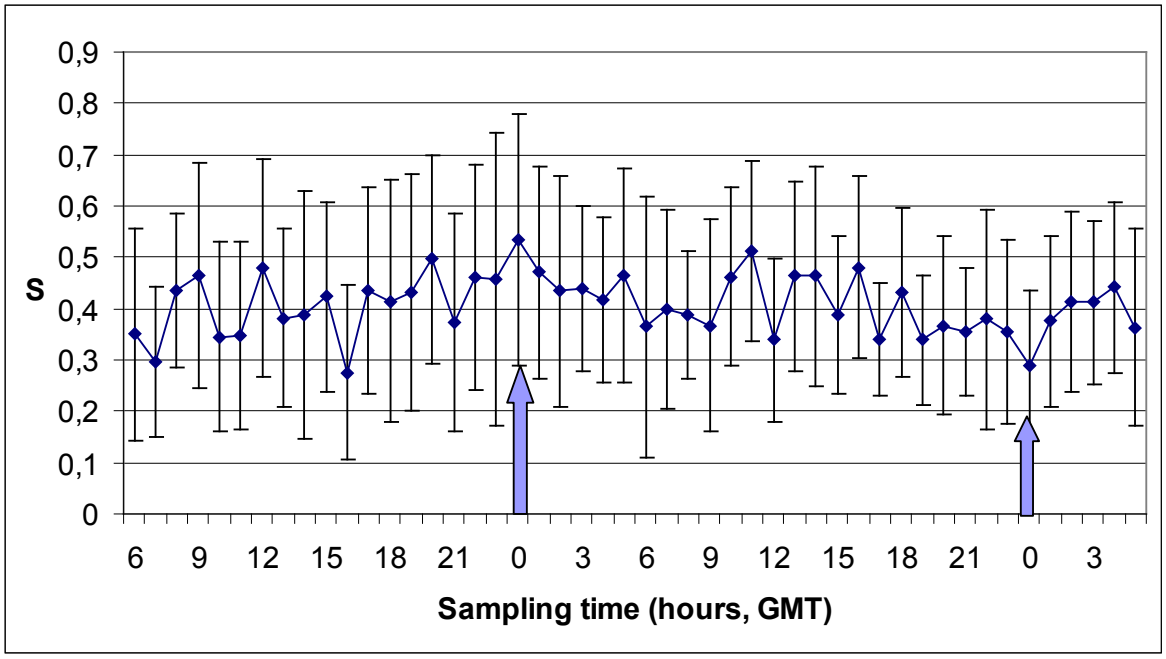

Figure 2. Mean incidence of Day 0 (left) and Day 1 (right) spawning cohorts during a 48 hour sampling period and visualization of over-sampling of active spawning females around midnight and parallel under-sampling of the Day 1 spawning cohort. GMT = Greenwich mean time; S = spawning fraction.

Ovarian Dynamics and Oocyte Growth Rate in the Atlantic Sardine, *Sardina pilchardus*.

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Introduction

Spawning frequency, *S*, in iteroparous fishes expresses the number of spawning events per unit time. Its equivalent at the population level is spawning fraction, i.e. the proportion of females spawning per day, whilst at the individual level *S* is expressed through the inverse of the interspawning interval, i.e. the time lag between two subsequent spawning events. Estimates of *S* are of primary importance in exploring temporal patterns in fish reproductive dynamics, they are used in estimating spawning biomass through the daily egg production method (DEPM) and in calculating annual egg production in indeterminate spawners. To date, most estimations of *S* in the wild are performed at the population level except from species-specific visual census or telemetry applications or in cases of fish where different spawning stages co-occur, e.g. co-occurrence of postovulatory follicles, POFs, of known age with migratory nucleus oocytes. The most popular method for estimating population *S* is the POF method first introduced for northern anchovy, *Engraulis mordax*, by Hunter and Goldberg (1980). Despite its popularity, the method can be quite inaccurate when its criteria are applied to other species and populations without prior validation (Stratoudakis et al. 2006). Furthermore, the method is quite costly and labor intensive, because it needs large number of adult samples, much histology and many work-hours from experienced personnel. In that respect, the development of alternative methodologies which could provide *S* estimates based on interspawning interval seems worthwhile.

Methods

Figure 1A presents a conceptual model for estimating *S* at the level of the individual. Specifically, knowing oocyte size at the beginning and the end of the spawning cycle, O_b and O_e respectively, and the rate of oocyte growth, *G*, *S* could be estimated using the formula:

$$\frac{1}{S} = \frac{O_e - O_b}{G} \quad (1)$$

In order to be valid, the model should satisfy the following prerequisites: i) female spawners at the beginning of their spawning cycle should be easily and accurately identified in order to measure O_b, ii) *G* should either be constant in the population or be a simple function of easily measured demographic or environmental parameters like body-size and temperature, iii) similarly, O_e should either be constant or predicted by easily measured life-history or physical parameters, and iv) there should be no unpredictable pauses during the spawning cycle, e.g. time lags between true vitellogenesis and hydration or 'ripe holding periods'.

Results and Discussion

We tried to apply the aforementioned method and test each of the four prerequisites in the Atlantic sardine, *Sardina pilchardus*, using histological and ovarian whole mounts data from two DEPM surveys held in 2005 and 2008. *G* was estimated by regressing the average size of advanced oocytes on the time elapsed from spawning in recent spawners with aged POFs. Oocyte growth rate did not differ significantly between the two years and equalled to 0.019 mm^2/day (Figure 1B). However, after measuring the average size of the spawning batch in hydrated females and regressing it on sampling time (Figure 1C), oocyte growth was shown to increase sharply at hydration (0.038 mm^2/h; Figure 1D). Because of this change in oocyte growth rate equation (1) was transformed to:

$$\frac{1}{S} = \frac{O_v - O_b}{G} + t_H \quad (2)$$

where O_v is oocyte size at the end of true vitellogenesis, just prior to the beginning of hydration and t_H is the time lag of oocyte hydration. As shown in Figure 1E the latter period was estimated to be less than one day and equalled 17 h or 0.7 d. Concerning O_v this was assessed by means of oocyte size frequency distributions. At the secondary stage of vitellogenesis a clear size gap developed between the spawning batch and the subsequent batches of smaller oocytes (Figure 2A). Oocyte growth seemed to be a continuous process throughout true vitellogenesis and oocyte size just prior to the onset of hydration was estimated to be 0.33 mm^2. This value was very close to that estimated not only for the Mediterranean population of *S. pilchardus* but also for the Chilean sardine (Claramunt and Herrera 1994) and the sardine of the ecosystem of Benguela (Le Clus 1979). These findings suggest that oocyte size at the end of vitellogenesis is fairly constant among sardine populations satisfying the third prerequisite of the conceptual model.

Hypothesizing that t_H is constant among individuals and since both *G* and O_v were shown to exhibit fairly constant values, individual *S* in the Atlantic sardine might simply be calculated by measuring O_b, i.e. the average size of oocytes of the spawning batch at the beginning of the spawning cycle. Based on previous findings for Japanese sardine, *Sardinops melanostictus*, the process of vitellogenesis is ceased during hydration (Matsuyama et al. 1994), implying that oocyte growth in the subsequent batch is also ceased during hydration. In that respect, O_b could be estimated by measuring the size of the oocytes of the subsequent batch in hydrated females. For the present work O_b was measured only in females from the 2008 DEPM survey; as shown in Figure 2B O_b values exhibited normal distribution and their mean was 0.16 mm^2. Given also that at least for sardine there is no pause between true vitellogenesis and hydration (Matsuyama et al. 1994) and no 'ripe holding period', the average individual 1/*S* using Eq. 2 was estimated to be 10 days. This value gives a spawning frequency of 0.01 which is very close to 0.09 which is population *S* estimated for 2008 using the POF method (considering only active spawners in the denominator). This match between the two estimates is quite encouraging for the validity of the method, suggesting that if each of the model variables are carefully explored (e.g. also measure *G* in other years and other populations of sardine to examine possible factors that affect it either physical or biological) then it could be applied for estimating *S* both in sardine and possibly other species with indeterminate fecundity. In

that case individual *S* could be easily estimated by capturing and instantly processing microphotographs of ovarian whole mounts from hydrated females which could also be used for batch fecundity measurements (e.g. Ganias et al. 2008). This combined estimation would much decrease both cost and labor in DEPM surveys.

A manuscript based on this presentation has been submitted to the journal Marine and Coastal Fisheries as part of the special module "Emerging Issues and Methodological Advances in Fisheries Reproductive Biology". Publication is anticipated in fall 2010. Web page: www.fisheries.org/mcf.

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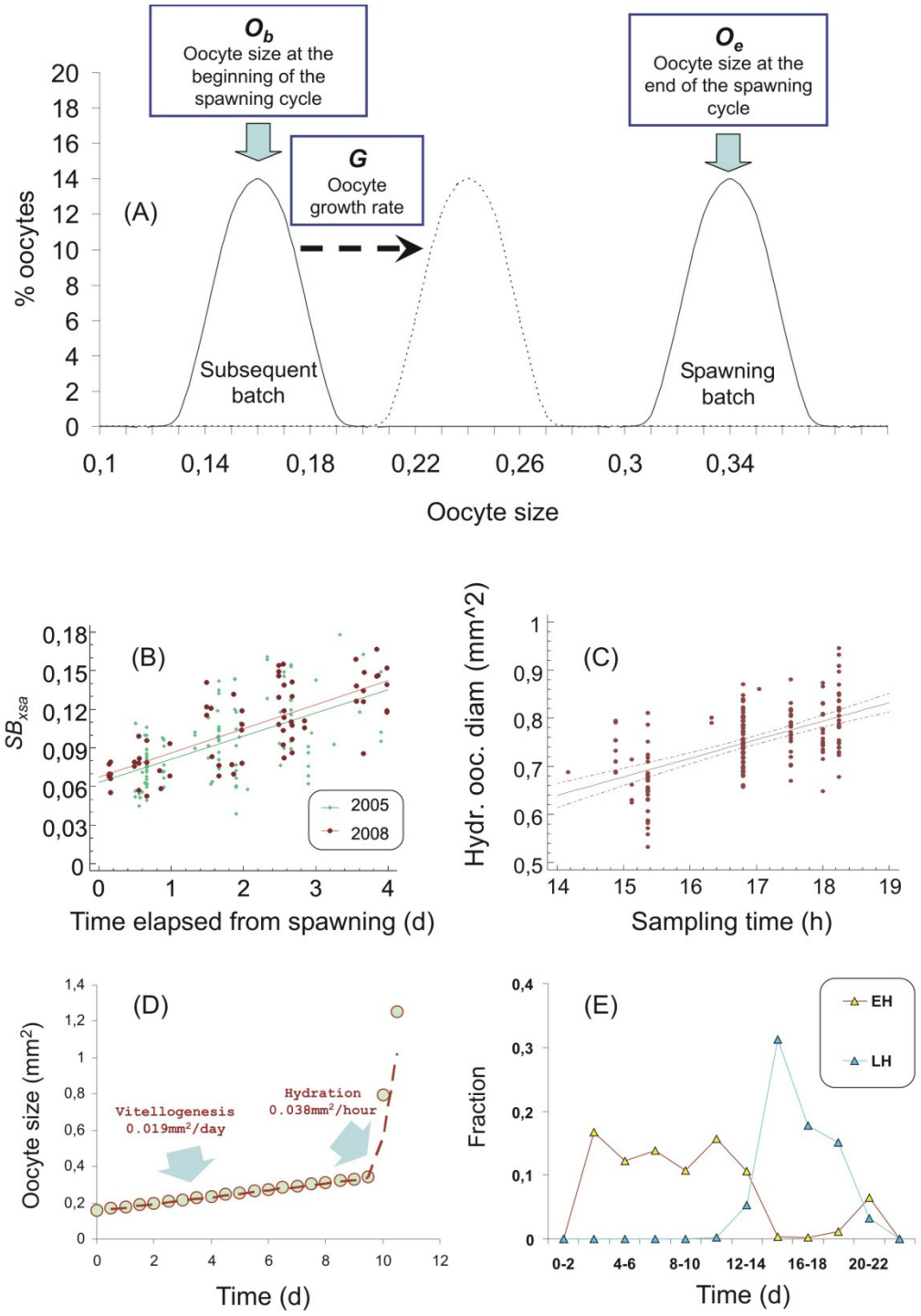

Figure 1. (A) Conceptual model for the estimation of individual spawning frequency illustrating parameters used in Eq.1; (B) Relationship between the cross-sectional area of the spawning batch (SBxsa, in mm2) and the time elapsed from spawning; (C) relationship between the size of hydrated oocytes and sampling time; (D) change in the rate of oocyte growth between true vitellogenesis and hydration; (E) hourly evolution of the fraction of early (EH) and late (LH) hydrated sardines.

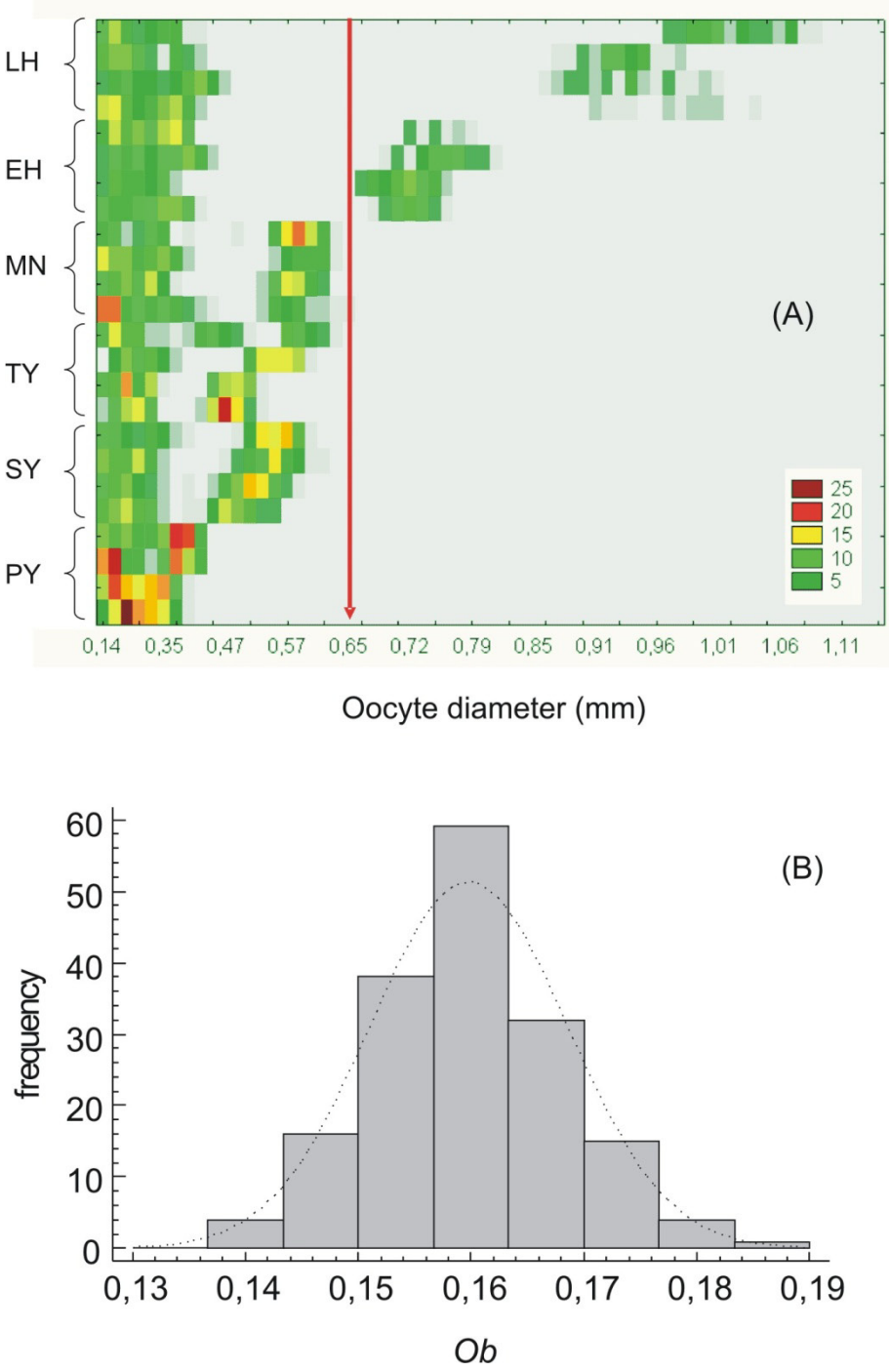

Figure 2. (A) Oocyte diameter frequency distributions in sardine ovaries of sequential developmental stages. PY: primary yolk stage; SY: secondary yolk stage; TY: tertiary stage; MN: migratory nucleus stages; EH: early hydration; LH: late hydration. (B) Frequency distribution of the averages of oocytes diameters of the subsequent batch (Ob, in mm2) in hydrated sardines from the 2008 DEPM survey.

Gametogenic Cycle and Size of First Sexual Maturity of Meagre, *Argyrosomus regius*, in the Gulf of Cadiz (SW Spain)

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Introduction

The meagre, *Argyrosomus regius* (Asso, 1801), is a member of the family Sciaenidae. *Argyrosomus regius* is a common species along the coast, although it is much more common in the western Atlantic and is scarce in the Mediterranean (Consejería Agricultura y Pesca 2001). Meagre is a nectobentonic species. It is characteristically found on shallow sandy bottoms, , but can reach depths of 200 m. As a euryhaline species, meagre can occur in the mouths of rivers and estuarine lakes. It is a gregarious species, moving in small groups.
The main objective of this paper is to study the gametogenic cycle and size at first sexual maturity of meagre in the Gulf of Cadiz using histological techniques and image analysis.

Methods

The specimens (n = 364) were obtained from the ports of Chipiona and Conil (province of Cadiz, Spain) between March 2006 and April 2008. Sections of gonads were fixed in phosphate-buffered 4% formaldehyde. Samples were dehydrated and embedded in paraffin in a vacuum and pressure tissue processor. Sections of 3 µm were obtained with a rotary microtome and stained with Hematoxilin-Eosin and Hematoxilin-V.O.F. (Gutiérrez 1967). Finally, they were observed using image analysis. The classification of microscopic states of maturity followed Wallace and Selman (1981) and Sarasquete et al. (2002) for females and Micale et al. (2002) for males. The size at first sexual maturity was determined using the software Origin 7.0: $Y = 1/(1+\exp(-k(x-L_{50}))$, where Y = percentage of mature individuals; x = total length; k = parameter and L_{50} = length at which 50% of individuals are mature.

Results

Argyrosomus regius is a species exhibiting the asynchronous type of oocyte development (Figure 1A). Its reproductive season in the Gulf of Cadiz occurs during spring and summer, and males have spermatozoa in the testis throughout the spawning season (Figure 1C)
The female gametogenic cycle began in the months of April and May. In the months April to July 2006 and April to August 2007, there was an increase in females in the spawning phase, with a maximum in May 2006 and April 2007. In 2006, the females were found in the post-spawning state from June through August (Figure 1B).

In males, the gametogenic cycle began between March and May, where there were individuals in the immature, development and maturation states. The spawning season is quite long, extending from March 2006 until July 2006 and from April 2007 until August 2007. In October 2007, males began to appear in the post-spawning state, and some post-spawning males were observed until March 2008 (Figure 1D).

The size at first sexual maturity was analyzed in both sexes separately. The estimated size at sexual maturity was 86.25 cm total length (TL) for females and 64.41 cm TL for males. Most female values were found at the bottom or top of the curve, because in this study we have analyzed the data in moths where the specimens were in reproductive condition (Figure 2A). Therefore, in these ranges, we found some small individuals in immature state and large individuals in spawning state. We saw a similar occurrence with males (Figure 2B), with a single point in the intermediate range belonging to an immature individual. Most of the specimens analyzed in this study were in a regressed state or in the spawning state.

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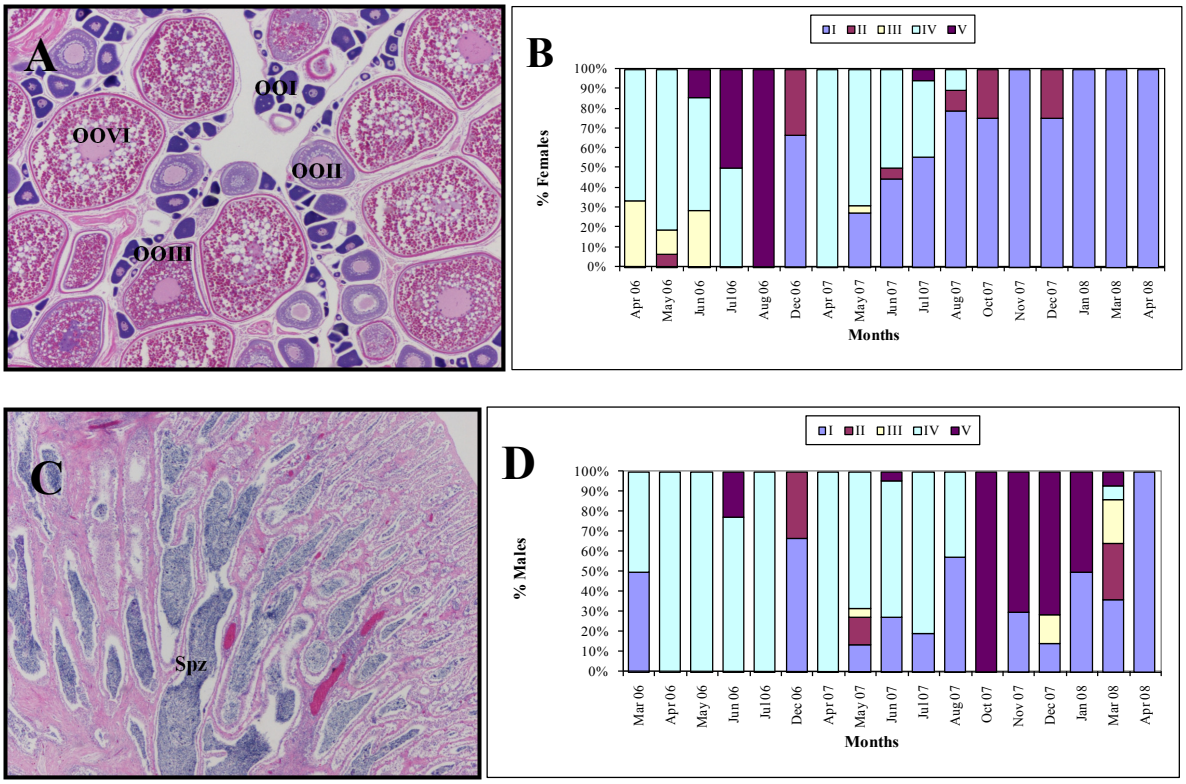

Figure 1: The reproductive seasonality of meagre. A. Ovarion tissue during maturation phase, showing asynchronous oocyte development. (OOI—primary growth oocyte; OOII—previtellogenic (cortical alveolar) oocyte; OOIII—vitellogenic oocyte; OOIV—mature oocyte). Stained H/E. 4x magnification. B. Proportion of the gonadal state of females during the months of study (I: Immature; II: Previtellogenic; III:Vitellogenic; IV: Maturation; V: Post-Spawning). C. Maturing male. (Spz: spermatozoa). Stained H/E. 4x magnification. D. Proportion of the gonadal state of males during the months of study (I: Immature; II: Development;III: Maturation; IV: Spawning; V: Post-Spawning).

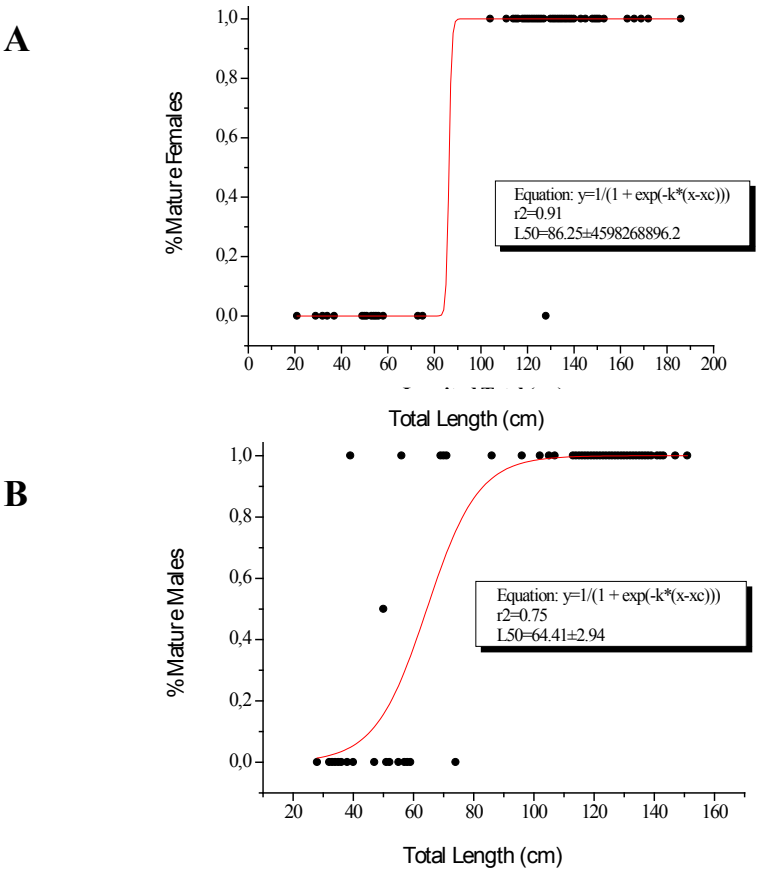

Figure 2. Estimation of the mean size of first sexual maturity: A. Females. B. Males.

Re-evaluating Size and Age at Maturity of Southern Flounder (*Paralichthys lethostigma*) using Reproductive Histology and Otolith Microchemistry

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Introduction

The southern flounder (*Paralichthys lethostigma*) is a large flatfish species that is distributed widely along the southeast US coast and throughout the Gulf of Mexico. The species is harvested both commercially and recreationally and has been the most valuable inshore finfish resource in the state of North Carolina for the past decade (NC Division of Marine Fisheries commercial harvest statistics 1998-2007). Currently, the NC stock is listed as 'depleted' due to a long period of elevated harvest rates beginning in the early 1990's. The NC fishery management plan of 2005 established several new harvest restrictions in an attempt to lessen the impact on the stock by reducing fishing mortality rates. While it is still relatively early to evaluate the harvest restrictions initiated in 2005, the results of a recent study suggest that fishing mortality rates may still be higher than management targets, at least in the river-based gill net segment of the fishery (Smith et al. 2009). An examination of the demographic traits of harvested flounder by Smith and Scharf (in review) also confirmed that the commercial catch is composed of young (primarily age-1) fish, many of which may be immature.

Results and Discussion

A size- and age-based maturity schedule for NC southern flounder was estimated for fish captured in the mid-1990's by Monaghan and Armstrong (2000). They estimated an L_{50} (length at which 50% of females are mature) of 345 mm (13.6 inches) and that approximately 73.5% of age-1 fish were mature. However, all but two of 19 running ripe females collected in ocean waters were greater than 414 mm (16.3 inches), and age-1 females failed to demonstrate a well defined peak in their gonadosomatic index (GSI) during late fall, as was observed for age-2 and older fish. These observations imply that the size- and age-at-maturity estimates of Monaghan and Armstrong (2000) may be low. More recent studies have provided new evidence that suggests maturation of southern flounder may occur at larger sizes and older ages (Figure 1). Smith and Scharf (in review) used macroscopic staging of the gonads of 185 southern flounder captured in the New River during Oct and Nov 2005-06 to estimate that only 30.3% of age-1 females were mature. Histological examination of a subset (n = 31) of the gonads validated the maturity classifications made based on macroscopic staging criteria. In a separate study, analysis of otolith microchemistry revealed that many southern flounder demonstrated chemical signatures in their otoliths consistent with estuarine residency until the end (fall) of their third year (i.e., age-2 fish about to turn 3), suggesting delayed participation in offshore spawning (Figure 2; Taylor et al. 2008).

We have just initiated a study to generate a comprehensive update of the size- and age-at-maturity schedules for southern flounder in North Carolina through an examination of the gonadal tissues of fishes captured from several locations throughout the state during two consecutive spawning seasons. We plan to prepare and examine histological samples for a considerable number of fish in order to validate macroscopic staging criteria. By partnering with local commercial fishers, fish dealers, and recreational spear fishers, we will obtain flounder from both estuarine and nearshore ocean habitats to more fully assess the reproductive state of young females as they transition to maturity. We also plan to analyze the chemical signatures of otoliths from a subset of collected fish to define the spatial resolution with which habitat designations (ocean vs. estuary) can be made, and determine whether chemical signature patterns can help to indicate the timing (age) of spawning related emigrations. Here, we present the cumulative evidence from several older and more recent studies which collectively indicate that southern flounder maturity may occur at older ages and larger sizes than previously assumed.

Conclusions and future directions

The monthly GSI data presented by Monaghan and Armstrong (2000) suggests a low percentage of mature fish at age-1, relative to older ages. Seasonal GSI data by age and size will be examined carefully in the current study to determine if GSI can be a useful predictor of maturity for southern flounder.

The most recent estimate of L_{50} predicts that most southern flounder less than 15 inches (381 mm) TL will not have reached maturity. The current minimum legal size for harvest in North Carolina is 14 inches.

The sub-sample of fish examined histologically by Smith and Scharf (in review) indicated that none of the fish categorized macroscopically as 'Developing' were mature. These fish did not possess vitellogenic oocytes. Whether fish with 'Developing' gonads during fall are capable of completing the maturation process spawning during the subsequent winter needs to be determined.

The otolith microchemical analyses support the notion that most southern flounder do not emigrate to oceanic waters for spawning until they are nearing age-3, which would indicate most fish being larger than 15 inches TL. Continued evaluation of otolith microchemical signals are planned in the present study.

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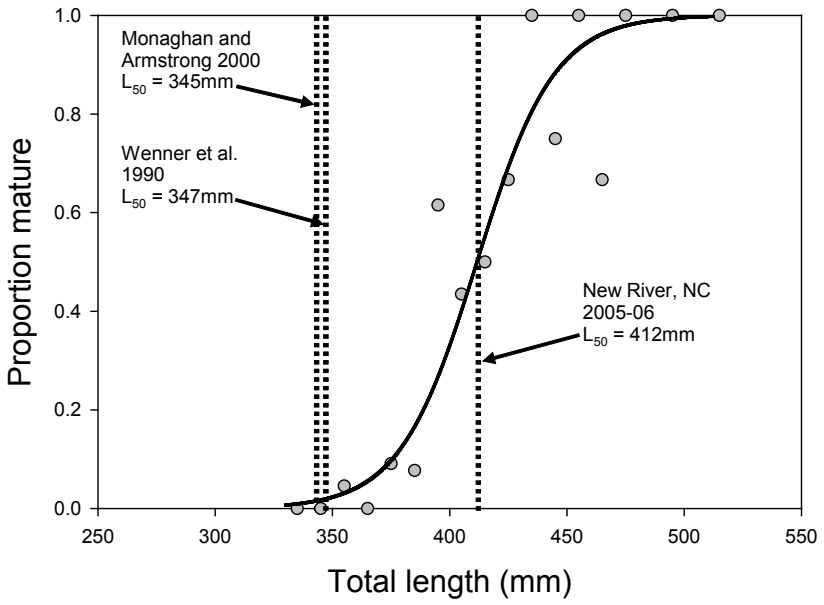

Figure 1. Proportion mature by total length for southern flounder collected during 2005-06 tag-return study (Smith and Scharf in review). Estimated L50 are presented for this data and also for previous studies in North Carolina (Monaghan and Armstrong 2000) and South Carolina (Wenner et al. 1990) waters.

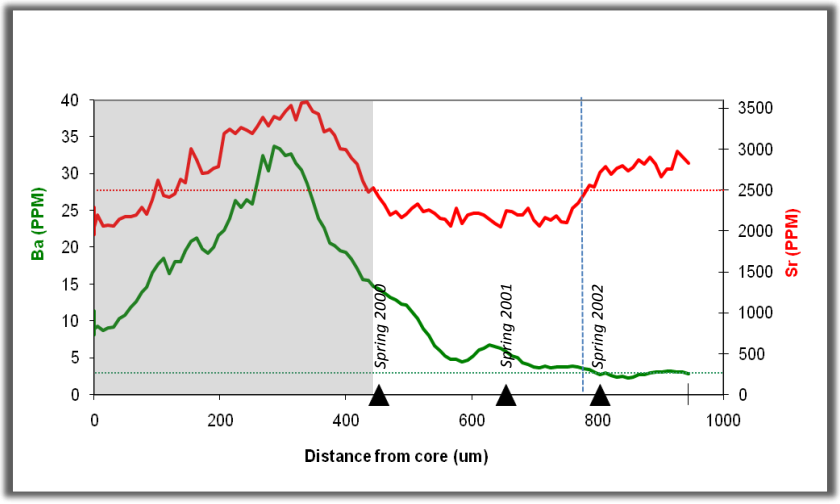

Figure 2. Barium and strontium concentration along laser transect for a male southern flounder captured on 22 May 2003. This fish was 355 mm TL, and nearly 4 years old, as indicated by the black triangles. The grey box represents the juvenile estuarine nursery period. Horizontal dashed lines show inferred boundaries for post-juvenile estuary/ocean residency (4 ppm Ba, 2500 ppm Sr). Vertical dashed line shows estimated time of first emigration from the estuary, which occurred near the end of the 3rd year.

Seasonal Reproductive Patterns and Recommended Sampling Times for Sentinel Fish Species Used in Environmental Effects Monitoring Programs in Canada

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Canada's environmental effects monitoring (EEM) program is currently in its fifth cycle of monitoring for the pulp and paper industry and second cycle of monitoring for the metal mining industry. More than sixty different sentinel fish species have been used in the EEM fish population surveys and reproductive impacts have been identified as an issue of concern in the pulp and paper program. A review of the literature was conducted to obtain details of the reproductive biology of each fish species that has been used in EEM studies in Canada. Using available data on seasonal changes in gonadosomatic indices, the seasonal reproductive patterns of Canadian fish species are divided into categories based on the number of spawning events per year and the timing of initiation of gonadal recrudescence. Recommended sampling times are developed for each reproductive pattern based on periods of temporal stability, minimum variability, and maximum value in gonadosomatic indices within a reproductive cycle. The reproductive strategy, spawning time, spawning temperature, and recommended sampling time are provided for the each sentinel fish species as well as life history characteristics including longevity, age and size at maturity, and mobility. Examination of the fish surveys using small-bodied forage species from the EEM pulp and paper program reveals that approximately 72% of these studies were not conducted at the developed recommended sampling times and the magnitude of impacts may be underestimated by failing to sample at the recommended time.

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Preliminary Data on the Ovarian Histological Structures Observed in Black Hakes (*M. polli* and *M. senegalensis*) Off Mauritania

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Introduction

The black hakes, *Merluccius polli* and *M. senegalensis*, are target species of a trawl Spanish fleet in Mauritanian waters. *M. polli* is a deep-water hake while *M. senegalensis* has a shallower distribution. Both species are gutted and mixed in the landings, although the deeper hake constitutes a greater fraction of the catch (85%) due to the fishing strategy of the trawl fleet. Scant information is available on the reproductive aspects of these species. The reproductive period extends from October to March, with a defined peak in December – January (Fernández-Peralta et al. 2007). Females of both species reached size at first maturity at the same length (39,2 cm) in 2003 (Fernández-Peralta et al. 2006). The present study is a first approach to the ovarian histology of *M. polli* and *M. senegalensis*, and is intended to provide basic knowledge for further detailed studies on the reproductive biology of these species, essential for an adequate assessment of the stocks.

Materials and methods

Female gonads were collected during an experimental survey carried out between November and December 2003 in the Mauritanian EEZ. Mature ovaries of 16 and 14 specimens of *M. senegalensis* and *M. polli* were analyzed respectively. The size range and the dates and the places of capture were similar for both species. Ovaries were preserved on board in 10% buffered formalin and a portion of the central part was processed by a standard histological technique. The samples were sectioned at 10 µm with a microtome and stained with Mallory's Trichrome Stain. Histological characteristics of ovarian tissues and oocyte stages were examined by light microscopy and photographed with a Leica photomicroscope. Oocytes of different categories were measured in three specimens of each species by means of an image analysis system (Image-Pro Plus V. 6.0)

Results

The histological examination of ovarian tissue from these species showed the oocyte stages described by Murua (2006) in European hake: perinucleolar, cortical alveoli, early vitellogenic, advanced vitellogenic, early migration (maturation), migration (maturation final) and hydrated (Figure 1).

The maturity stages observed in the ovaries showed different developmental stage frequencies in these hakes. In the case of *M. polli*, 1 was early vitellogenic, 8 advanced vitellogenic, 3 early migration and 2 in the hydrated stage. None of the *M. senegalensis* were staged as early

vitellogenic, but 4 were advanced vitellogenic, 3 early migration and 9 in the migration or hydrated stages.

The observed index of atresia was low in both hakes. Postovulatory Follicles (POFs) were absent in all gonads of *M. polli*, unlike *M. senegalensis* that showed a high presence of POFs in different stages of deterioration.

The oocyte size distribution suggests an indeterminate fecundity in both hakes (Figure 2).

Discussion

In the shallow-water hake, *M. senegalensis*, the abundance of POFs indicates a previous batch spawning episode and the presence of oocytes in the final maturation stage in the majority of their gonads indicates an imminent spawning event at the time of capture. However, the deep-water hake *M. polli*, did not present POFs in any of the analyzed gonads, and oocytes were less developed than in *M. senegalensis*, with the exception of two gonads with hydrated oocytes. This finding could indicate the commencement of the spawning period of the deep-water hake, *M. polli,* at that very moment of sampling.

Although few samples were analyzed, the results of this study indicate that the females of *M. senegalensis* showed an earlier spawning in comparison to *M. polli*. This fact has been described by other authors (Wysonkinski 1986) and is in agreement with the GSI values of both species in these waters 2003-2004 (Fernández-Peralta et al. 2006).

The incidence of atretic stages was low because the sampling period corresponds to the beginning of the spawning season, which occurs between October and March according to several authors (García 1982; Sobrino et al. 1990; Fernández-Peralta et al. 2007).

The delay of the beginning of the spawn period in *M. polli*, as well as differences in the depth of spawning habitat (Fernández-Peralta et al. 2007) could be mechanisms to reduce the potential for hybridization between these two species. An environmental study in their zone of distribution could help to explain the reproductive pattern in these hakes. The species are multiple batch spawners with asynchronous development of oocytes and probably indeterminate fecundity as suggested by their oocyte size frequency distribution. It is still considered necessary to continue with reproductive studies to confirm the reproductive time lag of approximately one month in *M. polli*.

The same size at first maturity in both species and the low abundance of the coastal hake do justify the joint evaluation of both species. Nonetheless, the estimate of batch fecundity for both species, as well as, the progress in the knowledge on their reproductive biology will allow us to consider whether the black hake complex is a single-stock entity for the purposes of assessment and management.

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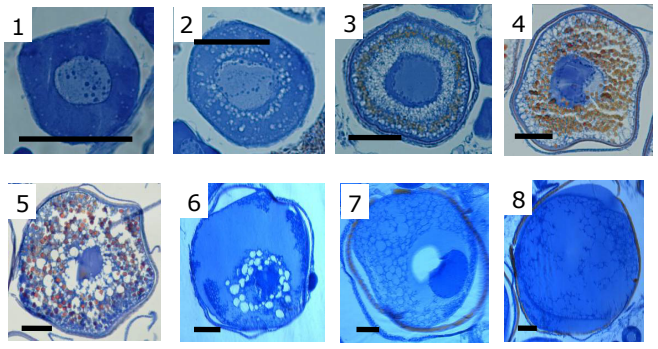


Figure 1. Oocyte development process observed in *M. polli*: (1) perinucleolar, (2) cortical alveoli, (3) early vitellogenic, (4), (5) advanced vitellogenic, (6) early migration (maturation), (7) migration (maturation final) and (8) hydrated stage; bar = 0.1 mm

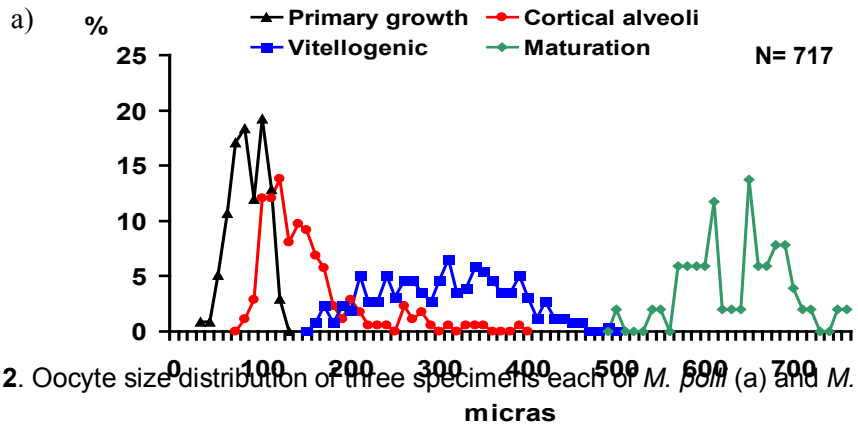
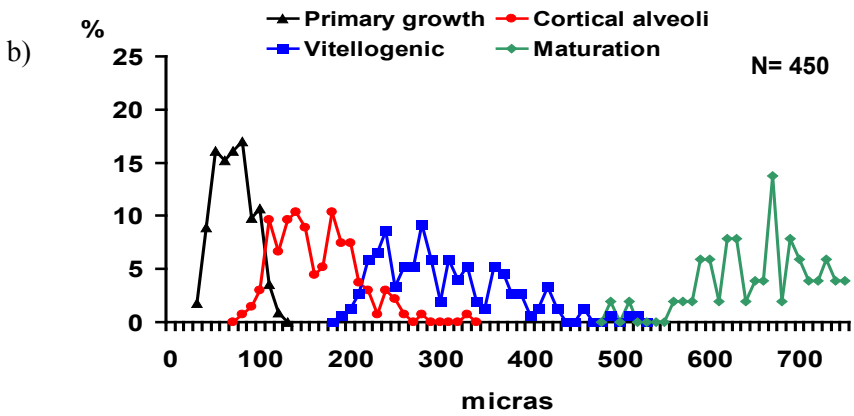


Figure 2. Oocyte size distribution of three specimens each of *M. poli* (a) and *M. senegalensis* (b).



Reproductive Cycle, Fecundity and Maturity Ogive of *Brycon guatemalensis* in Lake Cocibolca

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Introduction

Machaca *(Brycon guatemalensis)* is a freshwater fish inhabiting inland waters from Mexico to Panamá and it is considered a native fish in the Lake of Nicaragua or Cocibolca. This lake is considered the most important fresh water body of the Central America isthmus, and has an area of 8001 km^2 and maximum depth of 40 meter. *Brycon gautemalensis* is the second most abundant species in the lake, and is the target of local communities as a food source and for commercial activities.

There are no specific regulations for *B. guatemalensis* in Cocibolca Lake, as the current regulations have been established for the protection of other, more commercially important species. These regulations have not been based on knowledge of the biology of the species inhabiting the lake but rather on fishing gear selectivity studies which take into account the morphometry and maturity stages of individuals based on macroscopic observation. Other sources of regulation have relied on local knowledge about the abundance and distribution of the species in the lake.

Materials and Methods

Monthly gillnet surveys from February 2004 to January 2005 were conducted in the north east part of Cocibolca Lake. During the study, 1,957 females were sampled and ovaries collected. Maturity was staged macroscopically considering three ovary conditions (immature, mature and spawned), modified from Holden and Raitt (1975). From the ovaries collected, 401 were histologically processed and oocyte development stages were determined histologically. Terminology followed Tyler and Sumpter 1996; Saborido-Rey and Junquera 1998; Murua and Saborido-Rey 2003, and Brown-Peterson et al. 2007.

Results and Discussion

Length–at-maturity

To estimate the length at which 50% of the females were mature, 399 specimens of *B. guatemalensis* collected in June and July were assessed macroscopically to determine a length-at-maturity of 34.9 cm. A more precise estimation was achieved with histological observation of the gonads of 370 specimens, and the length-at-maturity of the species was estimated at 27.0 cm. (Figure 1). The determination of the length-at-maturity is very important to establish the fishing gear mesh size regulations. However, it is known that length-at-maturity based on macroscopic observations of the gonads often produces biased estimations.

Reproductive cycle

The observed type of oocyte development showed that almost all the oocytes advance together from one development stage to the next, i.e. a group-synchronous type (Murua and Saborido-Rey 2003). Thus, it would be expected that prevalence of different oocyte development stages in the gonad would be similar throughout the year. However, in every month females in the Developing phase predominated (mean of 42.6% throughout the year; Figure 2), indicating inadequate sampling of Spawning Capable and Actively Spawning females, probably because fish in these phases are less available to gillnets during the spawning period.

The few individuals caught in the Actively Spawning phase and, to a lesser extent, in the Spawning Capable phase, is likely due to i) changes in swimming fish behavior during spawning since the species spends time excavating in the sand bottom to deposit eggs (Greenfield and Thomerson 1997), making them unavailable to capture by the passive fishing gear used (gillnet); or ii) because spawning grounds or feeding areas are in areas not surveyed, i.e. freshwater marshes that are available for Machaca when the lake water level raises.

Although the Developing phase prevails throughout the entire year, the appearance of fish in the Spawning Capable and Actively Spawning phases is observed after the rainy period has started (Figure 2), which probably corresponds with an increase in the lake water level. The spawning season extends through the first half of the dry period which is also probably related to the lake water level which still remains high months after the precipitation has diminished.

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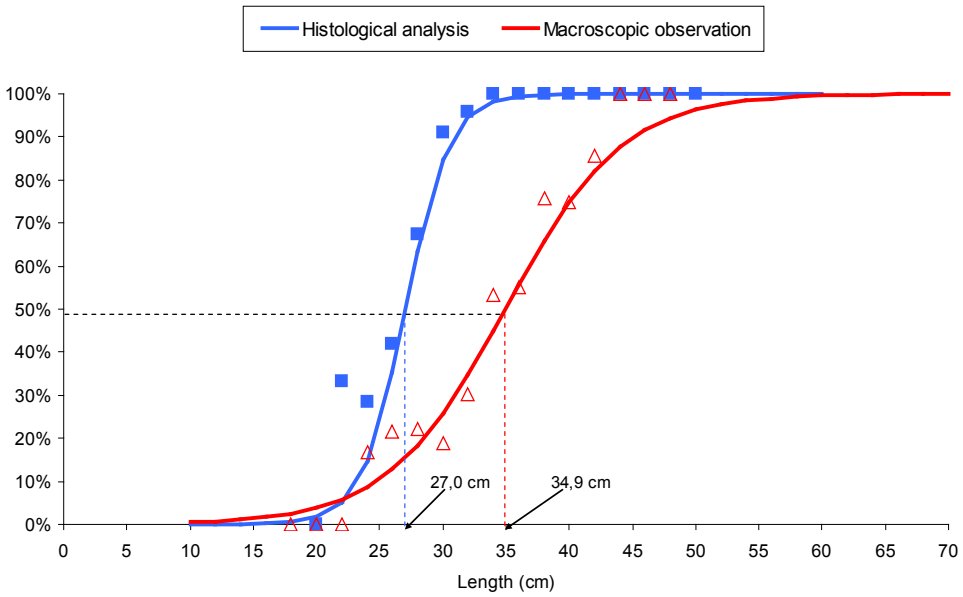

Figure 1. The estimated length-at-maturity of *Brycon guatemalensis* by mean of macroscopic and histological observation.

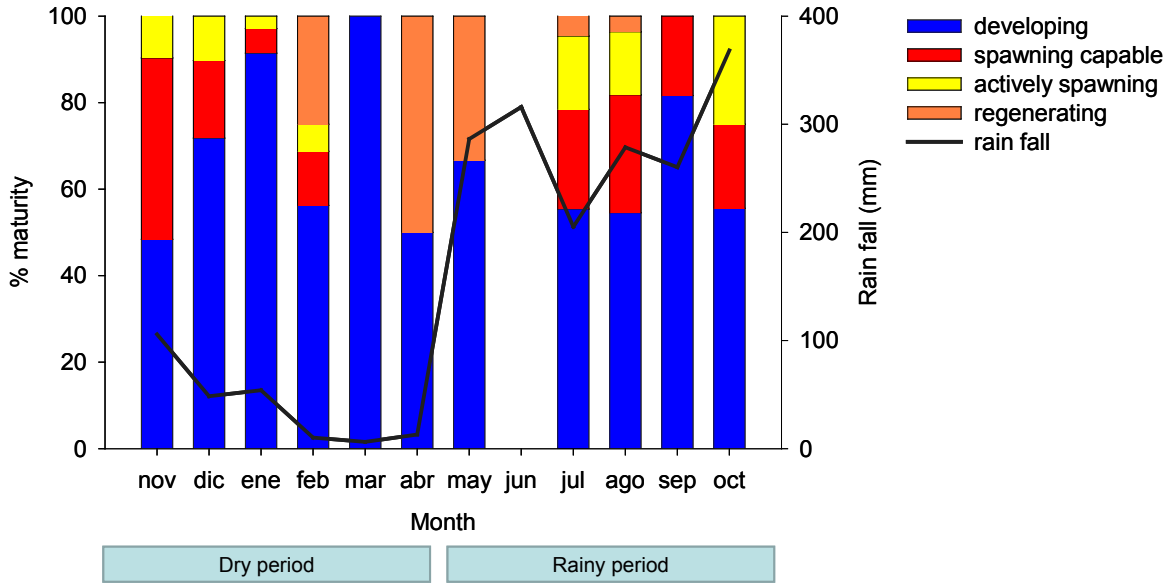

Figure 2. The seasonal reproductive cycle of *Brycon guatemalensis.* Dry period corresponds to winter and rainy period corresponds to summer.

Timing of Reproductive Events in Natural Pike (*Esox lucius* L.) Population

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Total amount of 316 pike (*Esox lucius* L.) specimens caught in the Danube River at river km 1175 and 1163 were analysed in this work. Samples were taken monthly during annual cycle. Activities of alanin aminotransferase (ALAT) and alkaline phosphatase (ALP) were determinated in pike blood sera. The values of reproductive score and oocyte diameter were estimated (based on analysis of histological preparates of gonads) as well as gonadosomatic (GSI) and hepatosomatic (GSI) indices. Significant negative correlation was revealed between reproductive score, oocyte diameter, HSI and GSI in relation to day length and water temperature in both sexes. This points out the fact that final phase of maturing in pike happens during the period of decreasing day length and water temperature. ALAT activity showed negative correlation with first order derivation of day length in both sexes indicating correlation between enzyme activity and changes in day length. The changes in ALP activity are connected with periods of spawning and active vitellogenesis in females. In this work attempt was made to explain relationship among serum enzyme activities, histology of pike gonads and photoperiod during annual cycle in order to ascertain the timing of reproductive events.

Seasonal Histological Changes of Testis in Males of Ocellate Freshwater Stingray *Potamotrygon motoro* of the Rio Negro Basin, Amazonas, Brazil

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Introduction

Potamotrygon motoro (Müller & Henle, 1841) is a species of freshwater stingray with a wide distribution in the Neotropical region (Carvalho et al. 2003). This species is well adapted to freshwater environments and presents a reproductive cycle controlled by hydrologic conditions of Amazonian rivers (Charvet-Almeida et al. 2005). Temporal changes of male gonadosomatic index (GSI) at the histological level are unknown for potamotrygonid species. The objective of the present study was to examine the annual variation in spermatogenesis of *P. motoro.*

Materials and Methods

Forty-five adult males of freshwater stingray were collected during the fishing season with hook and line from November 1998 to October 2003. For comparative purposes, two samples sites were established in the Rio Negro Basin located 140 km up river (area 1: 0°29'12.00"S; 63°15'02.00"W) and 30 km down river (area 2: 0°51'54.00"S; 62°46'42.00"W) from the city of Barcelos. Samples from both testes were preserved in 10% buffered formalin for histological examination. Tissues were embedded in paraffin, sectioned at 5 µm, and stained with haematoxylin and eosin. The gonadosomatic index (GSI) for each mature male was calculated using the formula: GSI= [Testis Weight (g)/Total Weight (g)] x 100. Based on GSI, annual variation in the spermatogenic cycle of P. motoro was examined by light microscopy.

Results and Discussion

Each testis was composed of 9 lobes embedded in the epigonal organ. Light microscopic examination showed that each lobe was arranged in zones, with the structural unit the ampulla or spermatocyst. Developing spermatogenic cells (spermatogonia (AI), spermatocyte (AII), spermatid (AIII) and spermatozoa (AIV) of the same stage were observed in each ampulla. A cross section of the testis lobe exhibits the dorsal-ventral development of spermatogenic cells, with AI in the dorsal area and AIV in the ventral area of the testicular lobe (Figure 1). The testis structure of *P. motoro* is a compound type of testis as described by Pratt (1988), and it shows structural similarities to other stingray species, including the presence of germinal papilla (Chatchavalvanich et al. 2005).

Monthly GSI shows annual variation (one-way ANOVA: F=4.235, p=0.00105), and is strongly related to the hydrologic cycle. The lowest value of GSI was observed for both areas during the flood period of the Negro River (end of April to August). The highest value of

GSI for both areas occurs during the low water period (September to February) (Figure 2). Seasonal variation of GSI has also been described for marine stingray species (Ebert and Cowley 2008).

Four distinct testicular phases were recognized based on monthly GSI and histological findings. A resting phase occured from February through May with decreasing of GSI value and testis lobes containing AI and AII; the recrudescent phase occured from June through September, it has an increased GSI, with rapid testicular growth. The maturation phase was characterized by a GSI peak in October in area I and in January in area II. Testis lobes during this phase exhibit AI, AII, AIII and AIV spermatogenic cells. During the copulation season, there was strong decline in GSI (November through January in area I and February through April in area II), and only AI, AII, and spermatozoa debris was observed in the testes. This decline is a consequence of spermatozoa moving toward the seminal vesicle. These findings are similar to the results for *Dasyatis sabina* (Maruska et al. 1996). There is a strong correlation between temporal patterns of testes development and hydrologic conditions in both areas. Nevertheless, the two month delay of the beginning of copulation season observed in area 2 is determined by local differences in water level of Rio Negro basin.

Epithelium variations of genital ducts were also observed. These histological findings mainly reflect changes in secretory activity on the epithelial surface. During the resting phase, immune cells present in the genital ducts are related to the elimination of residual spermatozoa.

Acknowledgements
The authors acknowledge numerous undergraduate students for participating in field sampling and animal collection procedures. This project was funded in part by Fundação de Amparo a Pesquisa do Estado do Amazonas (Process N° 925/2003), ACEPOAM and CNPq.

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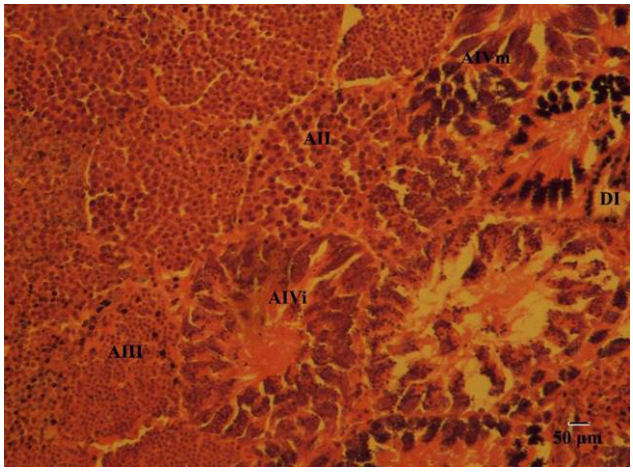

Figure 1. Sperm cells from adult male *Potamotrygon motoro*. DW= 45.0 cm, captured in area 1, October, 2000. AII—spermatocytes; AIII—spermatids; AIVi—immature sperm; AIVm—mature sperm; DI—intratesticular duct.

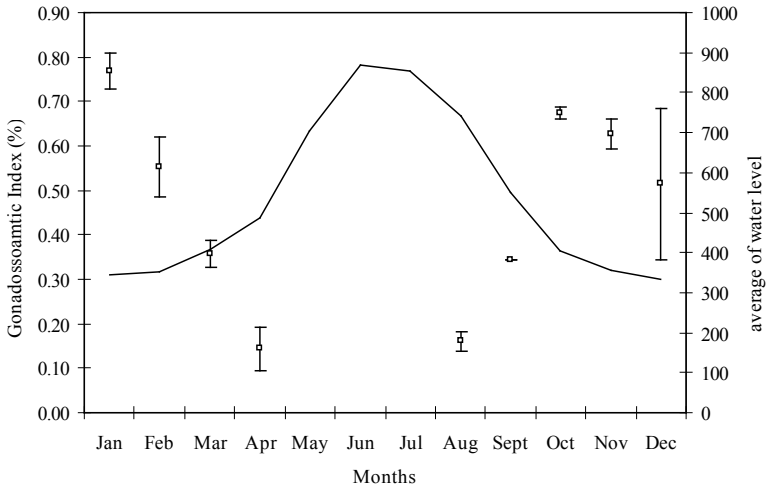

Figure 2. Monthly distribution of the gonadosomatic index (mean GSI ± standard error) for male *Potamotrygon motoro* and annual variation of Rio Negro water level (line) at Barcelos Municipality for years 1998-2003 (centimeters above sea level, a.s.l.). Source CPRN.

Environmental Influence in Oocyte Size, Fecundity, Egg Biochemical Content and Hatch Success in *Engraulis ringens* off the Chilean Coast

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Introduction

Engraulis ringens, an endemic small pelagic species from the Humboldt Current, has a long latitudinal distribution (4 to 42°S), spawning in a wide variety of environmental conditions. Three larger stocks have been identified along the species range: the northern Peru stock (the largest), a south Peru-North Chile stock (mid size) and the central Chile Stock (the smallest). A number of inter-population differences in life history traits have been recently reported, particularly between the northern Chile-southern Peru and the central Chile stocks. For instance, reproductive traits such as the proportion of hydrated females at the same size during the peak spawning month have been reported to vary between populations (higher proportion in northern Chile females; Claramunt et al. 2006). Batch and relative fecundity also differ between stocks (higher fecundity at a same female length in northern Chile; Leal et al. 2009). Planktonic egg size (smaller in northern Chile; Llanos-Rivera and Castro 2004), larval length at hatch, yolk sac size at hatch, and larval length at the end of the yolk sac stage (smaller in northern Chile; Llanos-Rivera and Castro 2006) differ between areas as well. From these studies it has become clear that the spawning environment may affect the reproductive output of widely separated populations in the Humboldt Current.

Less attention has been given to variations in reproductive traits in different temporal scales within single populations. Currently it is well known that throughout the anchoveta distribution range, their populations have experienced strong fluctuations in abundance in association with El Niño or La Niña events and also with longer term environmental fluctuations. In these long term scales, changes in fecundity have already been documented off Peru (Ayon 2000). Reports on variations in reproductive outputs (egg traits) in these time scales or shorter are less frequently observed in the literature. In this study we investigated reproductive traits such as hydrated oocyte size, egg volume in the plankton, biochemical composition of the eggs in the plankton, and egg hatch success, by determining their variability in different time scales: inter-annual (Normal vs. La Niña years) and intra-seasonal (along the spawning season) in the northern spawning area off Chile.

Methods

Reproductive information on adult female and hydrated ovaries were obtained from the anchoveta fish stock assessment yearly cruises in northern Chile (Daily Egg Production

Method). Oocyte size (diameter) at the same developmental stage (hydrated ovaries) was obtained from subsamples of ovary tissues by measuring the longest and shortest axis of those oocytes through digital image analysis. Egg volume of spawned eggs was obtained throughout the spawning season (July – November) from plankton collections carried out off Iquique (northern Chile) in 2004. Subsamples of those eggs from the plankton were deeply frozen fresh in liquid Nitrogen for weight and biochemical determinations (wet and dry weight, total lipids, TAG, cholesterol and total proteins). Since egg size has been reported to change during the spawning season (Llanos-Rivera and Castro 2004) and to avoid as much as possible the effect of egg size when making comparisons inter-monthly, we express the biochemical content of the eggs as per egg dry weight. Finally, other two subsamples of eggs were obtained for incubation and hatch experiments in all sampling dates throughout the spawning season.

Results

Inter-annually, our results show that hydrated oocyte sizes varied according to environmental conditions. During La Niña years (2007; 14 ºC on average compared with 16 ºC average in "normal" condition) hydrated oocyte diameters were longer than oocytes collected in normal years (Figure 1a). Eggs collected from the plankton during four years at the peak spawning month (August) in northern Chile showed the same trend: during La Niña 2007 the anchoveta egg volumes were larger than in normal years (Figure 1b).

Intra-seasonally, our results show a decrease in egg volume as the spawning season progresses, from July through October (Figure 2a). The biochemical content of the eggs also changed throughout the spawning season with higher lipid and protein contents early in the spawning season (mid winter, July; Figure 2b). Hatching success also decreased during the spawning season, and correlated positively with egg volume, lipid, and protein contents (Figure 2c-d).

Discussion

We believe that these differences in fecundity, hydrated oocyte diameter, egg volume in plankton samples, and biochemical content of the eggs are determined by the predominant environmental conditions of the habitat by affecting females during oogenesis. Seawater temperature and the entire plankton community (i.e. the adult female feeding environment) have been reported to change between normal and La Niña or El Niño years. In our study, during the cold La Niña 2007, both oocytes from hydrated ovaries and eggs in the plankton were larger than in normal years during the peak spawning months (Leal et al. 2009). At a shorter time scale, the environmental conditions also vary yearly during the spawning season from mid winter (colder water, higher turbulence, lower larval food availability) to late spring. These changes have been shown to be even stronger in higher latitude spawning areas such as in Talcahuano (37^{o}S) where environmental conditions are more severe than in northern Chile. Here, mid-winter eggs are larger, contain more lipids, essential fatty acids, and proteins than in late spring, and their hatch success is higher than later in the season (Castro et al. 2009). Hence, female anchoveta seem to adapt their reproductive strategy, producing eggs in sizes, quantities and biochemical content that favor offspring survival under the environmental conditions in which they are to develop.

Acknowledgements
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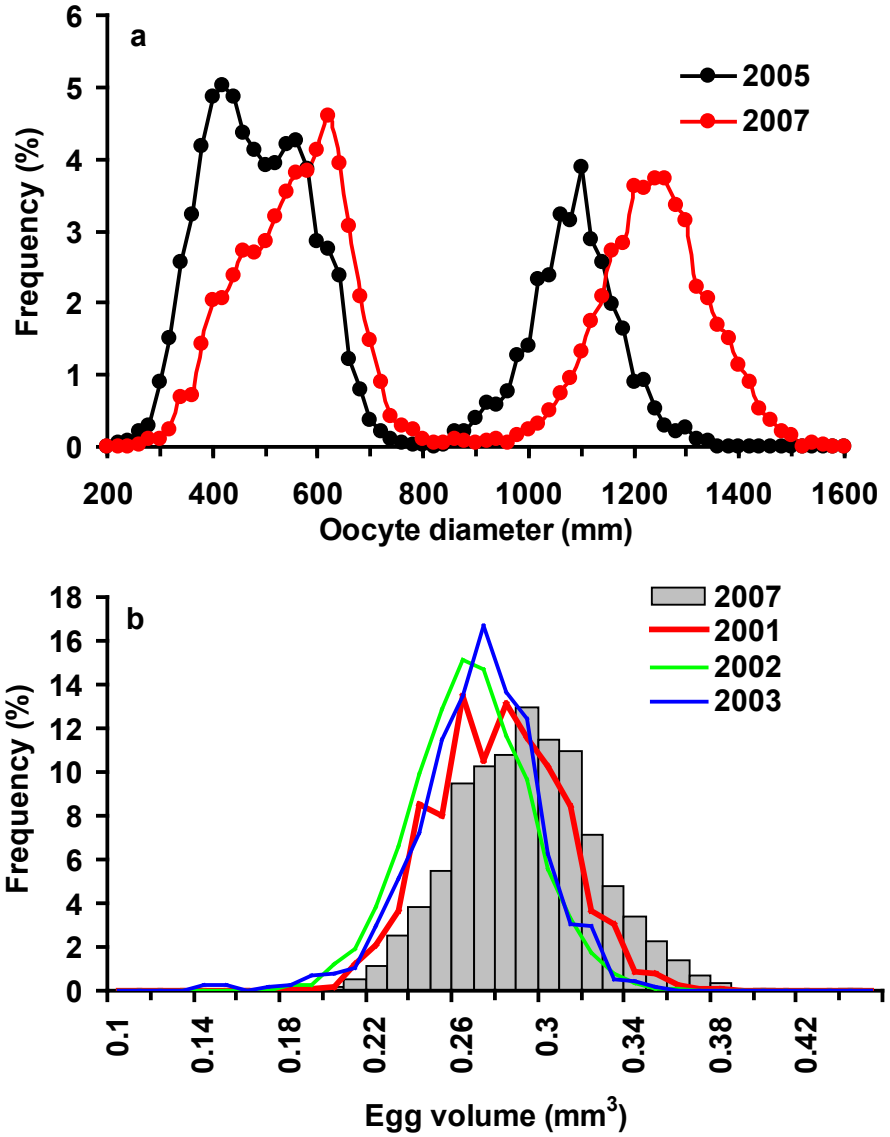

Figure 1. a) Oocyte diameter frequency distribution of the hydrated ovaries (first mode is vitellogenic oocytes and second mode is hydrated oocytes). b) Volume frequency distribution of the eggs collected in the plankton. Year 2007 corresponds to La Niña condition (cold period) with an average of 14 ºC. Others years are "normal" condition (Neutral ENSO) with 16 ºC on average.

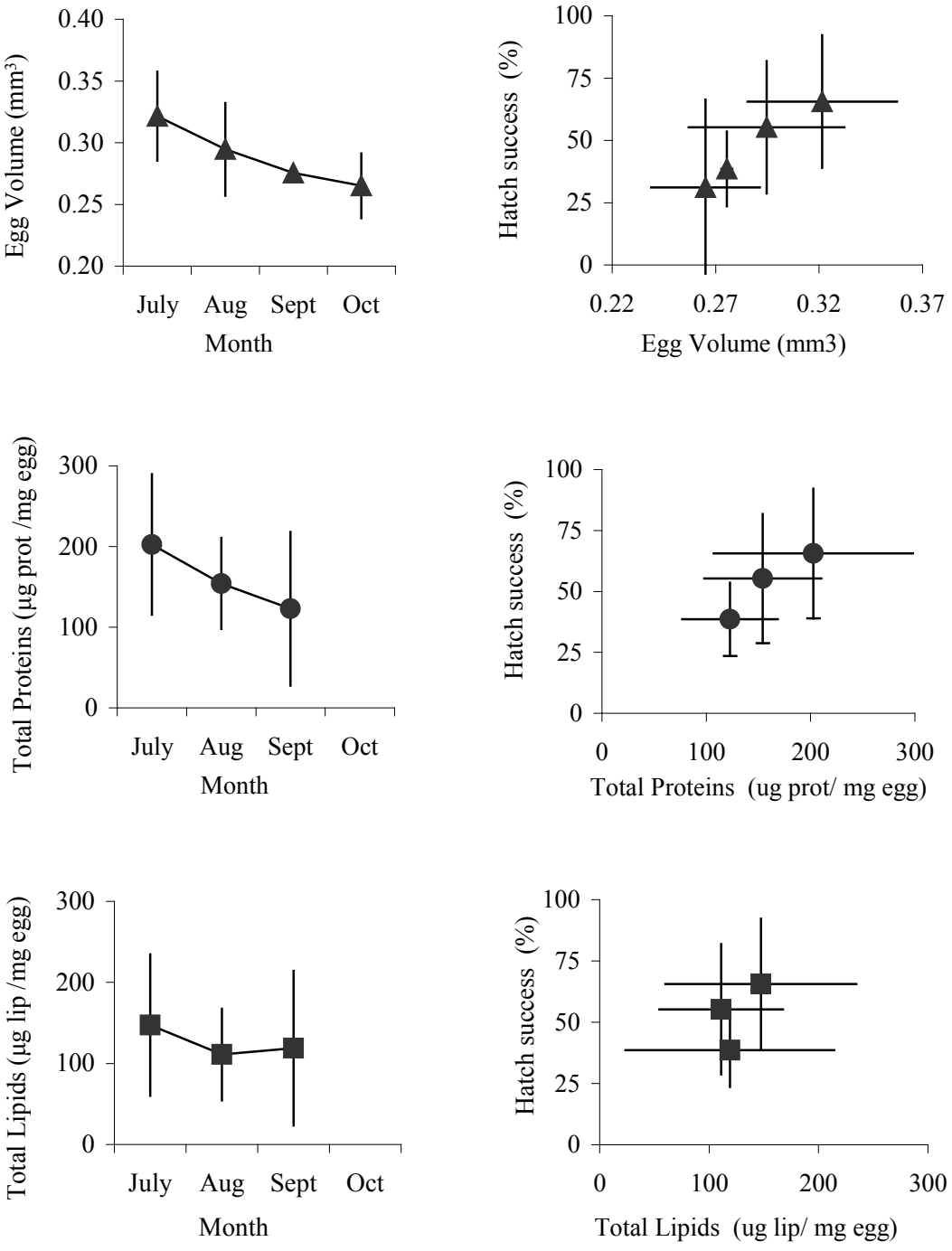

Figure 2. Variations in egg volume, total protein and lipid contents in anchoveta eggs throughout the main spawning season off northern Chile and the relationship of these traits with hatch success.

A Model Based Method for Estimating Daily Spawning Fraction in *Engraulis ringens*

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Introduction

How many eggs do stocks of fish with multiple spawning and indeterminate fecundity produce on a given period, say a year? (Total egg production). This question is highly relevant for population dynamics and fisheries biology because many commercially important fish stocks show indeterminate fecundity. For example, one of the major issues in the field is the existence and nature of a relationship between the abundance of spawners and recruitment. This issue can be clarified by considering total egg production, instead of spawning biomass, as best representing the true reproductive potential of a stock, and then searching for its relationship with recruitment. This is not just a change of focus since spawning biomass and total egg production in fish with indeterminate fecundity are not expected to be proportional. So how can total annual egg production be measured in fishes with indeterminate fecundity? Information concerning size-specific spawning fraction is not usually available because it must be estimated from the proportion of females with postovulatory follicles, which is a time-consuming, biologically intensive histological methodology that can only be applied on samples obtained over short periods, regardless of age or size structure of the samples. Alternative methods based on indirect procedures and large samples sizes are therefore desirable. Claramunt and Roa (2001) developed theoretical and statistical arguments for a new method, previously outlined by Claramunt and Herrera (1994), which utilizes the Gonadosomatic Index (GSI) to estimate spawning fraction. The main objective of this work is to validate this method through the comparisons with direct estimation of spawning fraction (histology) for several years in *Engraulis ringens* from Chile.

Methods

The information and data used in this study were obtained from two different sources: a) application of the Daily Egg Production Method (DEPM) for *Engraulis ringens* in northern and southern Chile, and b) from the historical database (1964 – 2006) consisting of monthly samples of the catch (sex, total length, total weight and gonad weight). For northern Chile (18º 20'S - 26º 03'S), DEPM was applied in 1995, 1996 and from 1999 to 2006. For southern Chile (33º 00'S - 41º 30' S), DEPM was applied from 2002 to 2005. The daily spawning fraction (S) of *E. ringens* in each DEPM application was estimated through the classification of postovulatory follicles (POF) into daily cohorts (0, 1 and 2 d).

The monthly averages of the GSI were estimated from the historical database of *Engraulis ringens* for northern and southern Chile to estimate the spawning fraction through the model of Claramunt and Roa (2001). The spawning fractions resulting from the model were compared with estimations of daily spawning fraction from histology (DEPM application).

Results

The model predicts a non-linear relationship between GSI and daily spawning fraction, which is not well explained by other simple non-linear models as power or exponential functions. The model renders a lower sum square error (SSE = 0.030) if is compared with power (SSE = 0.044) and exponential (SSE = 0.073) functions.

To validate the model, spawning fraction was estimated using the GSI average from the historical database centered in July (month that DEPM surveys start). The relationship between the daily spawning fraction from the DEPM surveys (i.e histology) and the model shows a linear tendency (Figure 1). The year 2005 for southern Chile was not considered in the linear regression because it is out of tendency due to the low number of specimens sampled in July to estimate the average of GSI (n = 75). The estimations of the spawning fraction from the historical database shows the same tendency and very close to the values obtained from the direct method (i.e. DEPM surveys), as shown in figure 2. Therefore, according to these results it is possible to estimate the spawning fraction and to study its fluctuations through the monthly average of GSI.

Discussion

The method that was applied to *Engraulis ringens* provides a straightforward way to estimate spawning fraction on a monthly basis once it has been calibrated against histological information. The results obtained with use of GSI showed the same tendency that the results obtained with intensive sampling design and time consuming laboratory process and analysis (i.e. histology) information. According to Parrish et al. (1986), the best approach for estimating spawning fraction is a combination of detailed and time-consuming histological analyses on a few fishes and macroscopic indices of reproductive activity from a large number of fishes. The method applied here falls neatly into the category described by these authors.

The biorhythm hypothesis (Hunter and Lo 1997) proposes that the interspawning interval estimated from active females may be a stable feature of a population under similar temperature conditions, and that the observed variations in spawning fraction may simply result from changes in the proportion of active fish in the mature population. According to Stratoudakis et al. (2006), this seems to provide some basis for the rather spectacular performance of the inexpensive method for daily spawning fraction estimation that was developed by Claramunt and Herrera (1994) and then redefined by Claramunt and Roa (2001). Ganias et al. (2003) expanded the biorhythm hypothesis to include a body-size effect: the frequency of spawning is relatively constant for the equally sized active females as long as the habitat conditions remain about the same. In the context of the Claramunt and Roa (2001) method, the relationship between body size and spawning fraction is explained because the proportion of active females is higher in bigger females than smaller ones.

Acknowledgements

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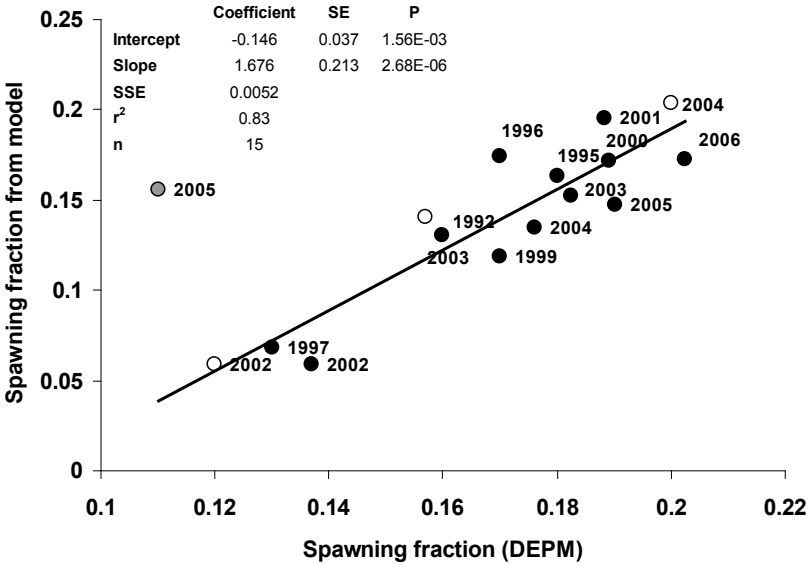

Figure 1: Relationship between spawning fraction from histology (DEPM surveys) and from the model. Open circles: Southern Chile; Solid circles: Northern Chile. Year is indicated for each point. Table inset: Parameters of the linear regression (Line). SE: Standard error of the coefficient; P: Probability coefficient = 0; SSE: Sum of square error.

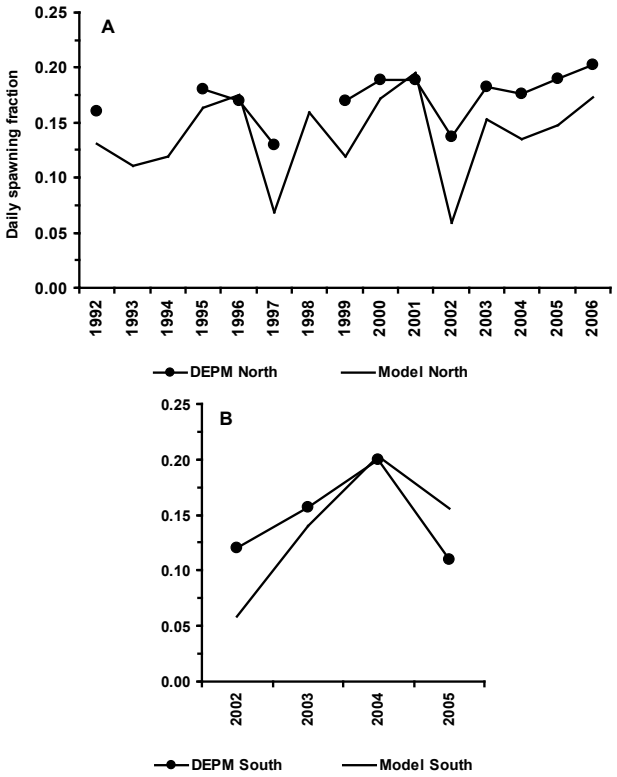

Figure 2: Daily spawning fraction estimated by the DEPM surveys (i.e. histology) and from the model in northern (A) and southern (B) Chile

Ageing of Postovulatory Follicles in Anchovy (*Engraulis encrasicolus)* by Means of DEPM Survey Data in the Strait of Sicily: a Critical Review.

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Introduction

The European anchovy (*Engraulis encrasicolus*) fishery represents the main world fishing resource and constitutes almost 30% of the total Mediterranean fish production (Lleonart and Maynou 2003). Anchovy is widely distributed along the European Atlantic coast from South Africa to the North Atlantic Ocean and in both the Mediterranean and Black Seas. In the Strait of Sicily the anchovy population has been investigated in the last decade by means of ichthyoplankton data and the Daily Egg Production Method (DEPM) which is based on the reproductive biology of adults (Somarakis et al. 2004). This method requires estimations of potential reproductive parameters such as fecundity and the spawning fraction of the adult population. The principal method adopted for the evaluation of the spawning fraction is the ageing analysis of postovulatory follicles (POFs). The POFs represent cellular residuals in the ovary, and the post-spawning resorption time is species-specific and changes according to the residence temperature. In the literature, the only studies which report POF duration vs. temperature were performed within different temperature ranges than in the Mediterranean Sea, and did not take into account the summer season temperatures when anchovy spawning takes place. Data collected during the 2007 and 2008 adult DEPM surveys were used here to study the temporal patterns of reproductive activity to estimate POF duration.

Methods

The anchovy samples were collected by the DEPM surveys in the Strait of Sicily in 2007 and 2008 during the peak spawning period (July-August). Additional samples were taken from commercial catches. The ovaries were analyzed using traditional histological techniques and stained with haematoxylin and eosin.

The anchovy POFs were aged using three different morphological features as identified in literature for this species (ICES 2004). The POF age was correlated to the catch hour and water temperature. The temperature along the water column was measured directly during the trawls by a temperature/depth net sensor. As for the commercial catches, for which no direct measurements were available, surface temperatures were obtained via satellite SST images (http://eoweb.dlr.de:8080/servlets/template/welcome/entryPage.vm). To obtain temperature measurements in the water column, the net sensor temperature data were plotted against SST for each trawl and the linear relationship was estimated.

The histological slides were used to estimate the size distribution of oocytes in the ovaries by an image analysis system. To estimate the POF resorption rate a second method, based on the decreasing proportions of specimens with POF in the trawls, was adopted. It was applied only to the nocturnal consecutive trawls. The POFs abundance for trawl was plotted against the time of capture and the slope of the regression line obtained was adopted as the resorption rate.

Results

Only two sampled ovaries were hydrated (2400; daily spawning time); this corresponded well with the observed diel distribution pattern of the early POF. The majority of POF 0 were observed between 2400 and 0800 (Figure 1A). Ovaries with POF 1 appeared in abundance starting from 0800 (Table 1). Ovaries with POF 2 were present in the samples starting from 1600, but these were not abundant, indicating that POF 2 is present also on the first day (< 24 h). The cumulative POF resorption time (0+1+2), as inferred from their distribution along the 24 hour daily cycle, extended more than 24 hrs (Figure 1A).

Only a few trawls among the survey data set met the requirements for the estimation of the resorption rate using the second method. The POF duration, obtained by plotting the differences in their decreasing proportion vs. the capture time, showed how the backcalculated duration of POF 0 was about 3.5 h while POF 1 was present much longer before resorption (11.5 h). There were not enough trawls available for computing the POF 2 resorption rate (Table 1). No POF older than POF 2, based on morphological features as defined in the literature as POF 3 (ICES 2004) were detected.
The temperature data obtained by SST for each trawl indicated a temperature range between 16-28 °C while the range in the water column was between 16-23°C.

The image analysis carried out by measuring the oocyte average diameter gave distinct results for POF 0 (147 µm) and POF 1 (167 µm), and their confidence limits do not overlap (Figure 1C). No significant differences between POF 1 and POF 2 in the advanced average yolked oocyte batch diameters were recorded (Figure 1C). The oocyte diameter distribution pattern showed an increasing trend during the day starting from 2400 h (Figure 1B), suggesting that this could be a proxy of the ovary regeneration from the last spawning event and consequently of the POFs degeneration. This method appears functional to discriminate recent from older spawning ovaries and thus supports the POF age assignments at least for POF 0 and POF 1.

Discussion

The oocyte diameter measurements were confirmed as a good predictor of POF 0, particularly when a preliminary study is conducted to define the diameter dimensional classes for the POF stage, as in this paper (Figure 1A). The POF 24 h daily distribution showed a presence of POF 0 for only a few hours (4 h) after spawning, while the presence of POF 1 in the trawls was observed between 0100 and 1200 (11 h) (Figure 1A). These results are in agreement with the POF duration from the resorption rate obtained by regressing the decreasing proportion of POF for consecutive trawls. These results, even if preliminary, are encouraging in the application of this method for POF ageing and furnish a basis for further investigations. Finally, the POF resorption lasted for less than 48 h within the observed 16-24°C temperature range in the water column. Furthermore, the resorption rate obtained for those consecutive trawls with decreasing POF proportion were of 19% h-1 for POF 0 at an average column

temperature of 16.7°C, and 17% h-1 for POF 1 at 16.3°C. On the basis of the new findings a critical revision of the past DEPM estimations would permit evaluation of how the differences in POF duration may affect the final biomass estimation.

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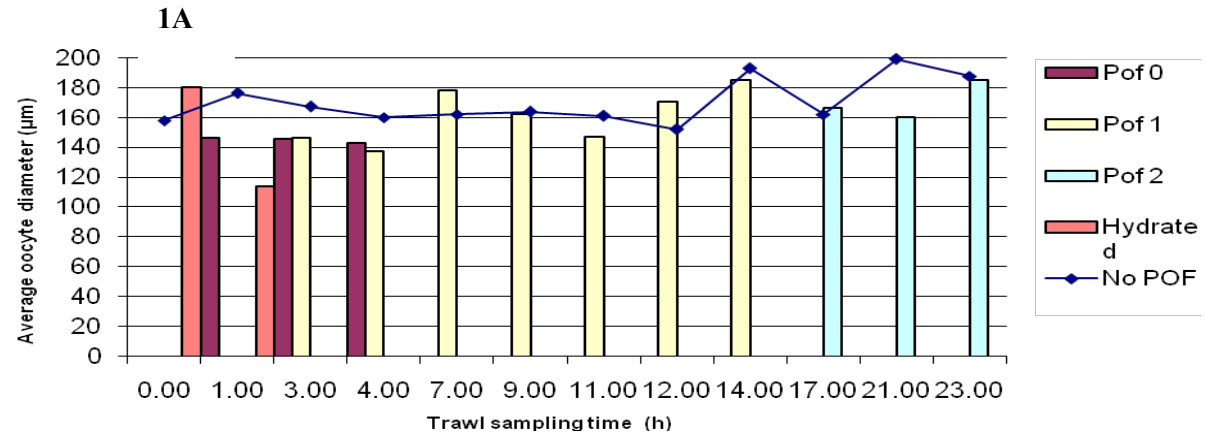
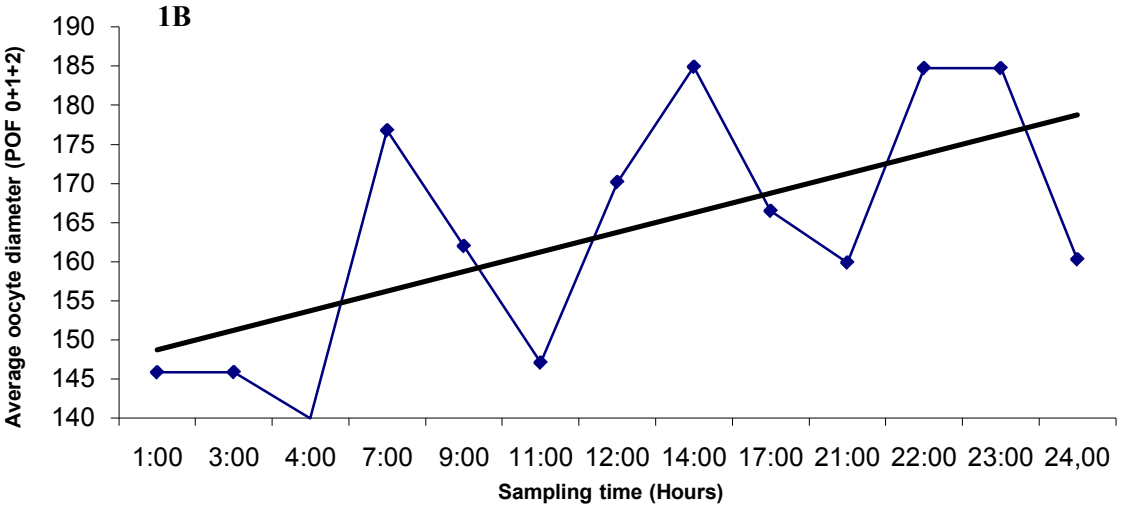
Table 1. Sampling time, number of specimens with POF 0, 1 and 2, and estimation of POF resorption rate by two different methods.

	TIME	#	Temperature (°C)		POF resorption rate (h:min)	
			Average SST	Average T in water column	Method 1	Method 2
POF 0	2400	1	**24.3**	**16.0**	**04:00**	**03:30**
	0100	5				
	0300	2				
	0400	5				
POF 1	0300	3	**22.5**	**18.9**	**11:00**	**11:30**
	0400	2				
	0700	16				
	0900	3				
	1100	16				
	1200	5				
	1400	34				
	1700	4				
	2300	2				
POF 2	1400	1	**22.0**	**18.4**	**06:00**	**-**
	1700	2				
	2100	1				
	2300	1				





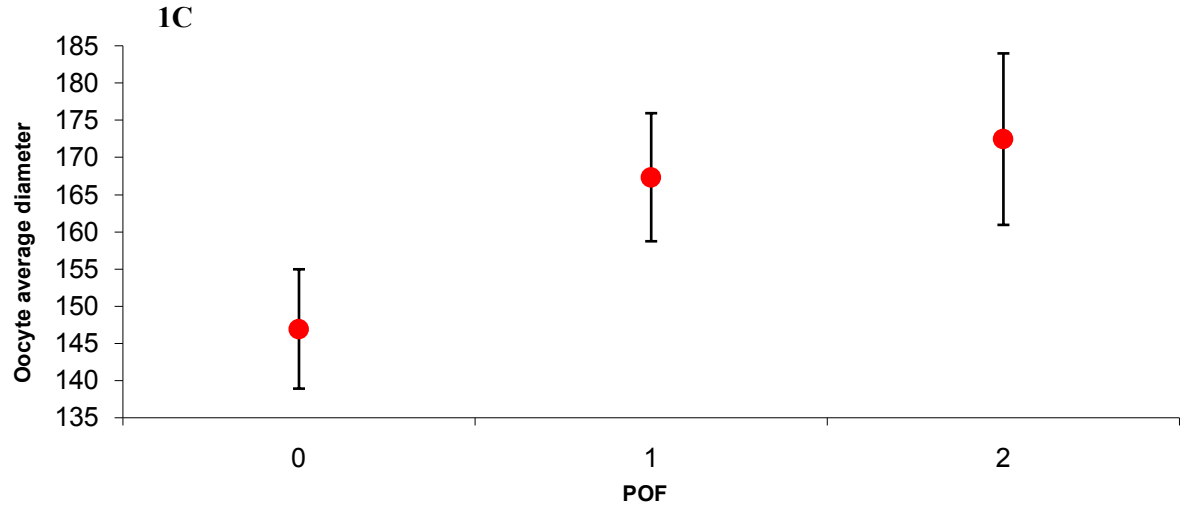


Figure 1: A. Distribution of average oocytes diameter in ovaries with different age POFs (POF 0, POF 1 and POF 2), hydrated oocytes (Hyd) and without POFs (No) in relation to sampling time. B. Oocyte diameter change with time, including regression line. C. Oocyte diameter (mean ± confidence limits) in ovaries with POF 0, POF 1 and POF 2.

Aging of Postovulatory Follicles and Oocyte Recruitment in Baltic Sprat

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Introduction

Baltic sprat, similar to many other small pelagic clupeoids, are indeterminate spawners, releasing several batches of eggs over a protracted spawning season (Alheit 1988). The indeterminate spawning strategy demands special requirements for assessing the spawning stock biomass of these species with egg production methods, e.g. the Daily Egg Production Method (Parker 1985). Prerequisite parameters for the aforementioned method are spawning frequency, batch fecundity and data on maturity and sex ratio. With respect to these parameter requirements, the duration of postovulatory follicles (POF) and the oocyte recruitment pattern are important measures. The first allows assessing the spawning frequency with the POF method, and the latter is important to give insight into the oocyte maturation process, which is relevant for fecundity estimations. Neither detailed histological investigations of Baltic sprat ovaries have been published before nor an attempt has been conducted to age POF of sprat. Because sprat show an asynchronous daily spawning pattern, it is difficult to age POF. Therefore, the spawning frequency, calculated with the hydrated oocyte method, and the oocyte development pattern were combined to get an estimate of the POF duration.

Materials and Method

Samples were collected in the Bornholm Basin during the spawning season in April 2007. Pelagic trawls were carried out during a 24h period every 3h. From each haul a 2 kg sub-sample ($n \approx 200$) of sprat was preserved in a buffered 10% formaldehyde solution. At least 20 females were taken randomly from each sample for histological analysis. Histological sections of 3µm were produced, and stained with the Hematoxilin-Eosin method. Sections were classified into different oocyte developmental stages. The presence of POF was checked for each section. Four different POF stages were defined according to their histological features (Figure 1). In order to analyse the oocyte development pattern, the whole mount method was applied and oocytes were counted and measured with image analysis software.

Results

The results of histological analyses revealed spawning activity of Baltic sprat throughout the day. The proportion of females with hydrated ovaries was 23%, which indicates a spawning frequency of approximately every 4 days. It was possible to classify four different stages of POF:

I. The POF has a clear convoluted shape and has a rather big lumen. It consists of two cell layers with clearly visible nuclei. The two cell layers are relatively thin probably due to the high pressure experienced during the hydration phase of the oocyte. The

cross sectional area is larger compared to that of the other identified POF stages. This type of POF occurred exclusively in vitellogenic ovaries (Figure 1a).

II. The POF still has a distinct convoluted shape but the lumen has become smaller. The two cell layers with their nuclei are still distinguishable but not as clear as in the first stage. Vacuoles are also becoming visible in the cells. The thickness of the two cell layers has clearly increased. This type of POF occurred exclusively in vitellogenic ovaries (Figure 1b).

III. The convoluted shape begins to disappear. The lumen is small or non existent. It is not possible to distinguish between two cell layers. The cross sectional area has clearly decreased compared to the two first POF stages. The thickness of the cell layer has decreased again due to degeneration. This POF type occurred in ovaries with early and advanced nucleus migratory oocytes or in ovaries with beginning hydration (Figure 1c).

IV. The convoluted shape is not visible anymore. Only a few cells are remaining. The shape has become more triangular. The cross sectional area is very small compared to the other stages. This POF type occurred in ovaries with advanced nucleus migratory and hydrated oocytes (Figure 1d).

The oocyte development pattern is asynchronous. However, two clearly distinct cohorts of secondary growth stage oocytes are visible: one fully recruited advances steadily from cortical alveoli to hydration, while another recently recruited remains in early vitellogenesis until the proceeding cohort is spawned (Figure 2). Both cohorts become distinct when the first is at the migratory nucleus stage. POFs degenerate (stages I to IV) in parallel with the development of the more advanced cohort (Figure 2), confirming the spawning frequency obtained by the hydrated oocyte method.

Conclusions

Although sprat show an asynchronous daily spawning pattern, the estimation of POF duration could be justified by the combination of the hydrated oocyte method and histological analyses. The spawning batch becomes visible from the migratory nucleus stage onwards. This pattern is similar to that of the Mediterranean sardine (*Sardina pilchardus sardine*), another indeterminate spawning clupeoid, as described by Ganias et al. (2003). The forming of a clear hiatus before hydration might provide the possibility to use non-hydrated sprat females for batch fecundity analyses. The results of the present study can serve as basis for future studies on sprat spawning frequency and batch fecundity, in order to compare these reproductive parameters between different stocks, years and seasons. The lack of adequate estimations of spawning frequency hampered former studies on the implementation of the daily egg production method (Kraus and Köster 2004).

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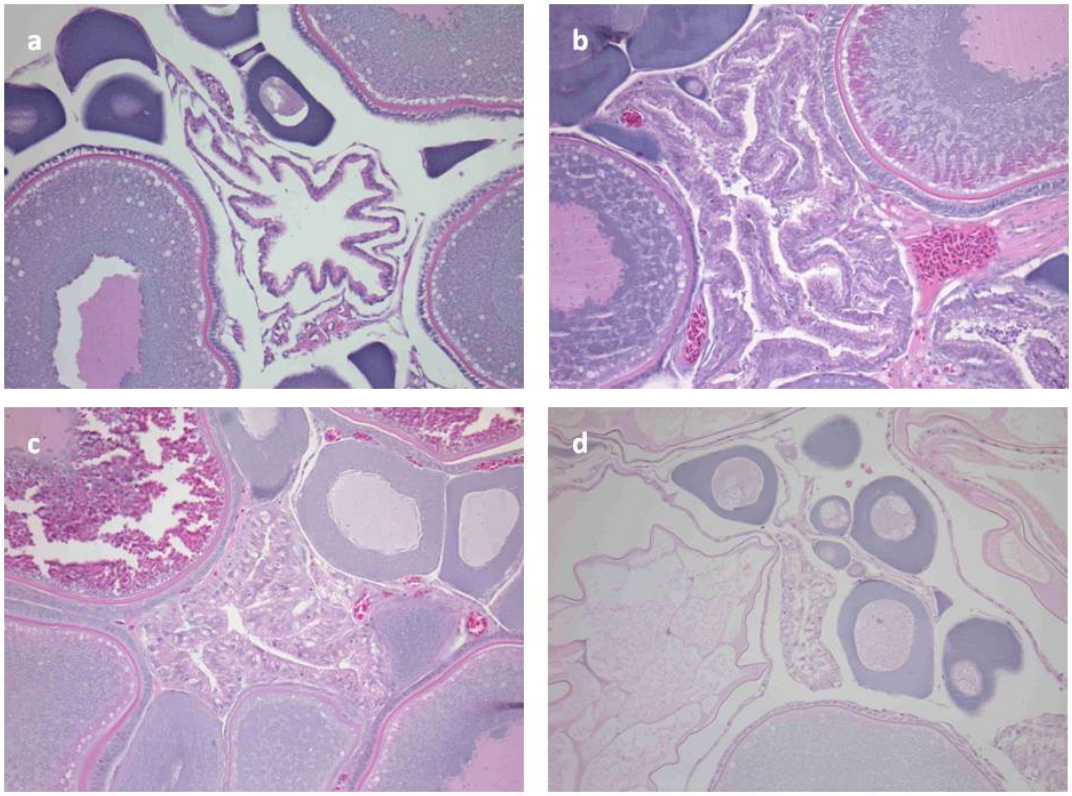

Figure 1. Different stages of postovulatory follicles of Baltic sprat. a) POF I < 24h, b) POFII, c) POF III and d) POF IV > 72h.

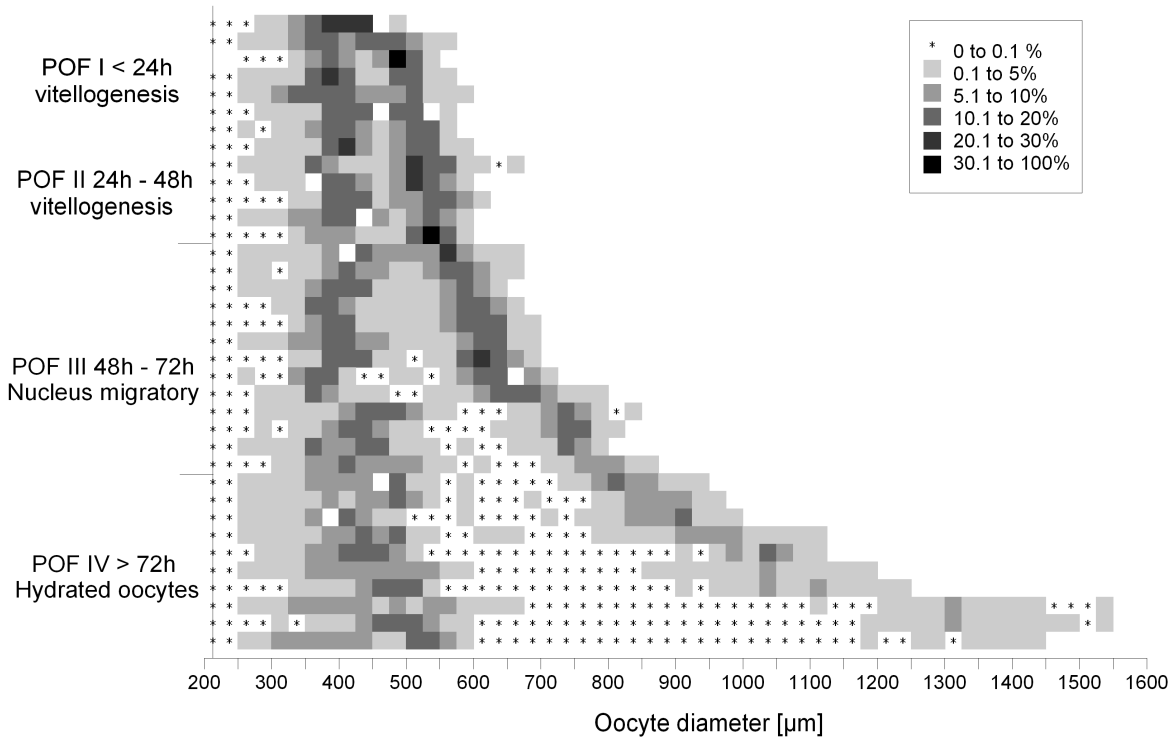

Figure 2. Oocyte development pattern displayed as oocyte size frequency distribution according to POF stages.

Effect of Atresia on the Spawning of *Helicolenus dactylopterus*

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The blackbelly rosefish *Helicolenus dactylopterus* (De la Roche, 1809) is a benthic species in the Family Scorpaenidae. It is widely distributed in the Atlantic Ocean and the Mediterranean Sea, between depths of 200 and 1000 m. Various studies of blackbelly rosefish reproduction have been carried out in the Atlantic Ocean (White et al. 1998; Sequeira et al. 2003; Mendonça et al. 2006), and especially in the Mediterranean Sea (Muñoz et al. 1999; Muñoz and Casadevall 2002). This research has led to a definition of the blackbelly rosefish as an oviparous-zygoparous species, with very long sperm-storage times in the ovaries, varying gestation periods and multiple spawning (Muñoz et al. *op. cit.*). Studying the seasonal variations in histological sections of gonads, the authors mentioned identified the various states of development and described the annual reproductive cycle of the blackbelly rosefish. They determined the presence of postovulatory follicles (POFs) at the end of the spawning process and examined atretic follicles in ovaries of various reproductive states.

Atresia is a degenerative process in which oocytes in various stages of development are reabsorbed into the ovary. Towards the end of active spawning, atresia is very common and it is necessary to differentiate between this type of oocyte, normal ones and the POFs. Studying the prevalence and intensity of the atresia lets us identify ovarian regression and predict the end of the spawning period (Hunter and Lo 1997).

The aim of our work was to characterize *H. dactylopterus* spawning as well as determine the prevalence and the relative intensity of atresia during the recently-spawning, actively spawning and regressing phases.

Between November of 2007 and June of 2008, a total of 200 individual specimens were captured (171 females and 29 males; LT between 163 and 365 mm) by the artisan fleet in the Gulf of Lion. A total of 137 histological sections were taken from the ovaries of recently spawning, actively spawning and regressing phases. Cross-sections of the center of the ovaries were obtained, preserved in formalin solution (4%), dehydrated and finally embedded in paraffin blocks. The blocks were cut into sections of between 5 and 25 µm, depending on the gonad maturation stage, and were then stained with hematoxylin-eosin, Mallory's trichromatic and periodic acid-Schiff (PAS) stains. The ovaries were classified according to the most developed stage of oocyte, and special emphasis was placed on quantifying the ovaries with POFs. The fraction of spawning females (S) and the spawning frequency (time between batches considering the percentage of spawning females in the population at a specific time) was estimated. The presence of POFs was analyzed and the prevalence (P_a: % of females with alpha atresia) and relative intensity of atresia (R_{Ia}: % of α-atretic vitellogenic oocytes vs. total vitellogenic oocytes) were determined.

The spawning period of *H. dactylopterus* in the Gulf of Lion is from December to May. The fraction of spawning females was 0.54 ± 0.17 with a mean spawning frequency (S) of two days. The highest values of S were registered from February to May, with an interval between batches of 1 to 1.5 days (Figure 1). No ovaries were observed during the initial stages of the cycle (primary and secondary growth) from February to May. On the other hand, developing ovaries were not observed after the beginning of March but from March to May the presence of ovaries in recently spawning, actively spawning or regressing phases was confirmed. Of the females analyzed, 54% had recently spawned (49) or were actively spawning (25) at the time of their capture.

A total of 74 specimens with POFs and 13 with α-atretic follicles (P_a of 9.15% and an average R_{Ia} of 7.90) were examined. The presence of α-atretic oocytes was confirmed from December to May in the maturation stage (n=2 and a R_{Ia} of 1.45%), recently spawning stage (n=8 and a R_{Ia} of 3.04%), actively spawning stage (n=1 and an R_{Ia} of 4.41%) and regression stages (n=2 and a R_{Ia} of 58.33%) (Table 1). In March, sixty percent of the analyzed females had α-atretic follicles, whereas in April 100% of the females were spawning and 14.3% showed signs of α-atresia. The R_{Ia} increased in ovaries with POFs throughout the spawning period, reaching 58.33% in May.

The results of this study indicate that the spawning period of *H. dactylopterus* in the area of study lasts from December to May, which is an extension the period defined in previous studies. The increase in the R_{Ia} in ovaries with POFs throughout the spawning period suggests that the female would sacrifice a certain number of oocytes to recover some energy and thereby ensure the maturation of the others. In May, the R_{Ia} was higher than 50%. The fact that the ovaries were in the regression phase indicates a possible residual effect, with the female trying to recover some energy to invest in the next reproductive cycle. The low levels of atresia would indicate that this mechanism does not significantly effect spawning in this species.

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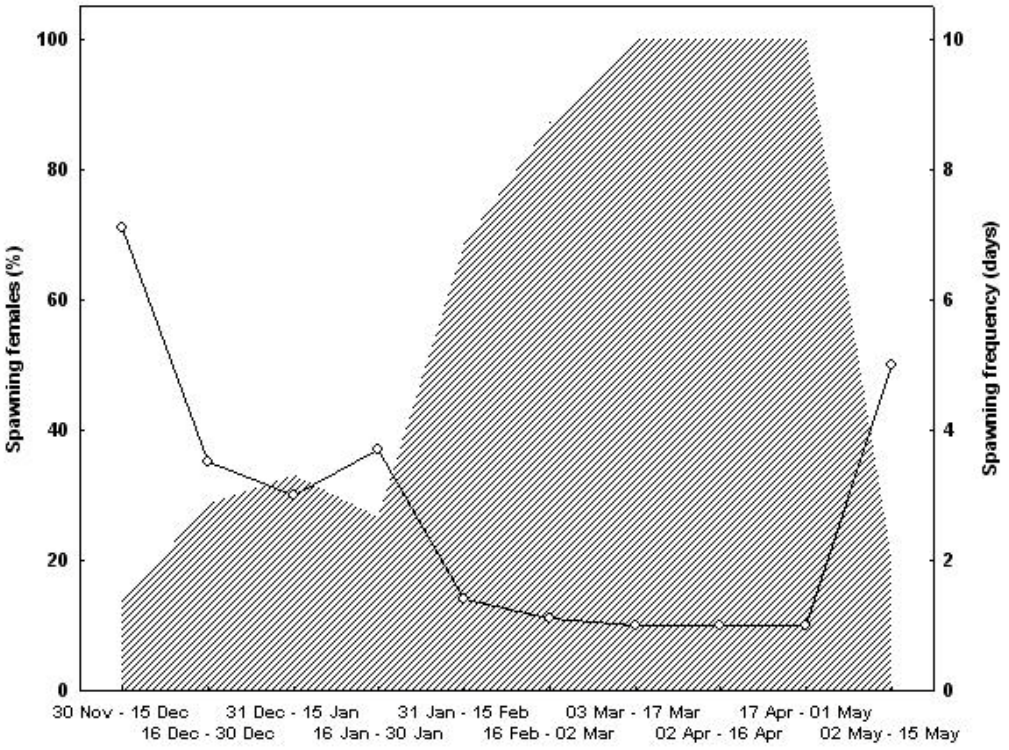

Figure 1: Fraction of spawning females and the spawning frequency of *H. dactylopterus* from November to May.

Table 1: Characteristics of individuals with atresia. N: Number of Individuals, Pa: Prevalence of Atresia, RIa: Relative Incidence of Atresia, SM: State of Maturity (M: maturation; RS: Recently Spawning; AS: Actively Spawning; R: Regression), LT: total length and P weight.

Months	N	% Females with atresia	Pa (%)	Characteristics of females with atresia			
				SM	LT (mm)	P (g)	R_{Ia}
DEC	17	5,9	5,9	M	270	349	1,56
JAN	39	5,4	5,1	RS	288	341,8	1,35
				RS	252	286	2,38
FEB	44	6,8	6,8	M	308	427	1,35
				RS	264	345	1,82
				RS	281	460	4,12
MAR	5	60	60	RS	313	589	-
				RS	365	844	2,17
				RS	302	525	3,85
APR	14	14,3	14	RS	256	362	5,56
				AS	292	437,2	4,41
MAY	20	10	10	R	333	576,1	58,33
				R	318	591	-

A Standardized Terminology for Describing Reproductive Development in Fishes

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Introduction

There are a number of terms and classifications used in the literature to describe reproductive condition in fishes, resulting in confusion and hindering communication among researchers in fish-related disciplines. This proliferation of terminology is often due to the needs of various disciplines to describe reproductive processes on either the macroscopic or microscopic level. For example, classification of ovarian condition has been based on methods such as 1) the macroscopic appearance of the gonad, 2) whole oocyte size, 3) the gonadosomatic index, and 4) histology (West 1990). Unfortunately, attempts to introduce standardization and consistency into reproductive classification (i.e., Hilge 1977, Bromley 2001) have met with limited to no success in the past, no doubt due to researcher's reluctance to adopt an unfamiliar nomenclature that may not be the best "fit" for the species of interest. Therefore, we suggest that rather than erecting a new classification system, communication among scientists studying fish reproductive biology can be improved by describing and naming the major phases within the fish reproductive cycle. Individual classification schemes can then be integrated into this framework of phases.

Methods

The terminology presented here was developed during discussions at the 3rd Workshop on Gonadal Histology of Fishes (New Orleans, USA, 2006) and has been further refined in relation to reproductive strategies defined by Murua and Saborido-Rey (2003). This terminology was developed using female marine teleosts, but is applicable to both sexes and all fishes. Three species with differing reproductive strategies (Atlantic herring *Clupea harengus*, a total spawner with determinate fecundity; Dover sole *Microstomus pacificus*, a batch spawner with determinate fecundity; spotted seatrout *Cynoscion nebulosus*, a batch spawner with indeterminate fecundity) are used to illustrate the phases of the terminology in females.

Results

A conceptual model of the terminology (Figure 1) demonstrates the major phases of the reproductive cycle. Once entering the cycle, all fishes proceed sequentially through the five phases. However, batch spawning fishes cycle between the spawning capable and spawning phases during the spawning season before progressing to the regressing phase. Sub-phases (such as early and late developing in the developing phase; imminent, active, recent and

embryonic development in the spawning phase; and sex transition and skip spawning in the regenerating phase) can be added to further describe important, species-specific reproductive events in each phase.

Reproductive phases in female teleosts

Immature: Macroscopically, ovaries are small, often clear, with indistinct blood vessels. Microscopically, only oogonia and primary growth oocytes present, with no atresia.

Developing: Macroscopically, ovaries are enlarging with blood vessels and some oocytes visible. Microscopically, oocytes in primary growth, cortical alveolar, and early vitellogenesis (early secondary growth) stages are present. No postovulatory follicles (POF) present. Some late vitellogenic oocytes (late secondary growth) can be present in the late developing sub-phase.

Spawning Capable (Figure 2A, B, C): Macroscopically, ovaries are large, blood vessels are prominent, oocytes are visible. Microscopically, all stages of vitellogenic oocytes can be present, with late secondary growth and full-grown oocytes predominant. POF and atresia can be present.

Spawning: Macroscopically, ovaries large (often maximum size), oocytes are visible and blood vessels are prominent. Microscopically, histological evidence of ovulation or recent/imminent spawning in the form of oocyte maturation (GVM), hydrated oocytes, or recent ($\leq$ 12 h old) POF. Atresia can be present.

Regressing: Macroscopically, ovaries are flaccid and blood vessels prominent. Microscopically, all stages of atresia are present. Some vitellogenic oocytes and POF can be present.

Regenerating: Macroscopically, ovaries are small and blood vessels are reduced. Microscopically, only oogonia and primary growth oocytes are present. Muscle bundles, late-stage atresia, and thick ovarian walls are also common.

Reproductive phases in male teleosts

Immature: Macroscopically, testes are thread-like, often clear. Microscopically, only primary spermatogonia are present, with no lumen in the lobules.

Developing: Macroscopically, testes are small, usually white. Microscopically, spermatocysts containing spermatogonia, spermatocytes, spermatids and some spermatozoa are present. No spermatozoa are present in the lumen of the lobules or sperm ducts.

Spawning Capable and Spawning: Macroscopically, testes are large and firm. Milt can be released with gentle pressure only in the Spawning phase. Microscopically, these two phases are indistinguishable. All stages of spermatogenesis can be present in the spermatocysts, and spermatozoa are present in the lumen of the lobules and in sperm ducts. Three sub-phases are defined based on the extent of the germinal epithelium (GE): early (Figure 2D), with a continuous GE throughout, mid (Figure 2E) with a continuous GE near the periphery and a discontinuous GE near the ducts, and late (Figure 2F), with a discontinuous GE throughout.

Regressing: Macroscopically, testes are small, flaccid and milt cannot be expressed. Microscopically, residual spermatozoa and widely scattered spermatocysts are present; spermatogonia nay occur in periphery.

Regenerating: Macroscopically, testes are small and thin. Microscopically, spermatogonial proliferation occurs throughout and no spermatocysts are present. Some residual spermatozoa can be present. Lumen of lobules is small if present.

Discussion

Terminology for hermaphroditic fishes (protandrous, protogynous) is the same as that described above for both the male and female phases, with the exception of the addition of a sex transition sub-phase to the phase in which sexual transition occurs; for most species, this is the regenerating phase. Terminology for simultaneous hermaphrodites follows that of female teleosts; development of the male portion of the gonad can be added in species-specific descriptions.

Terminology for live-bearing fishes (teleosts and elasmobranchs) is as described above, with the exception that fishes carrying embryos are considered to be in the spawning phase regardless of ovarian development. A series of spawning sub-phases describing embryonic development has been defined as ovulation/fertilization, early-celled embryo, optic vesicle formation, retinal pigmentation, and yolk depletion. Species exhibiting multiple stages of embryo development may require species-specific modification of these sub-phases.

The reproductive phases terminology can be adapted to all species and all reproductive strategies through the addition of sub-phases to describe species-specific reproductive events or the elimination of a phase when necessary (see Serra-Pereira 2009). Adoption of this terminology by fish biologists will simplify descriptions of reproductive biology and greatly facilitate comparisons of reproduction among species.

A manuscript based on this presentation has been submitted to the journal Marine and Coastal Fisheries as part of the special module "Emerging Issues and Methodological Advances in Fisheries Reproductive Biology". Publication is anticipated in fall 2010. Web page: www.fisheries.org/mcf.

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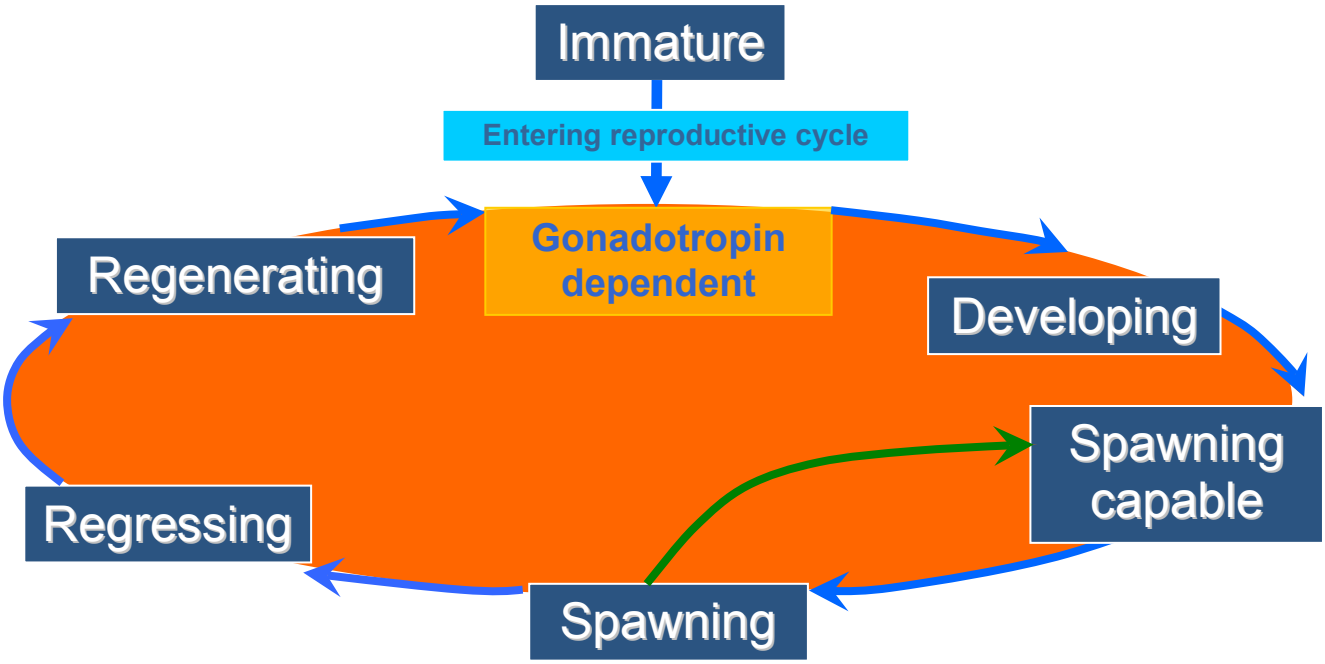

Figure 1. Conceptual model of reproductive phases terminology. Batch spawning species cycle between the spawning capable and spawning phases throughout the spawning season (green arrow).

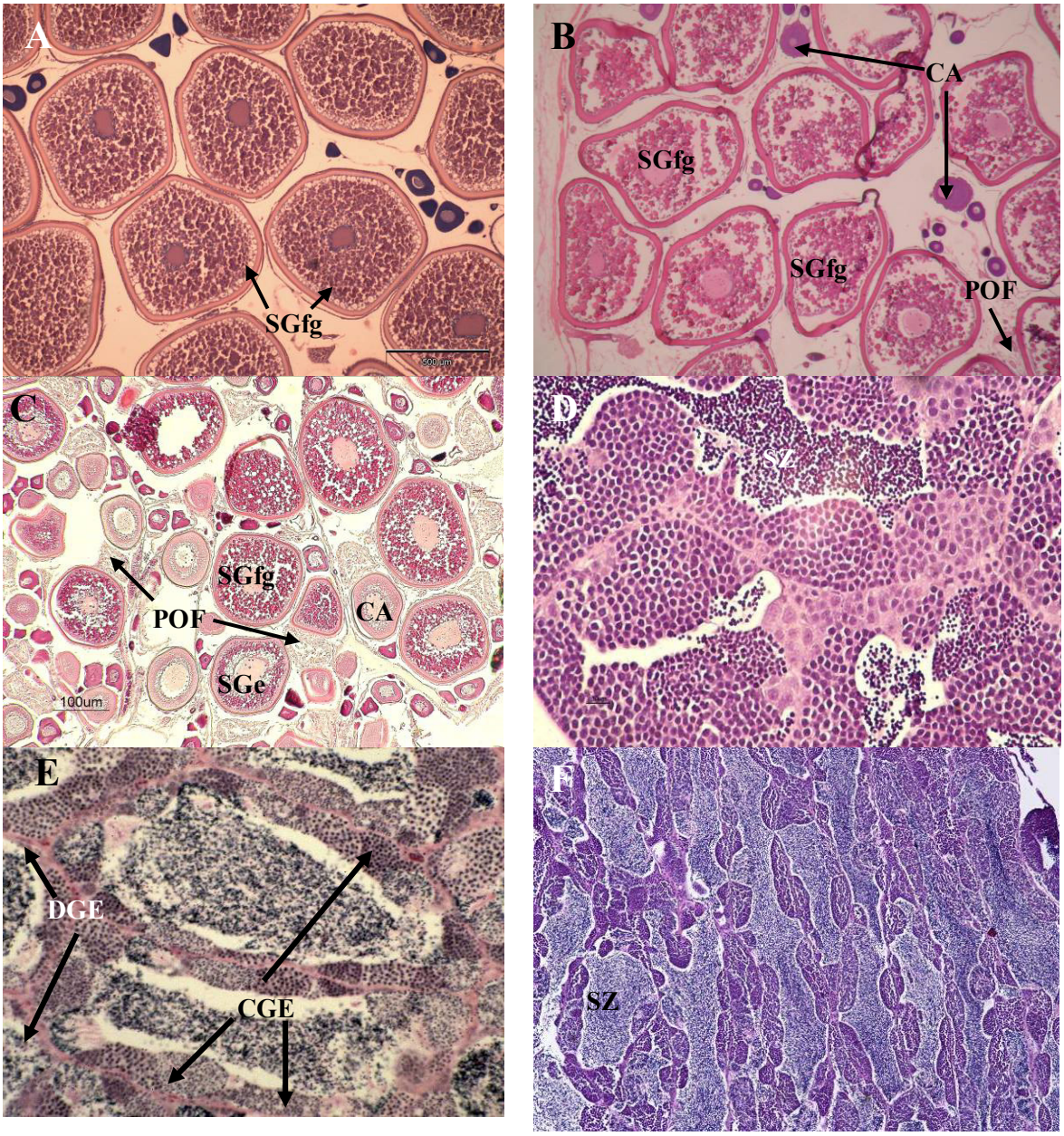

Figure 2. Histological photographs of female and male teleosts in the spawning capable phase. A. female *Clupea harengus*, a total spawner with determinate fecundity; B. female *Microstomus pacificus*, a batch spawner with determinate fecundity; C. female *Cynoscion nebulosus*, a batch spawner with indeterminate fecundity; D. male *Pimephalus promelas* in the early sub-phase; E. male *Rachycentron canadum* in the mid sub-phase; F. male *Archosargus probatocephalus* in the late sub-phase. CA—cortical alveolar oocyte; CGE—continuous germinal epithelium; DGE—discontinuous germinal epithelium; POF—post ovulatory follicle; SGe—secondary growth early vitellogenesis; SGfg—secondary growth full-grown oocyte; SZ—spermatozoa. Oocyte terminology follows Grier et al. 2009.

Standardisation of Methods and Terminology Applied in the Determination Maturity of Gadoids and other Marine Fish Species in the Northeast Atlantic

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The decline of many common fish stocks has urged the need for reliable data on fish reproduction capacity to establish a sustainable management of marine living resources. The need for standardised sampling methods and processing of samples as well as their interpretation has been to focus of a series of Workshops organised by The International Council for Exploration of the Sea (ICES) and promoted by sampling programs directed by the European Commission. These international workshops address a range of commercially exploited marine fish species. This presentation illustrates and discusses a workshop with focus on the reproduction of four gadoids species: cod, whiting, haddock and saithe. The participants examined histological preparations of own samples that had been collected on research surveys and macroscopically staged according to national routines. The perception and interpretation of macroscopic gonadal traits were compared to histological criteria using photographs of fresh gonads and processed gonadal tissue. The first macroscopic judgement of maturity deviated considerably from the following histological determination. A common terminology and classification scheme was developed to standardize maturity determination for stock assessment purposes and illustrated manuals were elaborated for each of the four species. The manuals include species specific descriptions and illustrations of macroscopic and microscopic characteristics to enhance consistence in judgements.

The classification scheme addresses the terminology associated with reproductive strategies and recommends that the estimates of the spawning proportion of the stock replaces a maturity ogive in ICES fish stock assessment. Skipped spawning and abnormalities such as intersex are identified as potential ecosystem status indicators to assist in management of marine fish stocks.

An Improved Description of the Development of Oogonia into Eggs

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Introduction

Understanding events during fish reproduction and life history is critical. However, numerous schemas are in use for describing egg development of pelagic fishes, most of which are classified within Perciformes. The commercial value of perciform fishes has resulted in fisheries science focusing primarily on the protection of fish stocks. Technological advances resulting in better information regarding ultrastructure, systematics, molecular biology, as well as molecular genetics makes possible the reduction of the variable terminology in current descriptive use. As new information is generated, especially regarding evolutionarily conserved processes of oocyte development, the classic nomenclature should be revised (Grier et al. 2009) in order to achieve greater clarity of expression, ultimately resulting in better understanding of reproductive events.

Methods

Ovaries from two species of saltwater fish, the common snook *Centropomus undecimalis* and red drum *Sciaenops ocellatus* were fixed in 5% phosphate-buffered formalin or Trump's phosphate-buffered formalin and glutaraldehyde. After fixation, they were embedded in glycol methacrylate and sectioned at thicknesses of 4 and 6 µm. Sections were mounted on acid-cleaned glass slides and stained with hematoxylin and eosin and periodic acid Schiff/metanil yellow/Weigert's hematoxylin.

Results and Discussion

The stages of development of fish oocytes were divided into previtellogenesis and vitellogenesis (Patiño and Sullivan 2002). However, with new morphological information, pelagic fish egg development has now been divided into six stages (Grier et al. 2009): oogonial proliferation (OP), chromatin nucleolus (CN), primary growth (previtellogenesis; PG), secondary growth (vitellogenesis; SG), oocyte maturation (OM), and ovulation (OV) (Table 1).

Oogonia, located within the germinal epithelium on the surface of ovarian lamellae, proliferate and then enter meiosis to become oocytes. This is the start of the chromatin nucleolus stage, consisting of four steps: leptotene, zygotene, pachytene, and early diplotene. During this last step—now recognized as part of oocyte development (Grier et al. 2009)—lampbrush chromosomes form and the germinal vesicle's nucleolus becomes prominent due to its production of ribosomal ribonucleic acid (rRNA). Meiotic arrest ensues in late diplotene, when the ooplasm also becomes basophilic at the initiation of primary oocyte growth.

Primary growth is divided into five steps: one nucleolus, multiple nucleoli, perinucleolar, oil droplets, and cortical alveolar. During this stage, the oocyte increases in size and cellular organelles are produced in preparation for secondary growth or vitellogenesis, which includes the receptor-mediated uptake of vitellogenin (synthesized in the liver) and its subsequent processing into yolk globules.

Secondary growth is divided into three steps: early vitellogenesis (only small yolk globules), late vitellogenesis (some maximum-sized yolk globules), and full-grown when the maximum oocyte size is reached for a given species. Secondary growth oocytes are opaque. Full-grown oocytes can enter oocyte maturation that includes ooplasmic and germinal vesicle changes leading to the resumption of meiosis.

Maturation has four steps, beginning with the eccentric germinal vesicle step when oil droplets coalesce and become oil globules, eventually fusing into a single oil globule. Oil globules displace the germinal vesicle to an eccentric position in the oocyte. This is followed by germinal vesicle migration, occurring coincidentally with yolk globule coalescence due to breakdown of yolk and resultant water uptake by the oocyte (Patiño and Sullivan 2002). The yolk clears as it becomes a pale yellow fluid. Germinal vesicle breakdown precedes the resumption of meiosis when the first meiotic division is completed. Meiosis then arrests again in metaphase of the second meiotic division when the oocyte is preovulatory and has a totally clear ooplasm.

The final stage of oocyte development is ovulation, at which time the oocyte leaves the follicle in which it developed, moving into the ovarian lumen to become an egg. Due to the uptake of water during maturation when yolk is clearing, the oocyte becomes less dense than saltwater; it becomes buoyant, a character of pelagic eggs.

All of these stages and steps of oocyte development were delineated in this study with common snook and red drum. One apparent difference was that basophilic ooplasm extended as thin bands through the fluid yolk of preovulatory oocytes in common snook whereas it was restricted to the oocyte periphery in red drum. Results of this study are schematically represented in Figure 1. In this development schema, upper case letters were used to designate stages and lower case letters to represent the steps, or the divisions within each stage (Table 1), instead of numbers as is common. These steps can be adapted and modified to suit the description of oocyte development in other saltwater Perciformes species, and also in non-pelagic fish species, such as the freshwater Ostariophysi (Quagio-Grassiotto 2009), a taxonomic group whose oocytes lack oil droplets and eggs lack oil globules. Therefore, this staging scheme is easily modified, highly adaptable, and can be widely applicable in describing oocyte development in any fish species.

Acknowledgements
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Table 1. An updated, comprehensive schema delineating oocyte development in fish. Oocyte development is divided into periods, stages, and steps. Periods are common to all vertebrates, while stages are common to fish and vertebrates that have yolked eggs. Steps are specific to fish that produce pelagic eggs, but can be modified to describe processes in other species. Letters, rather than numbers, are used at the stage level, and codes for both stages and steps are indicated by use of upper case letters for the stage, and lower case letters for the step. The nuclear (germinal vesicle) event steps are listed first during oocyte maturation (OM), as they are conserved among species. The ooplasmic events are shown in italics, as they vary among pelagic and non-pelagic fish. From Grier et al. 2009.

PERIODS	MITOSIS	ACTIVE MEIOSIS I	ARRESTED MEIOSIS IN LATE DIPLOTENE OF THE PROPHASE I												ACTIVE MEIOSIS II & 2ND ARREST IN METAPHASE II
STAGES	OOGONIA PROLIFERATE	CHROMATIN NUCLEOLUS (CN)				PRIMARY GROWTH: (PG)					SECONDARY GROWTH: VITELLOGENESIS OR YOLKED OOCYTES (SG)			OOCYTE MATURATION (OM)				OVULATION (OV)
STEPS	FREQUENTLY FORM CELL NESTS	LEPTOTENE	ZYGOTENE	PACHYTENE	EARLY DIPLOTENE	ONE-NUCLEOLUS	MULTIPLE NUCLEOLI	PERINUCLEOLAR	CIRCUMNUCLEAR OIL DROPLETS	CORTICAL ALVEOLAR	EARLY SECONDARY GROWTH OR EARLY YOLKED OOCYTES	LATE SECONDARY GROWTH OR LATE YOLKED OOCYTES	FULL-GROWN OOCYTE	ECCENTRIC GERMINAL VESICLE. *OIL DROPLETS COALESCE BECOMING ONE GLOBULE*	GERMINAL VESICLE MIGRATTION TO THE ANIMAL POLE. *OOCYTE HYDRATES*	GERMINAL VESICLE BREAKDOWN. *OOCYTE HYDRATION ALMOST COMPLETE*	MEIOSIS RESUMES. 2ND ARREST. *OOCYTE HYDRATION COMPLETE*	OOCYTE EMERGES FROM THE FOLLICLE, BECOMES AN EGG
CODE	OP	CNl	CNz	CNp	CNed	PGon	PGmn	PGpn	PGod	PGca	SGe	SGl	SGfg	OMegv	OMgvm	OMgvb	OMmr	OV

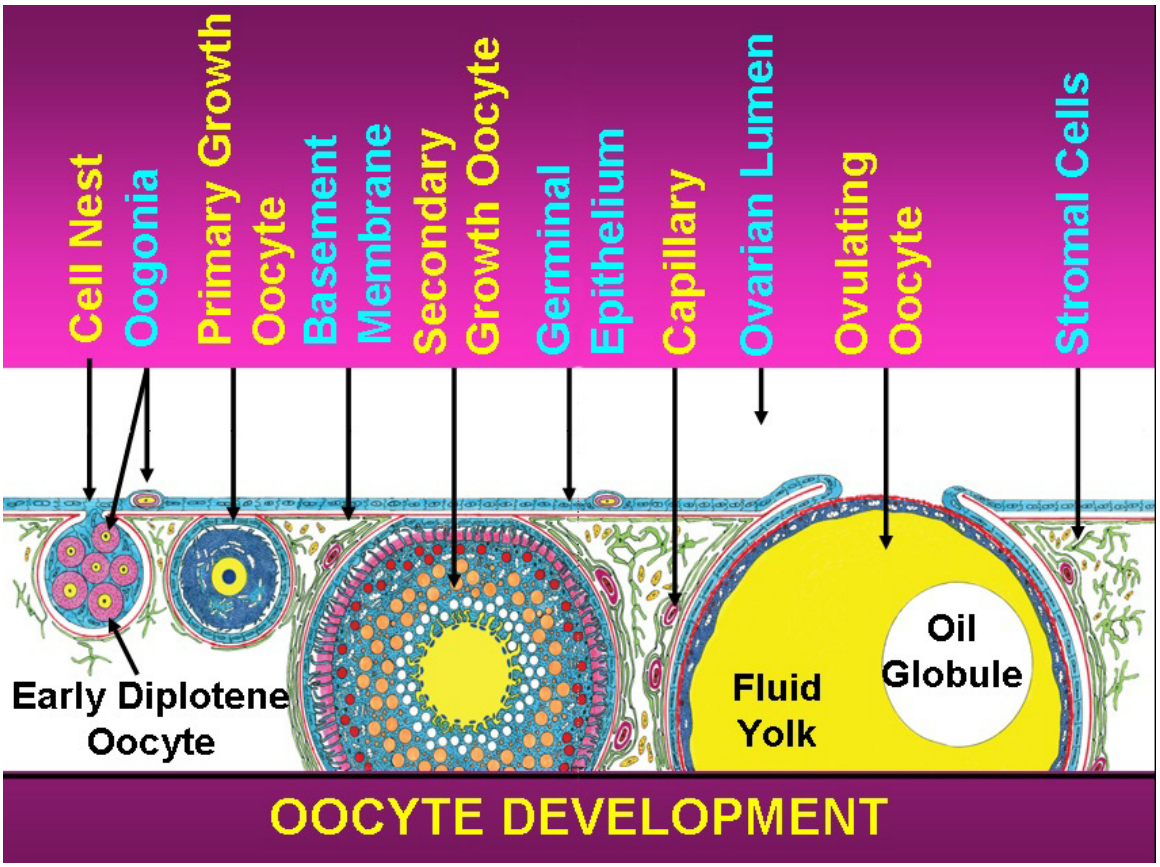

Figure 1. A schematic drawing illustrating oocyte development in fish that produce pelagic eggs. Oogonia are scattered among epithelial cells in the germinal epithelium represented in blue. When these cells become associated with oogonia that are initiating meiosis or within a cell nest, along with early diplotene oocytes, they are referred to as prefollicle cells. As development proceeds among cells, basement membranes (in red) increasingly surround individual germ cells to produce a follicle in which a primary growth oocyte is observed with a single nucleolus within the yellow germinal vesicle. Multiple nucleoli are observed in the secondary growth oocyte's germinal vesicle, along with numerous yolk globules (tan) and peripheral cortical alveoli (red). The oocyte is surrounded by a pink zona pellucida outside of which are blue follicle cells that were derived from epithelial cells in the germinal epithelium. In secondary growth oocytes, the follicle is composed of the oocyte, zona pellucida and follicle cells. It is surrounded by a red basement membrane and green theca with capillaries. An ovulating oocyte has yellow fluid yolk and a single oil globule. The zona pellucida is red and forms the outer, acellular layer of the egg. At ovulation, the follicle cells become joined to the epithelial cells of the germinal epithelium, as does the follicle basement membrane.

Freshwater Ostariophysi: From Oogonia to Eggs

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Introduction

Most of the knowledge on oocyte development in fish comes from higher Teleostei, primarily a few marine Perciformes (see Le Menn et al. 2007 and Grier et al. 2009 for reviews). The structural aspects of folliculogenesis and most of oogenesis appear to be constants throughout oocyte development. However, studies regarding the freshwater Ostariophysi differ significantly in specific details from the higher fish. The Ostariophysi is an assemblage of basal Teleostei comprising the Cypriniformes, Siluriformes, Characiformes, and Gymnotiformes.

Material and Methods

In order to make comparisons to marine perciform oocyte development, spawning females of the Characiformes, *Gymnocorymbus ternetzi*, from a fish farm in Florida, USA, were studied. In addition, females of the Characiformes *Serrasalmus spilopleura* and *Schizodon nasutus*, the Siluriformes *Pimelodus maculatus* and *Sorubim lima*, and the Gymnotiformes *Gymnotus cf. anguillaris* were collected during their annual reproductive cycles in the Paraná-Paraguay Basin, Brasil. Ovaries of these species were fixed in Trumps´s or Karnovsky's solutions. The material was embedded in historesin and sections were submitted to different staining methods. Post-ovulated and fertilized oocytes of the Cypriniformes *Cyprinus carpio* and *Hypophthalmichthys nobilis*, from a fish farm in São Paulo State, Brazil, were also studied. Oocyte development in ostariophysian fish ovaries was described and compared to the schedule of oocyte development of the saltwater Perciformes as proposed by Grier et al. (2009).

Results and Discussion

Development of freshwater ostariophysian fish eggs, as occurs in saltwater perciform fish eggs, begins when oogonia (Figure 1A), located within the germinal epithelium on the surface of ovarian lamellae, enter meiosis and become oocytes. Associated epithelial cells (Figure 1A) become prefollicle cells (Figure 1B), and the chromatin nucleolus stage (Figure 1B) commences during which there are four steps of meiosis: leptotene, zygotene, pachytene, and early diplotene when lampbrush chromosomes form and the nucleolus becomes prominent, producing ribosomal ribonucleic acid (rRNA) (Figures 1C-D). As in perciform fish, meiotic arrest ensues in late diplotene when basophilic ooplasm is observed histologically, designating primary growth that is divided into four steps: one nucleolus (Figure 1E), multiple nucleoli (Figure 1F), perinucleolar (Figure 1G), and cortical alveolar (Figure 1H), one less step than occurs in Perciformes since ostariophysan oocytes lack oil droplets. As in Perciformes, the "cellular machinery" is produced during primary growth that prepares an oocyte for secondary growth or vitellogenesis.

Most oocyte growth occurs during vitellogenesis as yolk globules fill the ooplasm. Secondary growth in ostariophysian oocytes follows the perciform pattern, being divided into three steps: early vitellogenesis with small yolk globules (Figure 2A), late vitellogenesis with some large yolk globules (Figure 2B), and full-grown (Figure 2C). Cortical alveoli formation**,** initiated at the end of primary growth, continues during early vitellogenesis (Figure 1I). Full-grown oocytes enter oocyte maturation, which includes the cytoplasmic and nuclear events leading to the resumption of meiosis. In ostariophysans, as in perciforms, maturation has four steps: eccentric germinal vesicle (Figure 2D), germinal vesicle migration (Figure 2E-F), which is coincident with yolk globules becoming fluid in some species but not all, germinal vesicle breakdown, and the final maturation step is the resumption meiosis. Ostariophysian oocytes lack an oil globule, unlike perciforms. Yolk globule fusion is partial in the species observed (Figure 2G-H) and does not occur prior to ovulation in some species. During the final stage of oocyte development, ovulation, the oocyte emerges from the follicle and enters the ovarian lumen, becoming an egg (Figure 2H). In ostariophysians, water uptake and liquefaction of yolk may occur post-spawning (Figure 2I) or be a post-fertilization event (Figure 2J).

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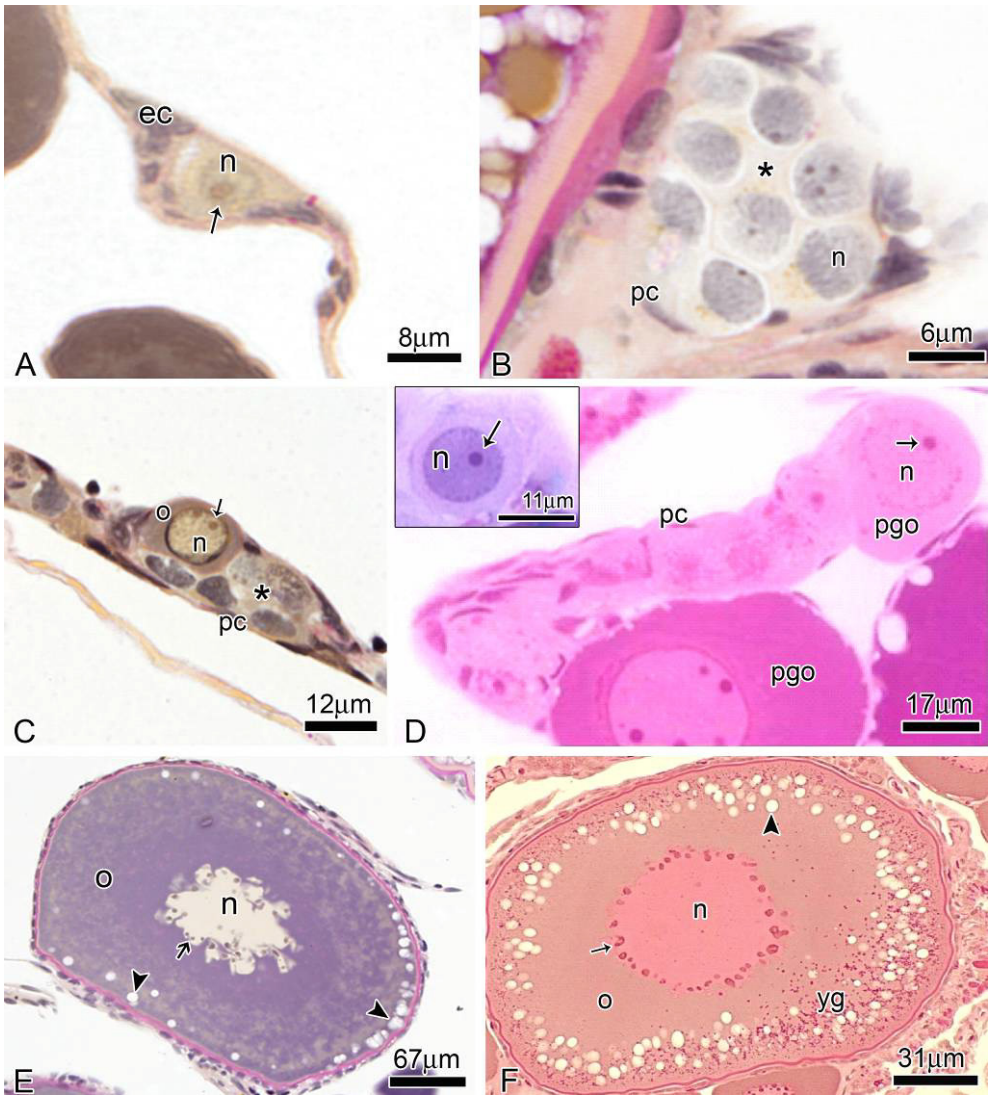

Figure 1. Oocyte growth and development in ostariophysian fishes. **A**. *Gymnocorymbus ternetzi*. Oogonia. **B**. *G. ternetzi*. Oocyte in chromatin nucleolus stage. **C**. *G. ternetzi*. Oocyte in early diplotene step of meiosis. **D**. *Schizodon nasutus*. Individualized oocyte. Inset: S*orubim lima*. Oocyte in primary growth: one nucleolus step. **E**. *G. ternetzi*. Oocyte in primary growth: cortical alveolar step. **F**. *G. ternetzi*. Oocyte in secondary growth: Cortical alveoli formation continues during early vitellogenesis step. ec - epithelial cells; n - nucleus; o – ooplasm; pc - prefollicle cells; pgo - primary growth oocyte; yg - yolk globule; arrow- nucleolus; arrowhead - cortical alveoli; asterisk - nest of oocytes.

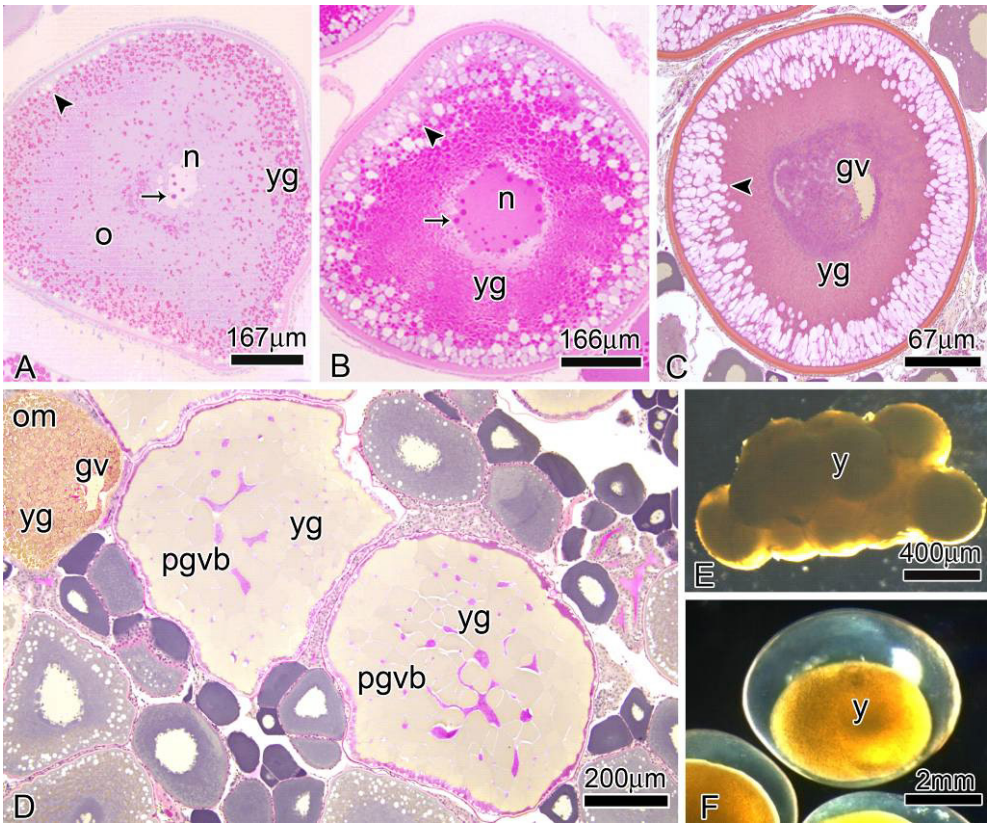

Figure 2. Secondary growth and oocyte maturation in Ostariophysian fishes. **A.** *Pimelodus maculatus*. Oocyte in secondary growth: early vitellogenesis step. **B**. *Serrasalmus spilopleura*. Oocyte in secondary growth: late vitellogenesis step. **C.** *Gymnotus cf. anguillaris*. Oocyte in maturation: germinal vesicle migration step. **D.** *Gymnocorymbus ternetzi*. Oocyte in maturation (om): prior to germinal vesicle breakdown step, and oocyte after germinal vesicle breakdown step (pgvb): partial yolk globule fusion. **E.** *Cyprinus carpio*. Post-spwning demersal adhesive eggs: yolk not fluid and hydration is discrete. **F.** *Hypophthalmichthys nobilis.* Post-fertilization pelagic eggs: yolk not fluid and high hydration. gv - germinal vesicle; n – nucleus; o – ooplasm; om - oocyte in maturation; pgvb - post germinal vesicle breakdown; y – yolk; yg - yolk globule; arrow – nucleolus; arrowhead - cortical alveoli.

Spermatogonial Stem Cell Candidates and their Niche in Zebrafish Testes (*Danio rerio*).

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The spermatogonial stem cell (SSC) has been defined as a subtype of spermatogonia capable of "infinite" self-renewal or differentiation. The balance between these two paths is regulated by the cellular and molecular composition of the microenvironment in which SSCs reside (niche). Since many aspects related to SSC biology are unknown in fish, it is our aim to establish zebrafish as a model for respective studies. Morphological analysis revealed two subtypes of single, undifferentiated type A spermatogonia: A_{und} and the suspected stem cell A_{und*}. Both subtypes show a large nucleus, poorly condensed chromatin, and 1 or 2 nucleoli, but A_{und*} has an irregularly shaped, A_{und} a smooth nuclear envelope. It is not clear at present if the two types are separated by a cell cycle. Analysis of BrdU (5-bromo-2'-deoxyuridine) incorporation demonstrated that a cell cycle of undifferentiated type A spermatogonia takes at least 10 hours. To characterize the SSC niche, we traced BrdU-retaining cells after 4 weeks of chase. We found that ~9% of A_{und*} were BrdU-positive. Intriguingly, 77% of them were situated near the interstitial compartment, but only 18% or 5% close to the intertubular area or the testicular capsule, respectively. Finally, we performed transplantation assays using as donors wild-type or *vasa::egfp* transgenic animals, and as recipient wild-type animals treated with busulfan to deplete endogenous spermatogenesis. Donor spermatogonia were enriched by density gradient centrifugation and subsequently stained with PKH26 (wild-type), and by FACS (*vasa::egfp* animals). In both cases, labeled donor germ cells were found in the recipient's testes 1 or 2 weeks post-transplantation. This indicates the presence of SSC and their capability to colonize a recipient's testes. In summary, we have taken steps towards the characterization of suspected SSC as a means to study the regulation of SSC physiology in zebrafish.

Staging Haddock (*Melanogrammus aeglefinus* L.) Ovaries: Implications for Maturity Indices, Estimation of Daily Spawning Timing, and Field Sampling Practices

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Introduction

The value of accurately determining fish reproductive maturity in fisheries surveys is often overlooked in the assessment and management process. To our knowledge there are no studies assessing the reproductive maturity staging of haddock, *Melanogrammus aeglefinu*s, from the Gulf of Maine or Georges Bank, an impediment to effective management. The purpose of this study was to develop a maturation index for female *M. aeglefinus* to improve collection of reproductive stage data in the field and to revise this stage index based on a comparison with a laboratory histological staging method.

Methods

Biological samples of *M. aeglefinu*s from the southwestern Gulf of Maine were collected in spring of 2006 (n = 15) and 2007 (n = 10). Fifty fathoms of longline was set and hauled 3 times in the same location twice during a 24 hour period. Samples from female *M. aeglefinu*s representative of all cohorts and reproductive stages from each longline haul were labeled and reserved on ice for later identification. To determine the accuracy of macroscopic maturity staging using our maturation index, histological analysis was conducted on tissue samples of a subsample of 169 ovaries.

Results and Discussion

Comparison of macroscopic and microscopic observations of ovaries helped improve the field index and methodologies, and provided useful insight into the reproductive biology of M. aeglefinus. The findings from this study underscore the problems of developing an accurate field maturation stage index based on macroscopic observation. There was good agreement between field and histological methods except for 'spent' and 'resting' stages; 46 % of the field-staged resting ovaries were assigned as spent during histological analysis. This is due to observational error, compounded by the short sampling period. As the ovary regresses into resting, it became easier to differentiate from spent. For future studies, it is recommended that the sampling occur well before and after the known spawning season and photographs of each ovary be compared with histology results.

Contrary to previous work (Robb 1981), our findings suggest that M. aeglefinus ovaries do not develop homogenously during the earliest stages of OM, although homogeneity was achieved by later development. The histology-based laboratory staging method underestimated the H1 stage because the non-homogeneous ovary was not adequately

represented in the tissue samples. In addition, developing ovaries were overestimated in the field as a result of this non-homogeneous stage of the ovary. We therefore recommend taking larger or multiple tissue samples to be used in histological examination in future studies. Noting the apparent longevity of POFs helped us understand the duration and cyclical process of OM in this species and potentially in other boreal or cold water fishes. Because reproductive maturation occurred over a prolonged period of time, OM occurred throughout three distinct field and histology stages (H1, H2, and H3). These findings support the conclusion that M. aeglefinus exhibits asynchronous maturation of individual groups of oocytes.

Criteria for staging in revised field index

Immature (I) Figures 1A, B

Macroscopic: Ovaries small, firm, about 1/8 the volume of the body cavity. Membrane thin, transparent, grey to pink. Length of fish helpful in distinguishing between immature and resting.

Microscopic: The ovary contains oogonia, and primary oocytes that vary little in diameter. The ovary wall is thin.

Developing (D) Figures 2A,B

Macroscopic: Ovaries larger, plump, 1/3 to 1/2 length of the body cavity. Membrane reddish, yellow with numerous blood vessels. Opaque eggs, giving ovaries a granular appearance. No hydrated oocytes present.

Microscopic: Oocytes increasing in diameter with accumulation of yolk.

Hydration Stage 1 (H1) Figures 2C, D

Macroscopic: Ovaries well developed, reddish yellow, at least 2/3 length of body cavity. Membrane opaque with prominent blood vessels. Large translucent oocytes in <25% of ovary. In early H1 stage, the ovary is not homogenous throughout.

Microscopic: Predominance of oocytes in early oocyte maturation (OM), with very early yolk globule coalesence and the the beginning of oil droplet formation. Few hydrated oocytes. Postovulatory follicles (POFs) may be present.

Hydration Stage 2 (H2) Figures 2E, F

Macroscopic: Ovaries well developed, reddish yellow, at least 2/3 length of body cavity. Membrane opaque with blood vessels prominent. Large translucent oocytes in 25-50% of ovary.

Microscopic: Predominance of oocytes in intermediate stage of OM, with yolk coalescence, oil droplet completion and nuclear migration. Some oocytes hydrated. POFs may be present.

Hydration Stage 3 (H3) Figures 2G, H

Macroscopic: Ovaries well developed, reddish yellow, at least 2/3 volume of body cavity. Membrane opaque with blood vessels conspicuous. Large translucent oocytes in 50-75% of ovary. Eggs flow freely with light abdominal pressure.

Microscopic: Predominance of oocytes in last stages of OM, with yolk coalescence complete. POFs may be present.

Spent (S) Figures 1C, D

Macroscopic: Ovaries soft, flabby, 1/4 the volume of body cavity. Membrane thick,

tough, purplish, bloodshot. Few eggs remain, gonad has a patchy appearance. No oocytes in OM present.

Microscopic: Many POFs present. Germ cells and primary oocytes more evident.

Resting (R) Figures 1E, F

Macroscopic: Ovaries small and firm, 1/3 the volume of body cavity. Membrane thin but less transparent, yellowish-grey. Oocytes microscopic, opaque.

Microscopic: Ovary wall thick. Often late stage atresia. Ovary contains previtellogenic oocytes that vary in diameter.

Conclusion

It is anticipated that the revised field maturation staging index presented here will be useful to *M. aeglefinus* resource managers. Hydration stages H2 and H3 appear to be useful indicators of spawning readiness for *M. aeglefinu*s ovaries in the field. Although there is hope that this revised field index will improve accuracy in the determination of the maturity stage of *M. aeglefinus* in the field, evidence has shown that field indices alone may not be enough to correctly stage a fish. However, our observations also demonstrate that determining the maturation of an ovary based on histological examination alone may not always be accurate, highlighting the importance of field staging. It is recommend that in addition to field staging with the resulting index presented herein, appropriate histological samples should be collected and used to ground truth problematic stages, especially when field data are used in assessing and managing a fish stock.

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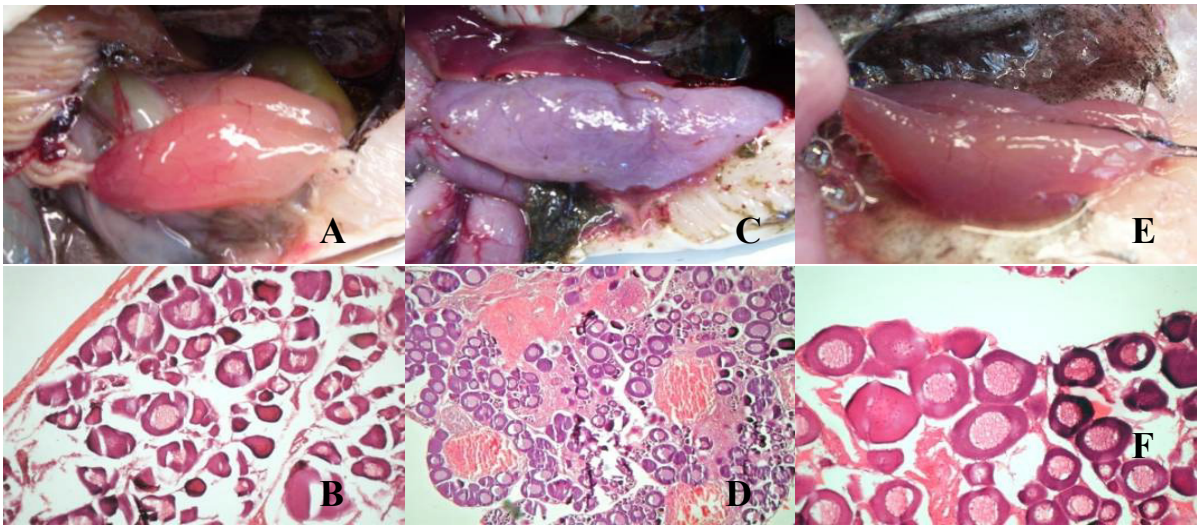

Figure 1. Histological photographs of reproductively inactive *Melanogrammus aeglefinus* ovaries. Photographs are combined with written descriptions for the final index. A. Immature, macroscopic view. B. Immature, microscopic view. C. Spent, macroscopic view. D. Spent, microscopic view. E. Resting, macroscopic view. F. Resting, microscopic view.

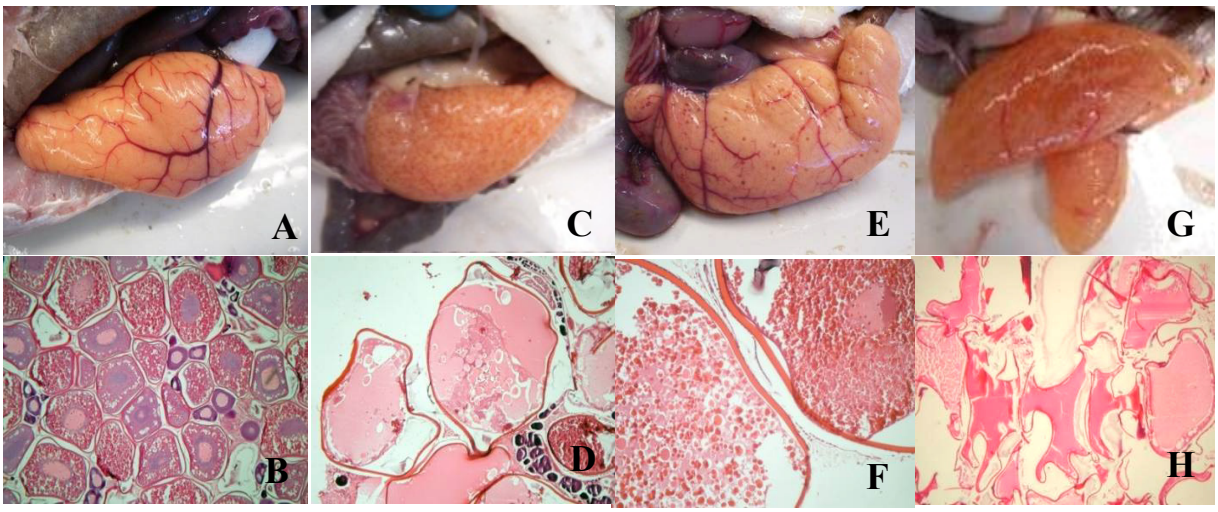

Figure 2. Histological photographs of reproductively active *Melanogrammus aeglefinus* ovaries. Photographs are combined with written descriptions for the final index. A. Developing, macroscopic view. B. Developing, microscopic view. C. Hydrated stage 1 (H1), macroscopic view. D. Hydrated stage 1 (H1), microscopic view. E. Hydrated stage 2 (H2), macroscopic view. F. Hydrated stage 2 (H2), microscopic view. G. Hydrated stage 3 (H3), macroscopic view. H. Hydrated stage 3 (H3), microscopic view.

Switching POCs For POFs

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Introduction

As the term is used in fisheries science, the postovulatory follicle (POF) consists of postovulatory follicle cells, derived from a germinal epithelium, a basement membrane that surrounded the follicle, and the postovulatory theca that is derived from ovarian stroma. In current usage, the "postovulatory follicle" describes a structure that is derived from two tissue compartments (epithelium and stroma), a fact that has not been previously considered. This usage is not consistent with the definition of an ovarian follicle that is composed of only an oocyte and surrounding follicle cells; the follicle is encompassed by a basement membrane and theca (Grier 2000; Grier and Lo Nostro 2000; Parenti and Grier 2004; Grier et al. 2009). Herein, post-ovulation ovaries from a saltwater perciform and a freshwater, basal characiform were compared regarding the morphology of postovulatory follicles using high-resolution light microscopy.

Methods

Within an hour of ovulation, ovaries from common snook *Centropomus undecimalis* (Perciformes) and black tetra *Gymnocorymbus ternetzi* (Ostariophysi: Characiformes) were fixed in Bouin's and Trump's fixatives, respectively. They were embedded in glycol methacrylate, sectioned at 4 or 6 µm and stained with either periodic acid Schiff and metanil yellow (common snook) or with the reticulin method (black tetra).

Results and Discussion

Periodic acid Schiff (PAS) stained basement membranes in common snook purple while the reticulin method stained basement membranes in black tetras black. Reticulin also stained basement membranes more reliably than did the PAS procedure and has great promise for future studies. After ovulation in both species of fish, the basement membrane that formerly surrounded the follicle is continuous with that supporting the germinal epithelium. Additionally, the postovulatory follicle cells become continuous with epithelial cells in the germinal epithelium, from which they were derived during folliculogenesis, and are separated from the postovulatory theca by a basement membrane (Figures 1, 2).

The germinal epithelium is an emerging constant among vertebrates (Parenti and Grier 2004). Germinal epithelia are homologous as are the ovarian follicles that are derived from them. An ovarian follicle is wholly derived from a germinal epithelium during folliculogenesis. In fish, this was first reported in common snook; a follicle is always separated from the theca by a basement membrane (Grier 2000). Neither the basement membrane nor the theca are part of a follicle (Grier 2000; Grier and Lo Nostro 2000; Grier et al. 2009) and should also not be part of a postovulatory follicle.

The postovulatory follicle, as is now evidenced in fish, has been redefined in light of new information regarding the germinal epithelium and the homology of gonad structure among vertebrates (Grier et al. 2009). The postovulatory follicle itself (POF) is composed of only the postovulatory follicle cells (Figures 1, 2). The redefined POF, the basement membrane and the theca are a postovulatory follicle complex (POC), a recently introduced term (Grier et al. 2009). The POC is derived from "follicle complex" (Grier 2000; Grier and Lo Nostro 2000) that is defined as a follicle (oocyte and follicle cells), basement membrane and theca. Following ovulation, a POC is a follicle complex without the ovulated oocyte, which has become an egg. Considering homology and the definition of a follicle, it is appropriate that the redefined POFs and newly defined POCs (Grier et al. 2009) should be incorporated into the fish literature in the future. In rainbow trout (Grier et al. 2004), the POC basement membrane was also continuous with that of the germinal epithelium as described here for black tetras and common snook. In trout, however, knowledge had not advanced to the point where POCs and POFs were defined. In rainbow trout, it was also shown that the germinal epithelium and everything derived from it (cell nests, follicles, and postovulatory follicles) are separated from the stroma by a basement membrane, and evidence to the contrary was not observed in either common snook or black tetras. Herein, specific examples are given for fish species in the orders Characiformes, Perciformes and Salmoniformes to support the redefinition of a POF to be part of a POC. The POCs of both the characiform black tetra and the perciform common snook are of identical morphology. Given new morphological awareness and knowledge, it is no longer appropriate to maintain the former usage of a POF that now has a more restricted definition. In the future, the term POC should be used for what has previously been termed a POF.

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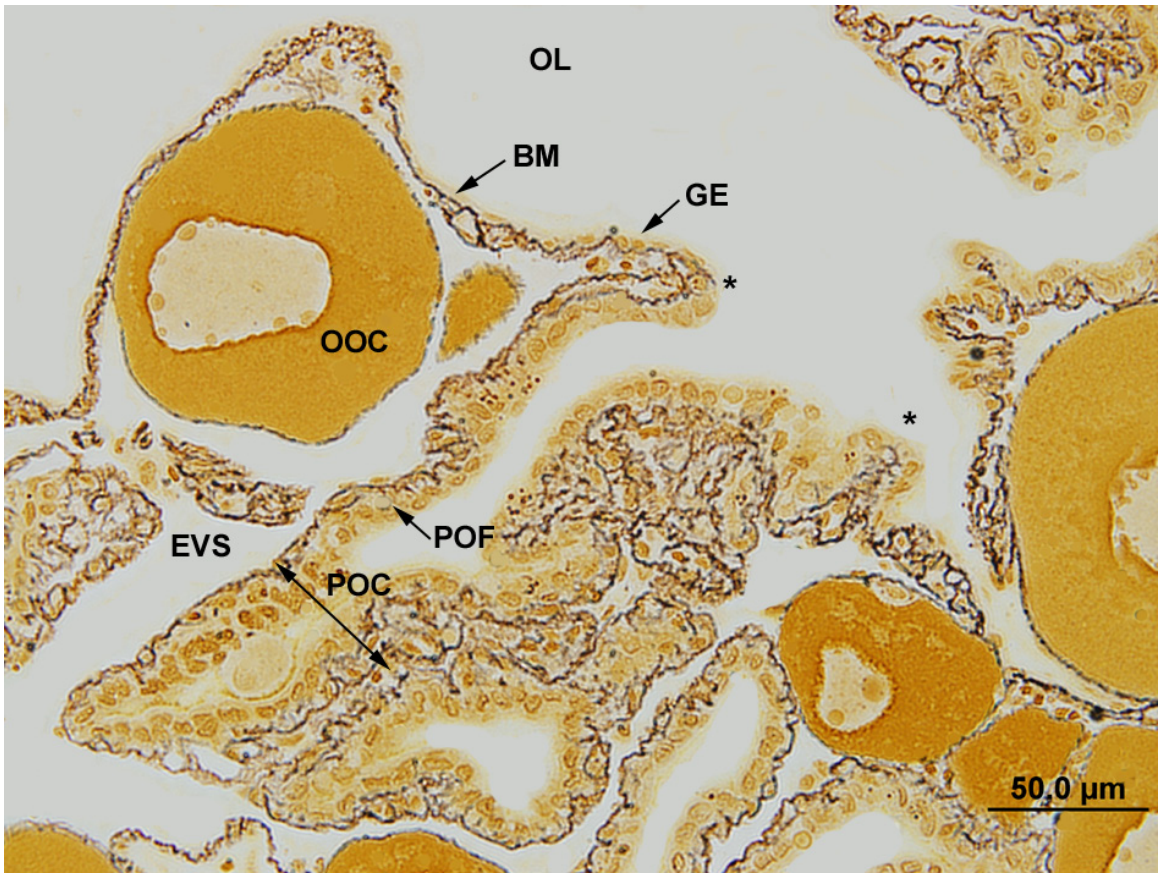

Figure 1. Postovulatory follicles from the black tetra stained with the reticulin stain that renders basement membranes (BM) black. The germinal epithelium (GE) rests upon the basement membrane and borders the ovarian lumen (OL). Asterisks designate where postovulatory follicle cells are continuous with epithelial cells of the germinal epithelium. The lumen enclosed by the postovulatory follicle (POF) was formerly occupied by an oocyte, and it is continuous with the ovarian lumen after ovulation. It can also be stated that the lumen enclosed by the POC is continuous with the ovarian lumen. The former thecal cells are poorly rendered in the POCs of the black tetra. EVS, extravascular space.

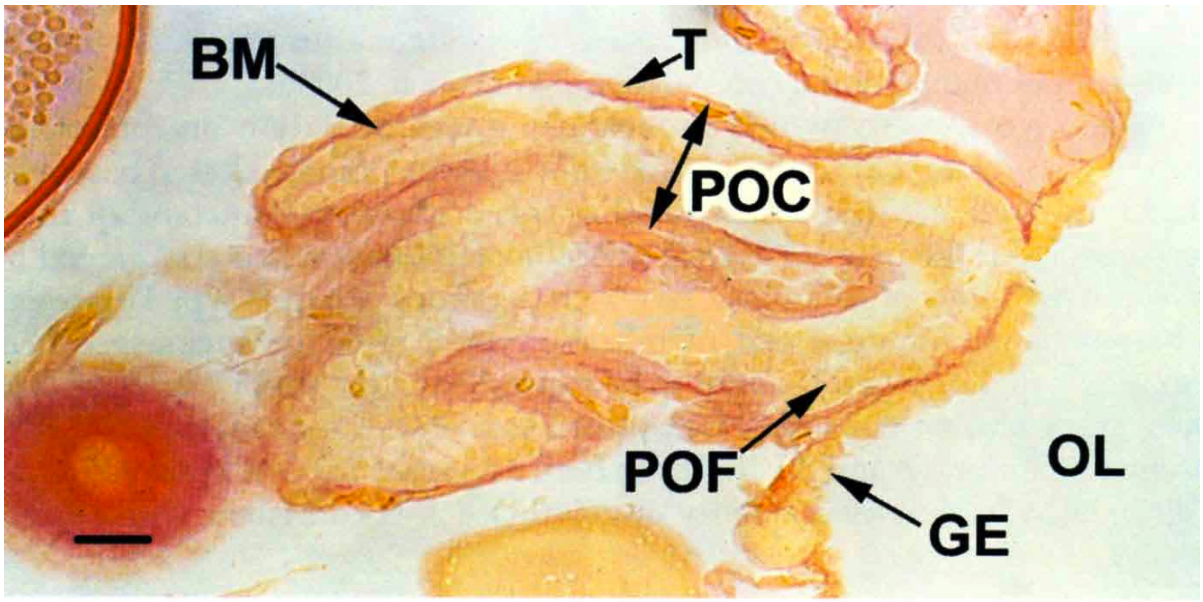

Figure 2. Common snook postovulatory follicle (POF) is composed of postovulatory follicle cells. A postovulatory follicle complex (POC) is composed of the POF, basement membrane and theca (T). The germinal epithelium (GE) and ovarian lumen (OL) are indicated. Note that cells of the POF become continuous with epithelial cells of the germinal epithelium. Bar = 10 µm. From Grier et al. 2009.

Comparative Analysis of the Results from Visual and Histological Estimation of Rockall Haddock (*Melanogrammus aeglefinus*) Maturity.

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In comparison to haddock from other areas, Rockall haddock are of a smaller size. The bulk of the population is made up of individuals 20-35 cm long, the portion of larger haddock is minor. Haddock of this area differ from other populations in terms of rates of growth and maturity.

As microscopic examination showed, in the period of trophoplasmatic growth, the oocytes of Rockall haddock had the same development phases as those ones of the other gadidae. At the beginning of prespawning stage, in the ovaries, the oocytes at different development phases may be observed simultaneously: the start of vacuolization and accumulation of yolk, intensive trophoplasmatic growth, the yolk filled oocyte. The post-spawning features could be observed in ovaries and testes until September. The residuals of post ovulatory follicles and spermatozoids serve as a reliable criterion to identify fish that had participated in the last spawning season. One more indication of completed spawning in females is the remnants of isolated large mature vitellogenic follicles and thickened ovary wall.

A comparative analysis of the results from visual and histological estimation of haddock maturity indicated that the ovary maturity stages were determined in sea conditions very accurately in prespawning and spawning periods when the gonads of mature and immature fish significantly differed in appearance. However, in the postspawning period, the results from field visual determination of haddock female gonad stage was prone to error compared to the histological staging.

The Rockall haddock population has an early rate of maturing. Visual examination of haddock gonads collected in the spawning period 2000-2006 showed that ML50=23.08 cm, according to histological examination ML50=23.02 cm. Almost all females (92.3%) with length 25 cm, and all males (100.0%) with length 24 cm and larger were mature. These data agree well with the results of recent Scottish research in compliance with which the majority of fish become mature at the age of 2 years.

Development of a Manual to Determine Gonadal Maturity of Herring (*Clupea harengus* L.)

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Gonads of herring (*Clupea harengus*) from the Baltic Sea were sampled during the period November to May for the purpose of elaborating an illustrated and histologically validated manual to determine gonadal maturity of female and male herring. Sampling was conducted on research cruises or caught on the spawning ground by fishermen. Female and male herring of different maturity were sampled. Ovaries and testes were weighed and photographed together with the individual fish and samples of oocytes from the fresh ovary tissue were photographed using light microscopy. Immediately after this, the gonads were preserved in formaldehyde for subsequent processing and histological examination in the laboratory. Based on microscopic characteristics of oocytes and spermatocytes in the histological sections, the ovaries and testes were categorized into 8 histological stages. A maturity scale including 8 developmental classes corresponding to the histological stages were then established and described using photographs of the gonads prior to preservation. An illustrated manual for visual determination of maturity was elaborated, describing the macroscopic characteristics of each stage. The manual also illustrates histological characteristics of ovaries and testes of different maturity stages and describe how light microscopy of fresh ovarian tissue can facilitate classification of maturity at sea or in the laboratory. The aim of the manual is to improve the quality of maturity data of herring, and thereby the basis for estimation of spawning stock biomass applied in stock assessment and management as an indicator of stock reproductive potential.

Evaluating Macroscopic Maturity Staging in *Centropristis striata* with Photographic Reassignment and Histology

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Abstract
Macroscopic assessment of gonads is a rapid and easy method for determining the sex and maturity stages of fish under field conditions, but is less accurate and precise than histology. We evaluate 6-stage and 4-stage macroscopic maturity staging keys developed for the protogynous hermaphrodite species black sea bass (Serranidae: *Centropristis striata*). Sampling was conducted along the coast of North Carolina, USA, through the entire spawning season. Fresh ovaries were macroscopically staged and photographed and specimens were later re-staged from photographs. Tissue samples were also preserved and analyzed histologically. Agreement between initial assignment and photographic reassignment was higher using a four stage key than a six stage key. Ripe and late developing stages were most consistent between macroscopic and histological staging. Most disagreements between macroscopic and histological staging occur among early developing, resting, and spent macroscopic stages. Use of a 4-stage macroscopic maturity key was more precise and accurate than a 6-stage key, though the poor identification of immature individuals was consistent. Many more samples have yet to be analyzed histologically in this ongoing project.

Introduction

In the study of reproductive biology we are often interested in estimating parameters such as length of spawning season, sex ratio, size at maturity and size at sex transition. Estimating these parameters usually involves the examination of gonads at the macroscopic and/or microscopic level. Histological methods allow for more precise maturity determination than macroscopic methods but are more complex and delay the availability of data (Tomkiewicz et al. 2003). Macroscopic staging can be accomplished in the field while histological staging requires samples to be collected, stored, preserved for several weeks and processed before analysis. The simplicity of macroscopic staging makes it so logistically preferable in many situations that it is likely to be practiced without prior validation. In such cases, sex and maturity data must be interpreted with caution. Ideally gross morphological characteristics used to determine sex and maturity stages should be species-specific and verified with histology. We present the progress of ongoing research to devise a species-specific macroscopic maturity key for female black sea bass (*Centropristis striata*) by comparing field assignment of gonads with photographic reassignment and histological staging.

Methods

Specimens were collected aboard a commercial trap-fishing vessel in Onslow Bay, North Carolina, at depths ranging from 13-34 m within an area of 350 square nautical miles. Samples were collected from February 15 to June 19 to encompass the known spawning season (McGovern et al. 2002). Fish were packed in ice overnight and sampled in the lab within 48 hours of capture. Gonads were photographed and preserved for histology analysis.

Sex and maturity stage was assigned to each specimen using a six stage maturity key modified from a general key used to macroscopically stage many fish species aboard NOAA Fisheries survey vessels (Burnett et al. 1989). Maturity of each specimen was later reassigned from photographs. The early developing, spent, and resting stages which appeared in the staging process to overlap, were combined into a single staged named inactive, to create a four stage key. A thin section of each specimen was mounted and stained with haematoxylin and eosin, and maturity was reassigned histologically. Independence of the maturity staging methods was analyzed with contingency tables (Zar 1999).

Results

Agreement between macroscopic staging of fresh gonads and photographs using a six stage key was strong for several stages (Table 1), though maturity stage was not independent of method (X^2 = 11.07, p = 0.002). When the six stage key was collapsed into four stages, agreement improved and maturity stage was independent of method (X^2 = 1.22, p = 0.748). Agreement between macroscopic staging of fresh gonads and histological staging was strong for several stages though immature, early developing and resting stages were in poor agreement (Table 2). Nonetheless maturity assignment was independent of method (X^2 = 7.27, p = 0.201). Histology confirmed that all specimens were correctly identified as females.

Discussion

The ability to reassign maturity to ovaries disassociated from accompanying morphological and temporal information, and to reexamine the entire range of maturity stages altogether, proved informative in evaluating the 6-stage maturity key. Of the six stages to which individuals were initially assigned, the early developing, spent and resting stages were found to be poorly defined and ambiguous for this species. Many fresh specimens classified as immature were reclassified from photographs as mature stages. In the staging of fresh ovaries, small fish are probably more likely to be classified as immature and bias the staging, because of fish size. Using a four stage maturity key removed a lot of disagreement among ambiguous stages, though misclassification of immature individuals remained a problem. Agreement between macroscopic staging of fresh gonads and histological staging was high for several stages but poor for others. Much of this disagreement would be removed by using a four stage key, though errors involving immature stages would still occur. Confirmation that specimens are being correctly classified as female is in itself an important success for macroscopic staging of this hermaphroditic species.

Conclusions

Reclassifying ovaries with photographs has allowed us to assess the repeatability of our maturity assignments and identify strengths and weaknesses of the staging key. Histological analysis has allowed us to assess the accuracy of macroscopic staging and identify stages that may need improved staging criteria. As we analyze more specimens histologically we will have a better understanding of the accuracy of our macroscopic staging keys. We will then be able to refer back to our library of gonad photographs to refine the staging criteria for inaccurate stages and develop an improved maturity key.

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Table 1. Agreement and error in restaging ovaries using a 6-stage key.

	I	**E**	**L**	**R**	**S**	**T**	***Total***
I	**17**	9				4	30
E	7	**64**	1			*24*	96
L		10	**73**	1		2	86
R			2	**16**			18
S		*5*	4		**4**	*4*	17
T	1	*4*			*1*	**14**	20
Total	25	92	80	17	5	48	267

NOTE: Rows represent initial macroscopic staging of fresh ovaries while columns represent restaging from digital images. Values in bold type in bordered cells indicate agreement between fresh and image-based staging. Values in grey italic type indicate misassignments that would not occur using a 4-stage key. I = Immature, E = Early Developing, L = Late Developing, R =Ripe, S = Spent, T = resting.

Table 2. Agreement and error in staging of ovaries between a 6-stage macroscopic key and a 6 stage histological key.

	I	**E**	**L**	**R**	**S**	**T**	***Total***
I	**3**	1					4
E		**1**	1				2
L			**18**				18
R				**9**			9
S					**4**	*2*	6
T	4	*5*			*1*	***3***	13
Total	7	7	19	9	5	5	52

NOTE: Columns represent macroscopic staging of fresh ovaries while rows represent histological staging. Values in bold type in bordered cells indicate agreement between macroscopic and histological staging. Values in grey italic type indicate misassignments that would not occur using a 4-stage key. I = Immature, E = Early Developing, L = Late Developing, R =Ripe, S = Spent, T = resting.

Oogenesis in the American Knifefish *Gymnotus cf. anguillaris* (Teleostei: Ostariophysi: Gymnotiformes)

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Introduction

Recent reports on the role of the germinal epithelium in ovarian follicle formation and on the functional events that drive oocyte development have provided a new understanding of folliculogenesis and oogenesis in fish (Patiño and Sullivan 2002; Le Menn et al. 2007; Grier et al. 2009). In a call for updating and standardizing the concepts and terminology utilized in studies on folliculogenesis and oogenesis in fish, Grier et al. (2009) are proposing a unifying terminology to be applied to fish oocyte development that may also apply to other vertebrates. However, most of the knowledge in this field comes from higher Teleostei, primarily a few marine Perciformes with an exception being one freshwater salmoniform, the rainbow trout (Grier et al. 2007). Cellular and structural aspects of folliculogenesis are constants between these species, but there are some differences between oocyte growth and maturation when marine Perciformes and Salmoniformes are compared. Studies regarding the freshwater Ostariophysi have also shown some differences in oocyte growth and maturation when compared to marine Perciformes. The Ostariophysi is an assemblage of basal Teleostei comprising the Cypriniformes, Siluriformes, Characiformes, and Gymnotiformes. Herein, we review follicle formation and oocyte development in Gymnotiformes in order to make comparisons to marine perciform oocyte development.

Material and Methods

Females of the Gymnotiformes, *Gymnotus cf. anguillaris*, were collected during their annual reproductive cycles in the Rio Bonito – Botucatu/São Paulo. Their ovaries were fixed in Karnovsky's solution. For light microscopy, the material was embedded in historesin and sections were submitted to staining method PAS+Hematoxilin+Metanil Yellow (MY). Oocyte development was compared to the saltwater perciform, the red drum, *Sciaenops ocellatus*.

Results and Discussion

The description of oogenesis in a basal ostariophysan fish, *G. cf. anguillaris*, confirms a new vision of ovarian follicle formation. Oocytes are contained within nests during the initial phases of meiotic prophase I where they advance to diplotene when meiosis is arrested. The oocytes enter primary growth and are subsequently individualized by prefollicle cell processes prior to the completion of folliculogenesis. The prefollicle cells are derived from epithelial cells in the germinal epithelium. Cortical alveoli formation is also initiated during primary growth and continues during vitellogenesis. In ostariophysan oocyte development, neither ooplasmic oil droplets are formed during oocyte growth nor complete yolk liquefaction occurs during oocyte maturation (Figure 1). This differs from marine species belonging to highly derived taxa such as Perciformes where numerous oil droplets form

around the nucleus during oocyte growth; these fuse to produce a single oil globule during maturation. The partial fusion of yolk globules in *G*. cf. *anguillaris* should occur just before ovulation takes place. The complete hydration and liquefaction of the yolk, as described for marine perciform species, does not occur. Although fused to each other and having unequal development, the ovaries are actually paired and of the cystovarian type with a central lumen. Considering the difficulties in communication generated by the usage of non-standardized nomenclature, this study has slightly modified the staging schema of oogenesis developed by Grier et al. (2009). The schema is based on the morphological and physiological events during fish oogenesis. It simplifies the comparison of oogenesis between lower ostariophysan fish, like *G*. cf. *anguillaris*, and higher perciform fish such as *S. ocellatus* (Figure 1).

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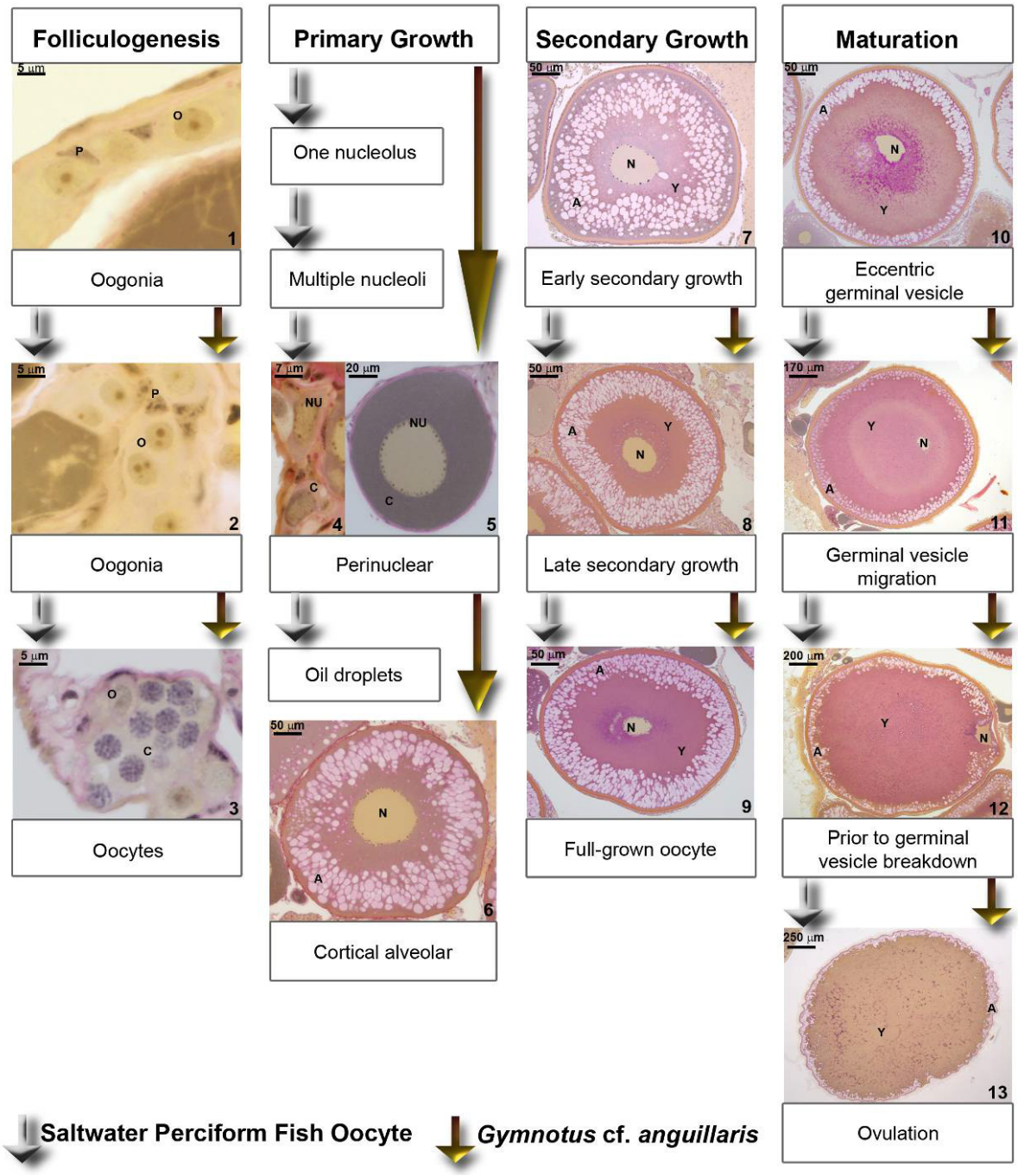

Figure 1. Comparison of oocyte development in saltwater perciform fishes and the freshwater Ostariophysian *Gymnotus cf. anguillaris*. Photomicrographs represent *G. cf. anguillaris*. A - cortical alveoli; C – oocyte; N – nucleus; NU – nucleolus; C – oocyte; NU – nucleolus; O – oogonium; P - prefollicle cell; Y - yolk globule.

Staining Techniques for the Improvement of Differentiation of Immature and Resting Fish

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Introduction

The estimation of maturity ogives is one of the most important aspects in fisheries assessments. Incorrect ogives will produce a wrong perception of spawning stock biomass, an incorrect forecast, and hence increase the risk of unsustainable fisheries. The main handicap to estimating proper female maturity ogives is the differentiation between immature and resting females.

The objective of the present study is to improve identification of these two maturity stages using different staining techniques (Quintero-Hunter et al. 1991; Sarasquete and Gutiérrez 2005) in order to identify quickly and precisely ovarian structures indicative of previous spawning activity (i.e., postovulatory follicles (POFs) and atretic follicles (AFs)), especially when they are in the advanced stage of resorption and visual identification is difficult. With this aim, different staining protocols have been tested in four species.

Materials and Methods

Ovaries of Atlantic cod *Gadus morhua*, four-spot megrim *Lepidorhombus boscii*, European hake *Merluccius merluccius* and pouting *Trisopterus luscus* were fixed in 3.6% buffered formaldehyde. One sample from the middle portion of fixed ovaries was extracted, dehydrated, embedded in paraffin, sectioned at 3 microns thickness. For each species, separate preparations were stained with Masson trichrome, Metanil yellow, tetrachromic VOF-Type III (Sarasquete and Gutiérrez 2005)and a routine method (Haematoxylin-Eosin). A Leica DM RE microscope was used for the histological examination and analysis. Protocols can be found at http://www.fresh-cost.org.

Results

Atlantic cod

It shows high affinity for most of the dyes that specifically stain structures indicative of previous spawning activity (AFs and POFs). Metanil yellow (Figure 1A) and Haematoxylin-VOF (Figure 1B) differentiate them clearly, the former stains them in pink because of the effect of the PAS reaction, and the latter in blue. Masson's Trichrome stains old AFs fuchsia-pink and POFs green (Figure 1C). With Haematoxylin-Eosin (H&E, the routine method), the results were not very good. To test the accuracy of these staining techniques, they all were applied to immature ovaries and indicative structures (AFs and POFs) were not observed (Figure 1D).

Four-spot megrim and European hake

In four-spot megrim and European hake, results were less clear. The best contrast was obtained with Masson's trichrome (Figures 1E and 1F), which distinguished old AFs and old POFs from the rest of ovarian structures, although differential staining (different colour) was

not observed; Therefore, the process of identification will be more difficult for inexperienced observer.

Pouting
Samples processed with new staining methods did not show differences when compared with routinely (H&E) stained samples. Even so, the new staining methods produced better contrast, a larger range of colours and hence a potential use for differentiation of immature and resting fish if protocols are adjusted properly.

Conclusions

This study proves the effectiveness of new staining methods to differentiate immature and resting females by recognition of structures indicative of previous spawning activity like AFs and POFs. However, it has been observed that the affinity of dyes is very different between species and is not universally applicable (Table 1). Results were promising with most of the dyes (Masson's trichrome, Metanil yellow and Haematoxylin-VOF), but diagnosis could be improved significantly by adapting staining protocol to the studied species. A greater effort to improve staining protocols will be necessary to achieve extensive use of these staining methods in a wide range of species.

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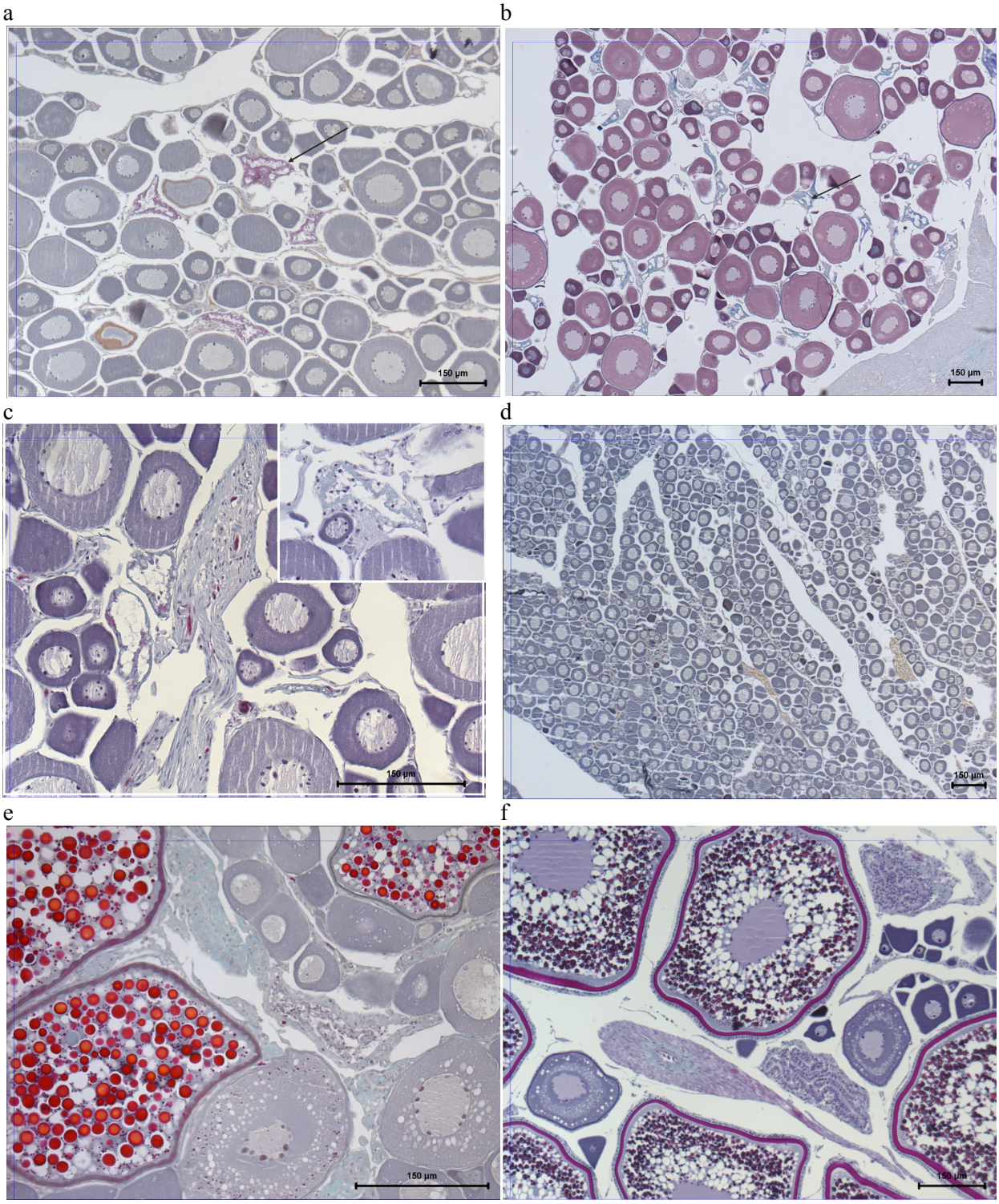

Figure 1. Images highlighting the most relevant results using different staining techniques for the improvement of differentiation of immature and resting fish. a) Metanil yellow (Cod), arrow indicating POF or AF; b) Haematoxylin VOF-III (cod), arrow indicates POF or AF; c) Massons Trichrome (cod), arrow in main image indicates AF, while inset image show a POF; d) Metanil yellow, immature ovary (cod); e) Masson´s Trichrome (Megrim); and f) Masson´s Trichrome (hake)

Table 1. Affinity of spawning activity structures (AF and POF) for each stain technique.

GOOD +++ FAIR ++ BAD +

AFFINITY BY COLOURS

	COD	HAKE	MEGRIM	POUT
Haematoxilyn -VOF	+++	+	+	+
Masson's trichrome	+++	+++	+++	+
Metanil yellow	+++	+	+	+
Haematoxylin -eosin	+	++	++	++

A Quick Method for an Easy Identification of Ovarian Structures: The Use of Autofluorescence

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Introduction

Estimation of fish stock reproductive potential has been of increasing importance in recent years. It involves the estimation of several key parameters on a routine basis, such as potential and/or batch fecundity, spawning fraction, atresia, etc. (Murua et al. 2003). Some of these parameters need a prior identification of the presence of postovulatory follicle (POF) structures within the ovary in order to estimate fecundity. However, expertise is necessary, as very often it is difficult to distinguish POF from late alpha- or beta-atresia.

Additionally, it is important to develop methodologies that reduce the time needed for estimation of fecundity, such as the automated detection of oocytes aided by image analysis used in combination with stereology. In histological procedures it is common to use autofluorescent stains (De Rossi et al. 2007) that previous studies have shown to be useful to detect these structures (Saborido-Rey et al. 2005). In this study, the utility of fluorescence as a method for a quick identification of key ovarian structures is analyzed.

Material and Methods

Ovaries of four different species (cod, *Gadus morhua*, hake, *Merluccius merluccius*, redfish, *Sebastes mentella*, and megrim, *Lepidorhombus boscii*) were sectioned (3µ) and stained with 14 staining protocols (see www.fresh-cost.org for protocols). Images of sections were taken with a Nikon Eclipse 90i using bright field illumination and direct fluorescence microscopy, using the filter B-2A EX 450-490nm and the G-2A EX 510-560nm filters.

Results

Differentation between POF and atresia

In hake, late-α or β- atresia (yellow arrow) and POF (blue arrow) are distinguishable using fluorescence with a G-2A filter (Figure 1a), although it is hard to discriminate between these structures with bright field with any of the stains tested. In atretic follicles, the remains of oocyte components are clearly visible under fluorescence.

Female cod were collected several months after spawning and analyzed. Using VOF-III stain, several unidentified post-spawning structures are visible (Figure 1b). However, fluorescence revealed two types of structures with and without red bright areas, respectively. More research is needed to assess if they correspond with old POF and β-atresia. Interestingly, these structures were not visible with the B-2A filter.

Table 1 shows the results with other species and staining protocols.

Detection of oocyte edges
The oocyte chorion is remarkably highlighted in hake using G-2A filter and Masson´s trichromic stain (Figure1c), while other ovarian structures are attenuated. This pattern is less clear with the B-2ª filter or bright field. In general, the chorion is highlighted in megrim, cod and hake using several of the tested protocols (Table 1). However, these results were not obtained in redfish probably because the chorion is thinner or has a different composition in this species due to its viviparity.

Conclusions
β-atresia and POF: Remains of atretic follicles, such as the chorion and residual yolk, are highlighted as bright areas under fluorescence. However, POF are less visible (attenuated) under fluorescence and therefore this technique allows a quick and easy discrimination between atresia and POF.
Edge detection: Enhancement of the chorion in relation to the rest of the ovarian tissue is possible using fluorescence. This will improve the results of the automatic detection algorithms currently in use or being developed.
Fluorescencence: The quality of the results is very much dependent on stains, protocols, and filters used. But most importantly, due to different tissue composition for each species during the breeding season and the differential affinity of dyes, the results varied among species and therefore research is needed to adjust protocols for each species under analysis.

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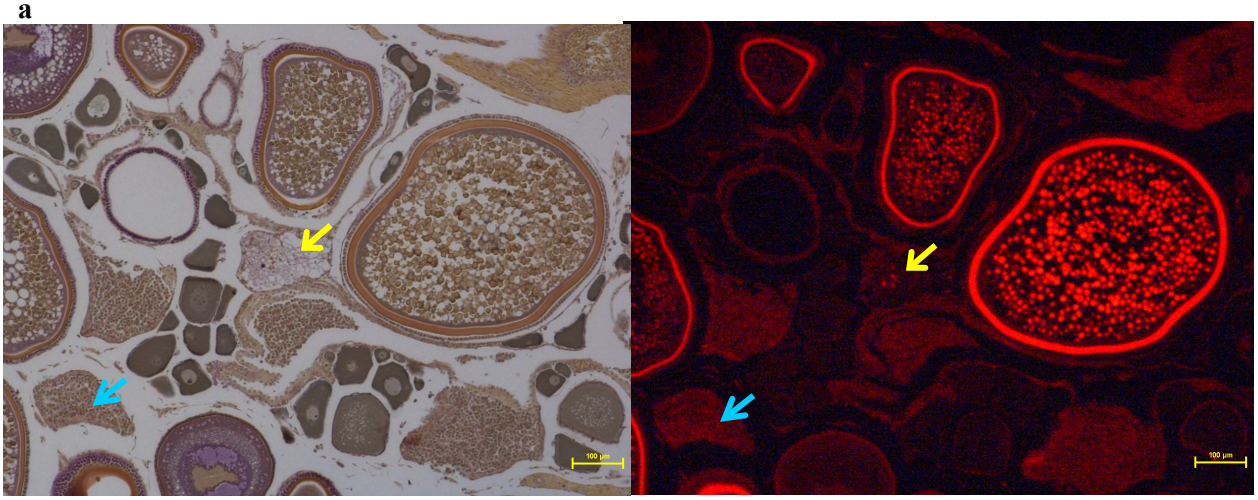
a



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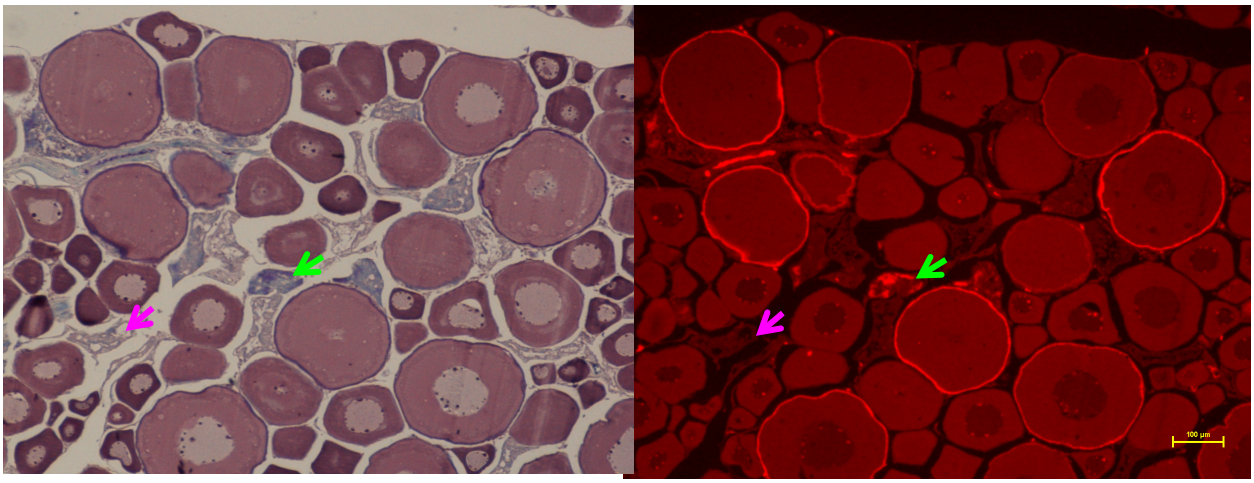

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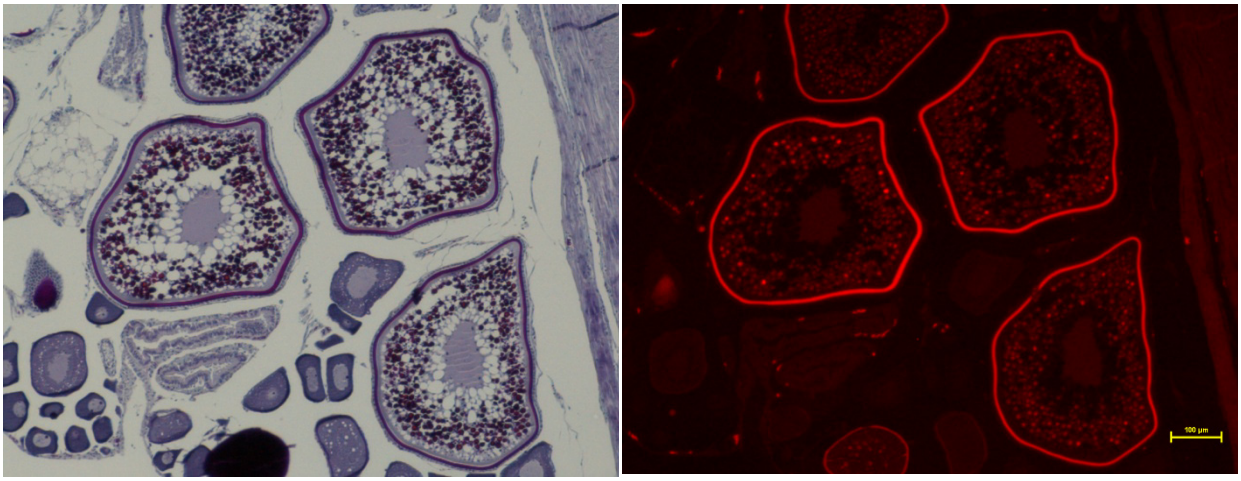

Table 1. Highlighted structures using fluorescence with G-2A filter for 14 stains and four species.Stains: V: VOF III; MT: Masson´s tricromic; MY: Metanil Yellow; P: Pas; E: Eosine; F:Floxine; FC: Fucsine; O: Orange G; A: Aniline blue; ER: Erythrosine; R/V: Bengal rose+ligth green; R: Neutral red; H: Hematoxylin. Results:c: chorion;;y: yolk, a: atresia; p: POF, - : negative results.

	Stains													
	V	MT	MY	AT	P	E	F	FC	O	A	ER	R/V	R	H
Megrim	c/a	-	c	c/y	-	c/y/a	c/y/a	y	c	c/y	c/y	c	c/y	-
Cod	c/a?/p?	-	p?/a?	c/y	-	c/y	c/y	-	-	c/y	y	-	-	-
Hake	c/y	c/y	c/y/a	c/y	c/y	c/y/a	c/y	c/y/a	c/y	y	c/y	c/y/a	-	-
Redfish	y	y	y	y	-	y	y	y	y	y	-	y	-	-

Staging and Ageing of the Postovulatory Follicles in *Engraulis ringens* from Northern Chile

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Introduction

In the Humboldt Current System, *Engraulis ringens* is an economically important fisheries resource. Since the Daily Egg Production Method (DEPM) to estimate spawning biomass in anchovy was developed in the late 1970's, the estimation of daily spawning fraction has become of importance for fisheries based on species with indeterminate fecundity. The daily spawning fraction (*S*) of *E. ringens* in each DEPM application is estimated through the histological preparation of the female ovaries by identifying and classifying postovulatory follicles (POF) into daily cohorts. Until year 2005 in northern Chile, POFs were classified in 0-, 1- and 2-days, where POF day 0 means that spawning occurred during the 9 hours before, POF day 1 were from 9 to 33 hours, and POF day 2 were from 33 to 56 hours from the spawning peak. However, no complete validation of the degeneration of the POFs has been made for this anchovy species. Since 2006, a new classification criteria was carried out based on 7 POF degenerative features that separated the staging from ageing, according to the methodology of Alday *et al*. (2008).

Methods

We used information obtained in Daily Egg Production Method (DEPM) surveys in the area off northern Chile (18º 20'S-26º 03'S), for the period 2004–2008 (n = 8153 adult females). We used the morphological characteristics of the 7 POF stages according to the methodology of Alday *et al*. (2008).

Results and Discussion

The peak of hydrated ovaries occurs between 16:00–18:00 h (Figure 1). There is a good correspondence in the distribution over the time between the eggs in the first stage in the plankton and the Postovulatory Follicle Stage I (POF I), indicating a spawning peak at 23:00 h (Figure 1).

Our results are very similar to Alday *et al*. (2008). There was a close agreement in the succession of POF stages over time after spawning. The first four stages of POF degeneration occurred in less than 24 h. A lower proportion of the stage IV, after the spawning peak (23:00 hour) probably did not come from the same spawning (i.e > 24 h). For Stage V it is difficult to separate daily cohorts due to the overlap; probably some proportion of the Stage V POF were still < 24 h. A high proportion of the Stages VI and VII occurred in the second day after spawning.

More research efforts are necessary to reach an objective and reliable method to classify the POF in daily cohorts, especially for stages IV and V. Variables to consider include the time of the capture, spawning peak and standard deviation in the age at the stage. A question to

still be answered is if the POF degeneration is faster in larger females than in smaller females. Meanwhile, the results show that ageing of POFs younger than 24 h (Stages I-IV) is essentially more objective and reliable than ageing older ones, because of the assumption required for the increasing overlapping periods of the most advanced degeneration stages (Stages V-VII) (Alday *et al.*, 2008).

An important consequence of this new classification method for staging and ageing POFs is that the spawning fraction of *E. ringens* would be higher than that reported using the old criteria (i.e POF day 1 or 2; Hunter and Goldberg, 1980) (Table 1).

Acknowledgments
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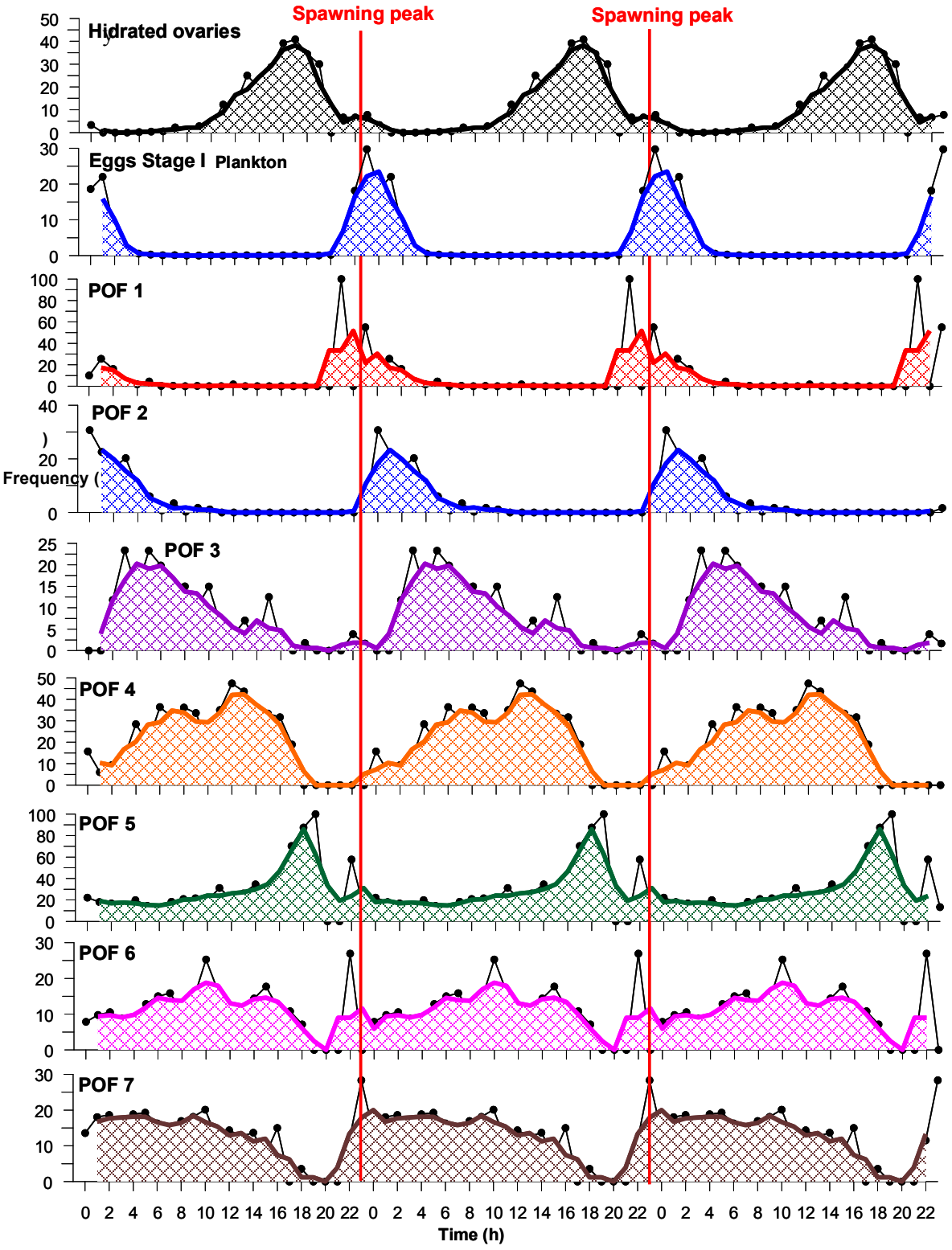

Figure 1. Percentage of occurrence of different stages over time: Hydrated ovaries, Eggs at the first stage (Stage I) and the seven stages of Postovulatory Follicles (POF).

Table 1. Daily spawning fraction estimated according to the old method (Old POF day 1) and the method of Alday et al. (2008) (New POF I-IV).

Criteria	2004	2005	2006
Old POF day 1 (CV%)	0.176 (7.16)	0.190 (6.7)	0.203 (5.2)
New POF I-IV (CV%)	0.147 (13.0)	0.234 (10.0)	0.277 (9.1)

Appendix

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Speaker	Oral Presentation
Alonso-Fernández	Three Different Hemafroditic Strategies on the Balearic Islands Shelf (North-Western Mediterranean Sea)
Barros Paiva	Characterisation of the Reproductive Trait Of Female Birdbeak Dogfish, *Deania Calcea* (Lowe, 1839), in Portuguese Continental Slope
Brown-Peterson	A Standardized Terminology for Describing Reproductive Development in Fishes
Brulé	The Use Of Histology in Defining the Pattern of Ovarian Development and Fecundity Type in Tropical Marine Teleost Fishes
Domínguez-Petit	Importance of Histological Information in the Assessment of Southern Stock of European Hake
	Comparative Analysis of Reproductive Strategy in Three Demersal From Galician Shelf
El Ouizgani	Histological Examination of Ovarian Development Of Anchovy (*Engraulis encrasicolus)* from the Agadir Bay (Morocco Atlantic Coast)
Ganias	Ovarian Dynamics and Oocyte Development Rate in the Atlantic Sardine, *Sardina pilchardus*.
Grier	Switching Pocs for Pofs
	Saltwater Perciformes: From Oogonia to Pelagic Eggs
	Freshwater Ostariophysi: from Oogonia to Eggs.
Hagstrøm	Stereology as a Tool to Assess Reproduction Strategy and Fecundity of Teleost Fishes
Japoshvili	Gonad Histology of Vendace *Coregonus albula* in Lake Paravani, South Georgia
Juanes	Staging Haddock (*Melanogrammus Aeglefinus*) Ovaries: Implications for Maturity Indices, Estimation of Daily Spawning Timing, and Field Sampling Practices
Kennedy	The Assessment of Maturity Stage in Greenland Halibut (*Reinhardtius hippoglossoides*)
Kjesbu	The Utility of Gonadal Histology in Studies of Fish Reproduction and the Subsequent Management of Fisheries and Ecosystems
	Pattern of Previtellogenic Oocyte Recruitment into Secondary Growth Phase in Indeterminate Reproductive Style Species
Kofoed	Histological Study of Hormonally Induced Spermatogenesis in European Eel (*Anguilla anguilla)*
Lowerre-Barbieri	Understanding Temporal Reproductive Patterns in Marine Fish: a Review Of Histological Approaches and Emerging Methodology
Medina	Quantification of Ovarian Follicles in Bluefin Tuna by Two Stereological Methods
Murua	Quantification of Seasonal Follicular Cycle of an Indeterminate Fish: The European Hake Model
	Revision of the Spawning Frequency Estimates for the Bay of Biscay Anchovy.
Nóbrega	Spermatogonial Stem Cell Candidates and Their Niche in Zebrafish Testes (*Danio rerio).*
Nunes	Seasonality of the Reproductive Activity and Resources Allocation in the Iberian Sardine
Rideout	Skipped Spawning: a Strategy for Maximizing Reproductive Output in a Variable Environment
Saborido-Rey	Female Reproductive Strategies: a Energetic Balance Between Maturation, Growth and Egg Production
	Impact of Mass Atresia in Reproductive Ecology, Maturity Ogive, Spawning Migration and Population Dynamics on *S. mentella* in Icelandic Waters
Sequeira	Investigating the Fecundity Type of the Zygoparous Species Helicolenus *Dactylopterus dactylopterus* (Delaroche, 1809)
Serra-Pereira	Oviparous Elasmobranchs: How Different Is Their Reproductive Cycle from Teleosts – the Case-Study of the Thornback Ray, *Raja clavata*
Tomkiewicz	Prevalence of Intersex in Eelpout (*Zoarces viviparus*) as an Ecosystem Status Indicator
Uribe Aranzabal	Ovarian Structure and Oogenesis in Viviparous Teleosts: Poeciliids and Goodeids
Vitale	Macroscopical and Microscopical Investigation in Kattegat Cod (*Gadus morhua*)
Wuenschel	The Reproductive Biology Female Winter Flounder (*Pseudopleuronectes americanus*): Validating Classification Schemes to Assess the Importance of 'Skip Spawning'