European Journal of Molecular & Clinical Medicine

ISSN 2515-8260 Volume 07, Issue 05, 2020

A Study on the Reproductive Biology and Gonadal Cycle of *Anabas testudineus*During Breeding Season

Golam Ziauddin

Assistant Professor, Fisheries Resource Management Department, Faculty of Fishery Sciences, West Bengal University of Animal and Fishery Sciences, PanchaSayar, Kolkata - 700 094.Email: golamziauddin@gmail.com

Abstract

The gonadal cycle constitutes a significant biological parameter to be investigated in the fields of Anabas testudineusreproductive biology and ecology and fisheries science. Maturity data may also be used to carry out temporary analysis of fish stocks' life-history characteristics. Maturity data was often used to characterise the species' gondola cycle and to track long-term transition in the reproductive cycle. That's why, according to the reproductive plan of the genus, the maturity stage should be calculated. In the current research, the gonadal cycle is observed by Anabas testudineus to maturity. The ripeness scales should ideally be based on quantitative and universal gonadal properties, which are measured macroscopically since they are less costly and labour intensive than microscope maturity stadiums.. The rate of ripeness should be standardised.

Keywords: Anabas testudineus, Reproductive Biology, Gonadal Cycle, Maturity Staging.

1. Introduction

The gonadal cycle constitutes a significant biological parameter to be investigated in the fields of *Anabas testudineus*reproductive biology and ecology and fisheries science. Maturity data are used to determine maturity ogives, which is necessary to calculate maturity period additionally by age (L50, A50), biomass spawning stock and overall egg development. Maturity data may also be used to carry out temporary analysis of fish stocks' life-history characteristics. Adequate estimates of the maturity level are thus a key phase in the reliability of such estimates and are primarily guided by biological parameters for fisheries assessment and management.

Maturity data was often used to characterise the species' gondola cycle and to track long-term transition in the reproductive cycle. Hence, according to the reproductive plan of the genus, the maturity stage should be calculated. The reproductive technique in marine fisheries is described as the blend of various breeding system components, such as the semelparous-iteroparous, complete spawning spawlers, external-internal fertilisers, and so on (Murua&Saborido-Rey, 2003). Note: in essence, the maturity sampling approach should be designed on the basis of the species' reproductive strategy. The time frame is significant in this case, as the chance of a neglected laying individual is greater during the pre-laying season than after the beginning of the laying season. Skip spawning ensures that the skipper

cannot be included in year spawning and is not included in the spawning biomass. In the sampling for maturity, it is often important to understand the behaviour of species.

The consistency and prejudice in the maturity stage is another significant concern. The ripening is mostly focused on ripening scales which are used without histological confirmation. Many published studies have used such maturity scales for particular organisms, of which little has related their scales to those described in other articles. But, as indicated in Núñez&Duponchelle (2009) it can be confusing to compare them, as they vary from basic measures of three to nine phases in more detail. These authors have made the classification of the maturing challenge in Anabas a universal scale to tackle this problem. A scale following the universal terms of the reproductive classification of fish as suggested by Brown-Peterson et al seems appropriate in order to support this already presumed standardisation need (2011).

If practicable, maturity levels for closely related species should be commonly measured. Standardisation of the maturity level, i.e. preferably the maturity scales should be based on objective and uniform gonadal functionality, which can be assessed macroscopically as the stage ripeness of these microscopes is cheaper and labour intensive.

2. Macroscopic Maturity Staging

In Anabas, typically, there are 5 stages of maturity, both in women and in men: immaturity, development, breeding and regression (Table 1). These phases are dependent in various phases of the menstrual cycle on the most general observable macroscopic features of the gonad for male and female fish. It should be noted that some phases of development are not macroscopically distinguishable in certain organisms, mostly because of a lack of distinctive recognisable structures, i.e. immaturity and regeneration are the most often deceptive phases, while in others it is difficult to discern immature or excluded spawning. Again, these erroneous phases are associated with the organisms' reproductive policy. Minor modifications may be required when extending this general classification to other species based on the reproductive biology of the individual species. In the female reproductive tract for short periods, zygoparous (a midphase from which fertilised ova is retained) and embryoparous (Before the releasing from the female reproductive tract to the external environment, the embryo develops into an advanced state) (Wourmsetcoll. 1988) may include modified descriptions and/or additions. But, as Falk-Petersen (2005) has pointed out, teleosts' fundamental development processes are similar; the distinctions are based on timing and genetically, physiologically and ecologically regulated oocyte and embryo developmental events. Falk-Petersen (2005) provides summaries and comparisons of early development experiments on fish, but several studies are published classifying the developmental phases of a species or genus.

Development stage	Description females	Description males
Immature	Ovaries are small and more or less translucent, sometimes lightly pink. Oocytes are not distinguishable to the naked eye. Transverse sections show little gonad volume.	Testes are small and more or less translucent, sometimes lightly pink. Sperm are not distinguishable to the naked eye. Transverse sections show little gonad volume.
Developing	Ovaries are increasing in size, becoming larger and more consistent. The ovary usually turns to a yellow, orange or pink color. Individual oocytes are still not macroscopically distinguishable. External blood vessels start to develop around the ovaries.	Testes are increasing in size, becoming larger and more consistent. The testes remain whitish. Individual sperm are still not macroscopically distinguishable.
Spawning capable	Ovaries are much bigger and voluminous; vascularization is very apparent. The granular consistency of the ovary can be appreciated externally, since yellow vitellogenic oocytes are individually distinguished.	Testes are much bigger and voluminous; vascularization is very apparent. In the testes, accumulation of sperm in the spermatic ducts is also macroscopically visible.
Actively spawning	The transparent hydrated eggs of the ovaries are visible through the ovarian wall.	The large amount of sperm in the testis is easily released towards the external medium when the fish abdomen is pressed, even very lightly.
Regressing	Ovaries are still large but almost empty. They are flaccid, show a wrinkled gonad wall, and are usually grayish in color.	Testes are still large but almost empty. They are flaccid, show a wrinkled gonad wall, and are usually grayish in color.
Regenerating	The macroscopic aspects of the ovaries in the regenerating phase are very similar to those in the immature phase, but the transverse section tends to be larger and the gonad wall thicker. They tend to be more opaque than immature gonads.	The macroscopic aspects of the testes in the regenerating phase are very similar to those in the immature phase, but the transverse section tends to be larger and the gonad wall thicker. They tend to be more opaque than immature gonads.
Abnormal	The ovaries may possess abnormal traits that causes at least partly reduced fecundity.	The reproductive tissue of testes may partly turn into adipose or only one lobe developed.

Table 1: Description of macroscopic characteristics of the gonadal phases in the reproductive cycle of female and male *Anabas testudineus* (Brown Peterson et al., 2011)

3. Microscopic Maturity Staging

While the sex and reproductive status of anabas is generally calculated by macroscope, a microscopic examination, that is histology and whole amounts, is also carried out to make the reproductive properties and annual cycle of the species more precise. Macroscopic examination is according to the improvements to the size and appearance of ovaries while histological approaches analyse cellular oocyte changes. Microscopic ripening is a helpful method to confirm macroscopic ripening and clarify developmental processes in gonads (Table 2). This is particularly helpful if macroscopically determined maturity levels are not allocated as sometimes would be achieved during some phases of the menstrual cycle. The microscopic maturity stage is therefore considered to provide a more accurate ripening evaluation as it removes misinterpretations, often the case, during macroscopic stages. Staging of microscopic maturity normally requires either histological preparation or whole mounting processes.

Development stage	Description females	Description males
Immature	Only oogonia and Primary Growth oocytes present. Ovarian wall thin and distribution of the oocytes very compact.	Only primary spermatogonia present. Testicular lobules without or with a very little lumen.
Early Developing	Only primary Growth and Cortical Alveoli oocytes.	Primary and secondary spermatogonia. Some primary spermatocytes.
Developing	Also primary and secondary Vitellogenic oocytes. No tertiary vitellogenic oocytes neither postovulatory follicles.	Also secondary spermatocytes, spermatids and spermatozoa within the cysts. No spermatozoa in the lobular lumen neither in sperm ducts. Germinal epithelium continuous through the whole test.
Spawning capable	Tertiary vitellogenic and/or germinal vesicle migration oocytes are present. In batch spawners postovulatory follicles can also be detected.	All stages of spermatogenesis can be present. Spermatozoa can appear in the lumen of the lobules and in the sperm ducts. Germinal epithelium can be discontinuous in lobules near ducts.
Actively spawning	Germinal vesicle breakdown and/or hydrated oocytes are present.	Large amount of sperm in the sperm ducts and lobular lumens. Germinal epithelium discontinuous throughout the testes.
Regressing	Atretic oocytes and postovulatory follicles. Some primary growth, cortical alveoli or vitellogenic oocytes can be present.	Residual spermatozoa and few scattered spermatocysts can be present in the testes. Regeneration of the germinal epithelium with spermatogonial proliferation in the periphery of the testes.
Regenerating	Only oogonia and Primary Growth oocytes present. Ovarian wall thick and distribution of the oocytes with some spaces. Degenerating postovulatory follicles can be present.	Only primary spermatogonia present. Testicular lobules with lumen. Germinal epithelium continuous.Very few residual sperm can remain in the testes.

Table 2: Description of microscopic characteristics of the gonadal phases in the reproductive cycle of female and male *Anabas testudineus* (Brown Peterson et al., 2011).

4. Gametogenesis

4.1 Oogenesis

The first stage in the development of eggs or in the meiotic cell division of oogonia into a primary oocyte at the sensu stricta phase is considered to be the Oogensis. (Wallace and Selman, 1981; Mayer etal., 1988); (Figure 1). However, the term oogensis is also used more widely to include all stages ranging from oogon division to the final ripening of oocytes (Kjesbu, 2009). Oogenesis refers to growth of oocyte and maturation processes from primary oocyte to hydrated oocyte.

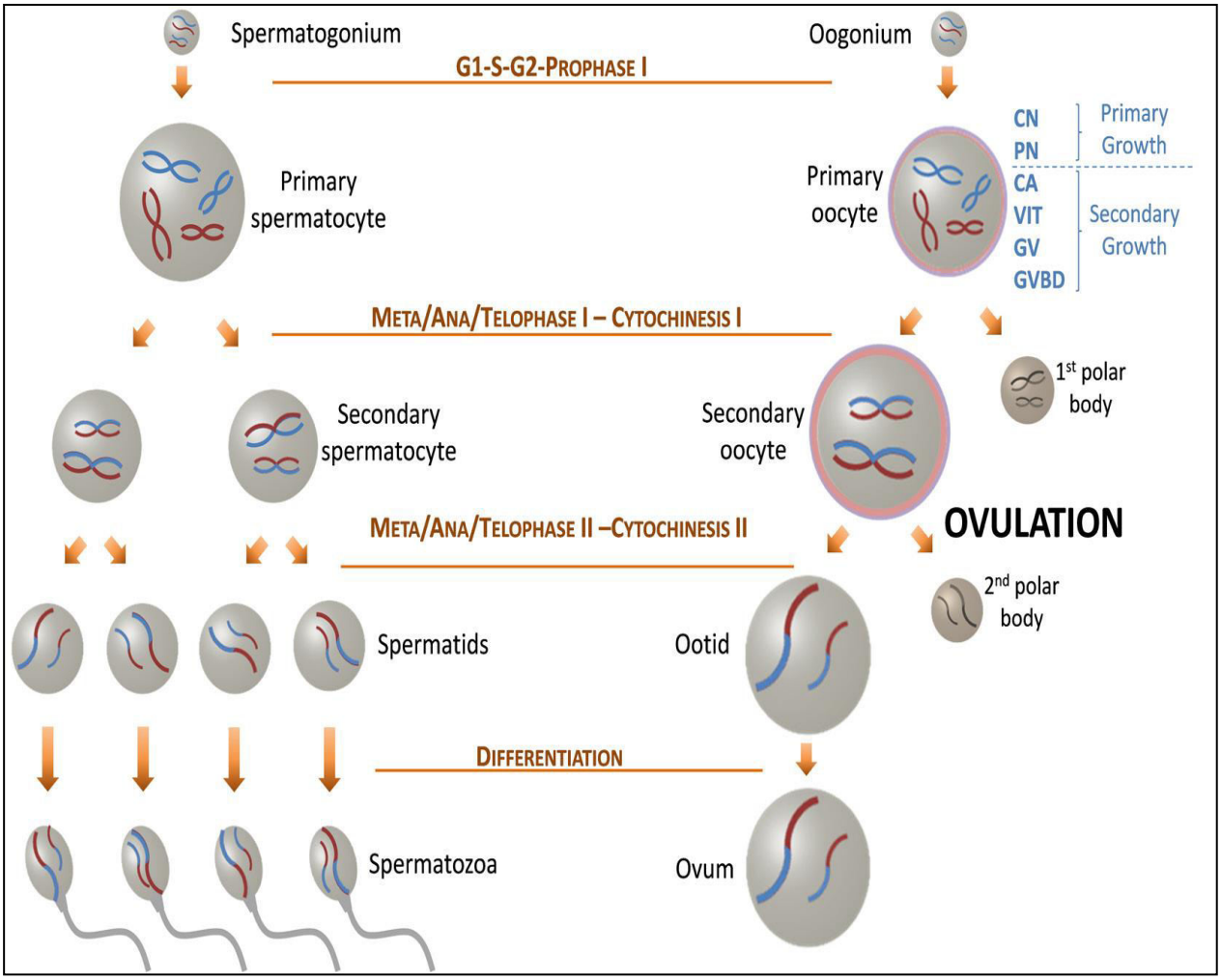

Figure 1: Gametogenesis process: Oogenesis and Spermatogenesis in *Anabas testudineus*.

The growth stages in oocytes typically meet the guidelines Wallace & Selman (1981) and West defined (1990). The chromatin nucleolar and the perinucleolar phases provide the primary growth oocyte level. Nucleolar oocytes of chromatin have a large nucleus which may hold many tiny nucleoli, but it is always remarkable. The scant cytoplasm is basophilic and uniform (Figure 2.A). In the perinucleolar oocytes, the nucleus consists of some peripheral nucleoli along the nuclear membrane. The cytoplasm loses its basophilia progressively (Figure 2.B). In the Cortical alveoli phase, secondary development of the oocyte begins and proceeds with the Vitellogenic phases. The oocytes present a granular, more acidophilic cytoplasm at the cortical alveoli level (Figure 2.C). Small lipid droplets may already be observed on perinuclear ooplasm prior to the forming of cortical alveoli in certain organisms. The very acidophilic zone of the radiate is visible. The first vitellogenic stage is a small yolk granule on the periphery of the cytoplastic. Cortical alveoli are higher than in the previous stage and lipid goutlets are bigger. The number, size and distribution of granules of yolk, which take up nearly the entire cytoplasm, is increased during the second stage of the

Vitellogene. The third stage of vitellogene reveals even thicker grains of yolk and lipid gout (Figure 2.D). In the secondary growth step, the oocyte maturation period is included but this process has also been named by some writers as the third stage of production (Grier, 2000). Maturation ends with nucleus migration through the animal pole (Figure 2.E), its dissolution and a further accelerated oocyte development due to fluid uptake (Figure 2.F) (Wallace & Selman, 1981). Ovulation happens during hydration as the follicle splits. The other follicles are called post-vulatory follicles (POFs). The postovulatory follicles behave as pliable constructs, which degenerate over various stages (Hunter &Macewicz, 1985). This function is known to be distinct from the atria (Wood & Van Der Kraak, 2003).

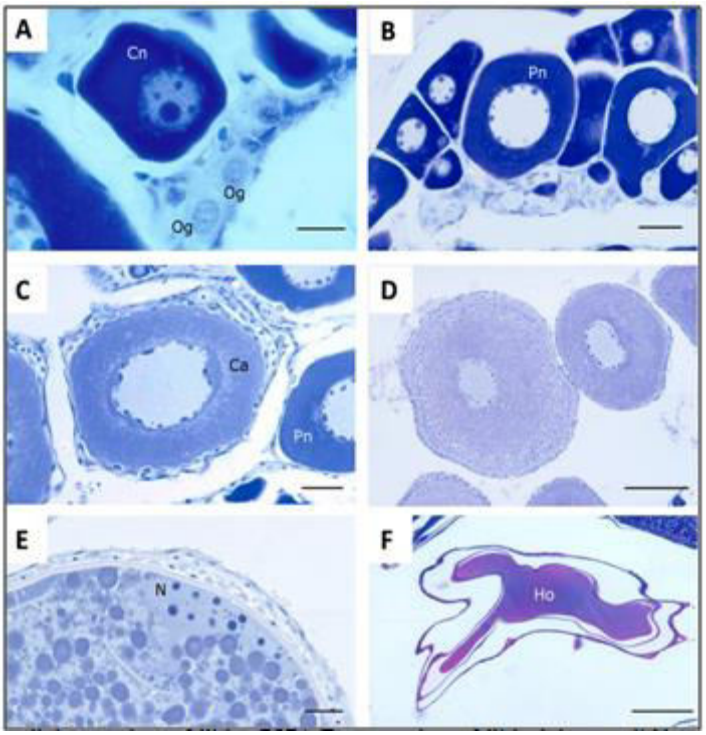

Figure 2: Oogenesis in *Anabas testudineus*.

4.2 Spermatogenesis

The spermatogenesis mechanism in most Anabas species is very similar (Figure 1). The male germ epithel usually consists of spermatocytes produced when the Sertoli cells enclose a single primary spermatogonia clone. Within these cysts, the germ cells develop synchronously. Afteropening of cysts, sperm are released into the lobular lumen at the end of the process. This famous form of sperm is called cystic because it takes place inside these cysts during the entire process. However, in all animals, spermatogenesis did not follow this trend. Until they become spermatozoa, sperm cells may open and expel germ cells to the lobular lumen. This semicystic spermatogenesis was first recorded in the Ophidion (Mattei et al., 1993) genera and later in several other closely isolated phylogenetic species such as Scorpaena (Muñoz et al., 2002c; Sàbat et al., 2009). Following Grier (1981) and Grier & Uribe- Aranzábal the growth stage of male gametes can be determined (2009).

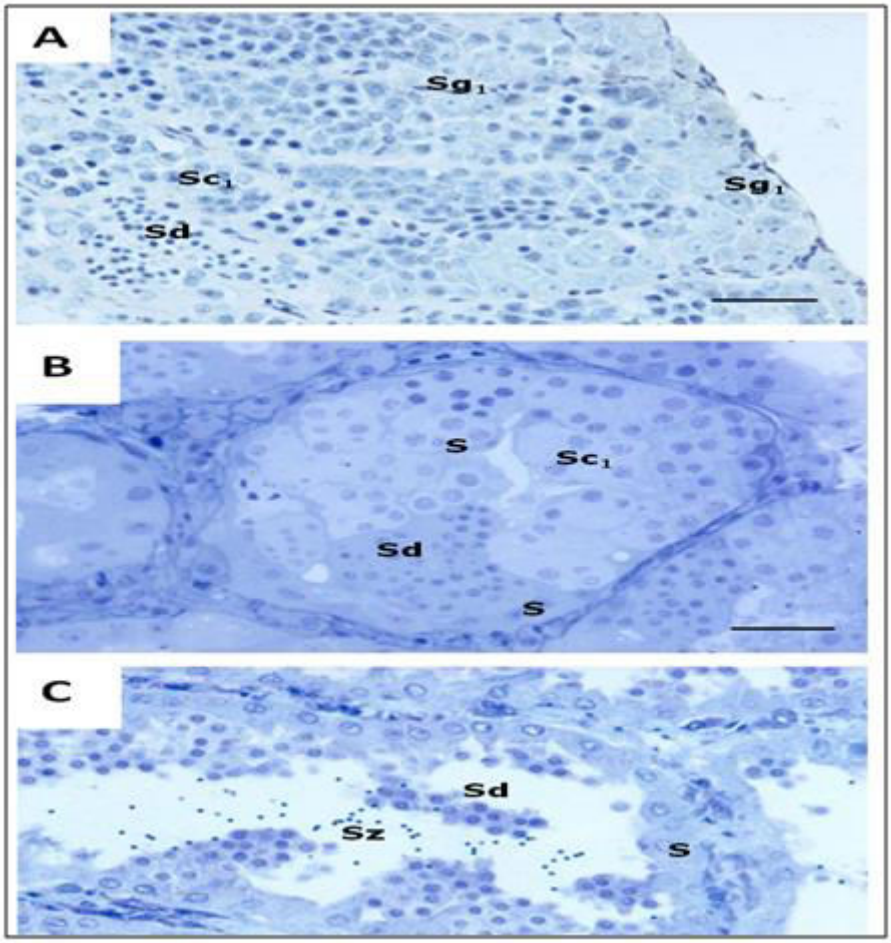

Figure 3: Spermatogenesis in *Anabas testudineus*.

A: Toluidine blue. Bar = 30µm. B: Methylene blue. Bar = 30µm. C: PAS. Bar = 30µm. S = Sertoli cell; Sc1 =primary spermatocytes; Sd = spermatids; Sg1 = primary spermatogonia; Sz = spermatozoa.

The knowledge acquired histologically can be replaced by transmission electron microscopy (TEM). This type of research is invaluable to analyse the sperm structure in this sense and considering the limited size of the gametes. A major circular nucleus may contain one or more nuclei, or the primary spermatogonia occurs in person or in small group (Figure 3.A). Secondary spermatogonia are more numerous and are often contained in a sertoli-circumcised cyst. Since the nucleus still has nucleolus, it is less than in the preceding point. The morphology is the most evident process of the pachytene phase characterised by synaptonemic complexes as the nucleus of the primary sperma cells continue into the meiosis prophase (Figure 3.A, B). The secondary nuclear-sized spermatocytes are not so possible to observe since the time is limited between 1st and 2nd division. The condensation of the chromatin in the spermatides means that the nucleus is thinner. The cytoplasm also greatly decreases. The flagellum begins to expand, but optical microscopy is hard to detect (Figure 3.A, B, C). Spermatozoa have a circular head and a little flagellum (Figure 3.C).

5. Histological Processing and Staining

A part of the ovary is embedded in wax or paraffin during histological preparation, and thin parts are cut and placed to see it in a microscope on slide. The parts are stained to emphasise specific structures within the ovary during processing. Fixing is the most critical step of histological treatment which can take place as soon as possible following tissue removal or soon after death. Formaldehyde is the most often used chemical in all regular fixations. In order to deal with it, it is carcinogenic, i.e. it requires appropriate protective precautions. Today, as with glyoxal, acrolein or carbide, non-carcinogenic fasteners are commercialised, but they can change marginally the composition of the gonad and should be closely checked for their effects on the examined tissue before either of them is replaced by formaldehyde.

When incorporated in resin, the cross-section of ovary is dehydrated for several hours (32,16,8) with increased intensity in alcohol solutions (70%,90%,96%) accompanied by resin penetration by upgradation (50%,100%) for several days (2, 2).

Finally, the ovary cross-section is put in a mould, incorporated into resin combined with hardener, to obtain blocks of resin. Sections are then removed from the resin block using a microtome (usually around 4 μm in width), which are thickened and placed on glass slides.

The treatment of wax miscible (chloroform, xylol) in a medium includes dehydration by ascending grades of alcohols, "clearing," and eventually impregnation with wax. Blocks are then cut into a particular thickness by a microtome based on their maturity level, since it is harder to have a very small segment of more advanced gona-ads. Parts of reducing alcohol sequence have to be hydrated before staining.

Owing to the simple and standardised use of hematoxylin-eosin methods, the majority of histological analyses for reproductive biology of fish are regularly stained but often knowledge with other stain methods needs to be supplemented. For example, the trichrome bleeding of the Mallory emphasises the zone radiata and makes its consistency easier to analyse, so that early atretic oocytes are detected (Muñoz et al. 2010).). Another illustration is evolutionary patterns in the direction of livelihoods which normally go hand in hand with small dimensions and the lack of cortical alveoli (Takemura et al., 1987; Muñoz et al., 2002a; b) which renders identification difficult. The periodic acid-schiff (PAS) stem therefore highlights this technique, making it extremely useable for detecting oocytes in zygoparous, embryoparous or vivipareous species at the cortical alveoli level. Spermatozoa can be emphasised with the Trichromic Tincture of the Mallory in males or in women with intraovarian sperm storage (Muñoz et al., 1999).

6. Maturity Staging from Whole Mounts

The whole mount techniques (Kjesbu, 1991) are normally clear and dependent on ovarian tissue distributed smoothly on the bottom of a petri dish and subsequently viewed in mild magnification under a microscope. Fresh tissue is stored in isotonic seawater (approximately 1.07%). It is easy to make by the combination of one portion of the seawater and two parts of

fresh water. When using fixed tissue, it is necessary to allow sufficient time to fix before measuring sizes is done, since fixation normally induces swelling or shrinking.

The entire scope of research can be considerably more effective in time and expense compared to histologic examinations for such reasons (Thorsen and Kjesbu, 2001; Alonso-Fernandez et al., 2009). Oocyte sizes can be easily determined using automatic photo-based particle analysis. Therefore the macroscopic stage of ovaries is frequently replaced by complete mounting methods. The leading cohort (LC) is a good proxy for the maturity of the ovary and the start of the breeding process especially in relation to the larger oocytes in the ovary (Kjesbu, 1991). The LC diameter of the preventive oocytes also allows for the detection of species that just finished a spawning year, as these oocytes are usually smaller than previously since they are not sexual maturation (Witthames et al., 2010).

In addition to calculation of oocyte size, oocyte clarity discrepancies for oocyte staging can also be used in whole mount techniques. Previtellogenic oocytes are highly translucent with advanced hydrated Oocytes while the cortical alveoli are semi-transparent with early vitellogenic oocytes, while the mid and late vitellogenic oocytes have no transparency (Figure 4). Transparency is especially important when separating early specimens (Figure 4.A), i.e. cortical oocytes or oocytes not visible from the naked eye in early vitellogenesis (separation). It may also be used for separating immature and regenerative specimens, where the latter contain hydrated oocytes (Figure 4.B) or atretic oocytes which remain throughout the spawning cycle (Figure 4.C). In addition to transparency, the oocyte type can be used in entire mounts to differentiate between ordinary and atretic oocytes (Figure 4.C). Atretic oocytes are more unusual in form and show a high clarity peripheral band (Óskarsson et al., 2002). Post-ovulatory follicles may also be identified on the basis of their form and contrast in entire mountings (Figure 4. A).

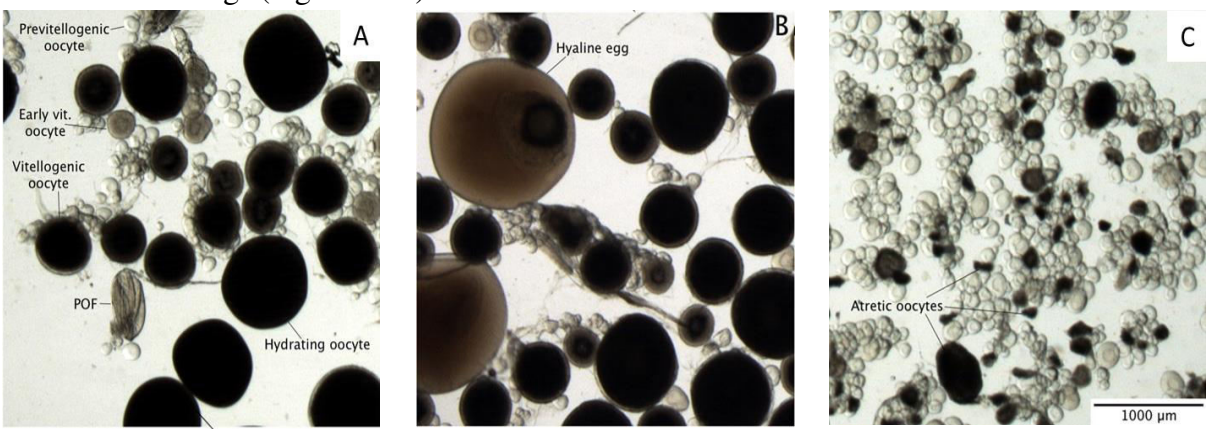

Figure 4: A)Pictures of whole mount formalin fixed ovary samples from *Anabas*. B) Samples from spawning fish. C) Sample from a spent fish.

Histograms of oocyte size provide valuable details on the ripe state of fish, the type and the type of ovary growth (Figure 5). The production of fish oocytes varies widely from species to species. On one end and on the other, we might imagine asynchronous ovary evolution.

However, crucial information on the nature and stage of the ovarian growth will be included in all oocyte size frequency histograms.

The growth and spraying of oocytes of Anabas is well studied. Thus, we can use Anabas to show how details on the frequency of the oocyte size can be interpreted to provide data on the maturity status of fish. Vitellogenesis is started in Anabas several months prior to spawning. The size of the vitellogeneic oocytes suggests the time for the start of spawning in the period between the beginning and spawning. The larger oocyte, the main cohort, seems to develop steadily in this period, making it particularly helpful to predict the time to start breeding (Kjesbu, 1994). The lead-cohort was identified in a number of studies as the mean of the 10% biggest oocytes (Thorsen and Kjesbu, 2001; Kjesbu et al, 2010) or the 95%-percentile.

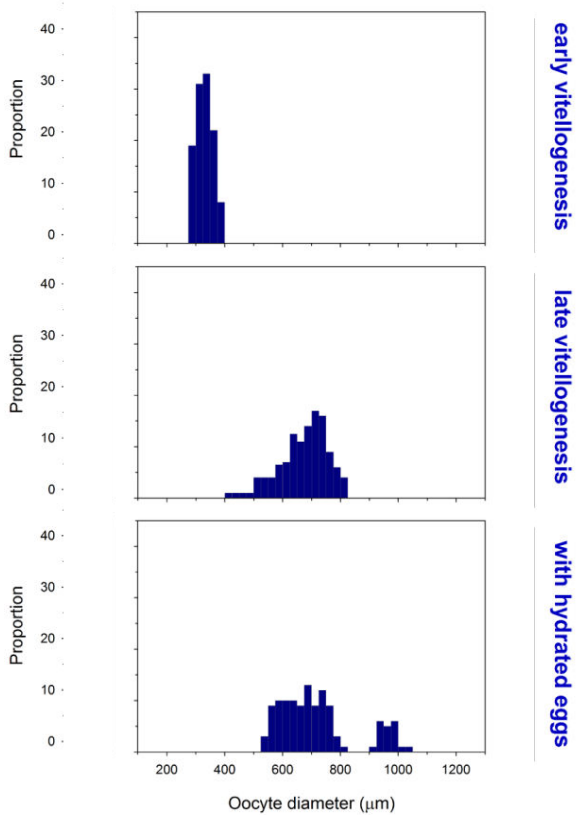

Figure 5: Follicle size frequency histogram for Anabas in different stages of ovary development.

Anabas is a certain batch spawder who generally releases 10 to 20 lots in a breeding season with an interval of 2-4 days (Kjesbu, 1989). Anabas ripening usually begins in autumn and is connected to oocyte formation. The first major maturation step of the cortical alveolus (Kjesbu&Kryvi, 1989). Typically, the cortical alveolus phase starts if the oocytes are around 200 µm diameter. The addition of a high volume of vitellin associates more development when the oocytes reach a diameter of 200-250 µm, and thus oocytes are said to be in the vital logensis stage from now on. In addition, they are ready for final maturation and eventual

ovulation or spawning when Anabas oocytes exceed 700-900 µm. As all these phases can be linked to oocyte size, the oocyte component analysis can offer the fish a fast ripening status (Figure 6).

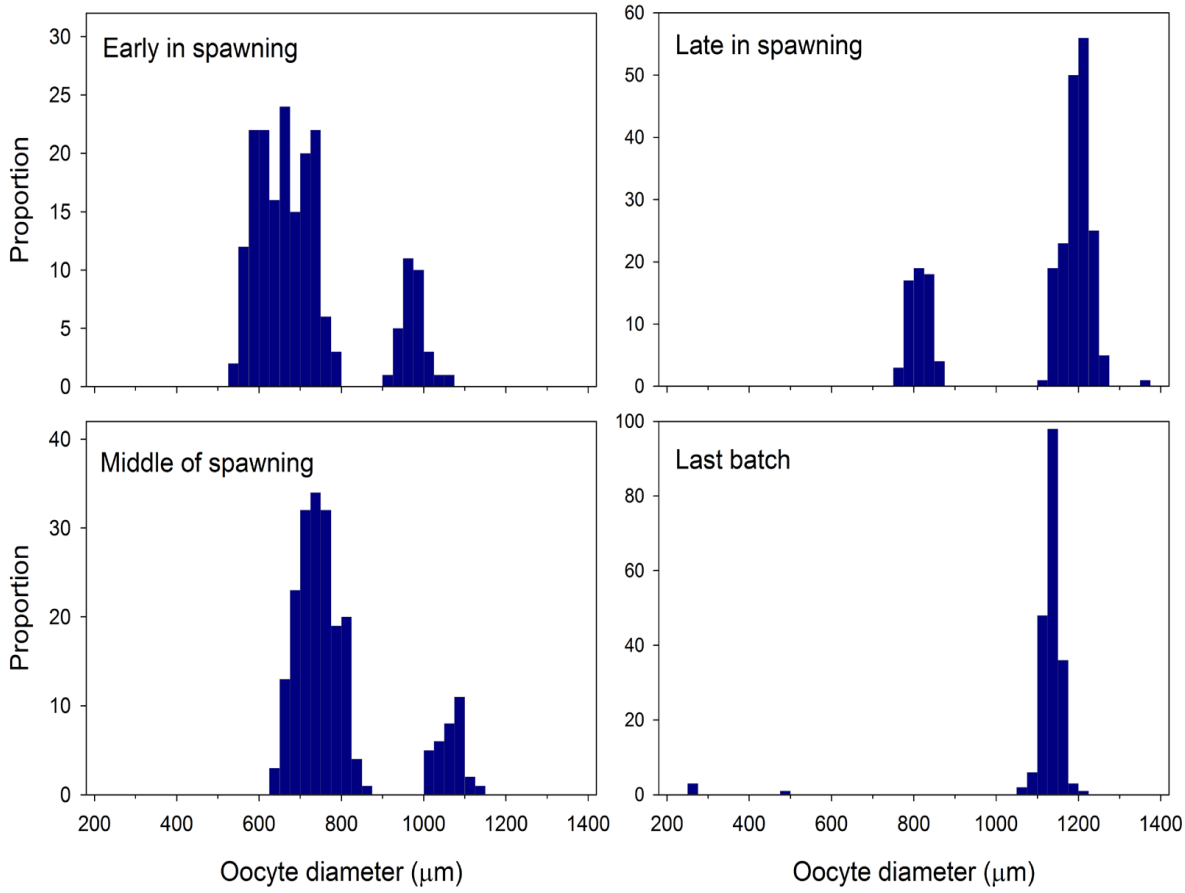

Figure 6: Follicle size frequency histograms from cod in the spawning period: A) early spawning, B) middle of spawning, C) late in spawning and D) last batch. All fishes displayed have a new batch (final maturation) coming up on the right-hand side. The size distribution of the vitellogenic pool on the left-hand side is broad early in the season but progressively narrows as the spawning progress.

Because anabas is a batch spawning agent, a new batch of oocytes greater than the remaining vitellogenic oocytes can be seen as an isolated community on a small histogram (Figure 6). In such instances, data on the scale of the frequency may also be used for batch fertility. The size frequency histogram can also show the proportion of spawned eggs during the spawning cycle; the breaking period is started (Figure 6.A), so as the oocyte size is broken down, the size range decreases and the mean oocyte diameter increases as the breeding process (Figure 6.B, C, D) (Kjesbu et al., 1990).

7. Validation and Methodological Calibration

The maturity steps conducted in various research institutions concerned with the same fish species are essential to calibrate and validate. Maturity studies for a certain species in many countries are mostly done by various laboratories. This data are then aggregated into a database and are used for inventory evaluation purposes. It is critical that the maturity scale of each species in laboratory/country participating in the sampling is consistent. Therefore, for calibration and confirmation, the maturity stage of individuals must periodically be reviewed.

A way to calibrate ripeness is via workshops where new samples are given for the maturity stage exercise among various laboratories. Frozen or set samples can be avoided because these samples lose any of the objective macroscopic feature used to allocate maturity as a result of the conservation process. The percentage of concord/disagreement between different laboratories is statistically determined and the accuracy and preference obtained during calibration exercise. Microscopic maturity stages are generally considered a basis for the identification of staging errors.

Recently, on-line resources such as webgr may be used for the calibration and validation of pictures from gonads by the organiser of a calibration workshop (http://webgr.azti.es/c/search/myce). Before the workshop, a strategy for collecting samples for the workshop should be developed. The workshop samples should closely resemble the samples of a real maturity analysis. The samples used during the workshop should also cover the whole spectrum of sampling sophistication and length. The biometric selection and processing of the gonads should be identical. Each participant scores the online maturity phase on a standard maturity scale. This detects differences in maturity stage and identifies much of the trouble phases. In addition, this is a simple and inexpensive method that only needs the compilation of high quality gonad images. However, it has clearly been seen that the maturity of images is harder than that of fresh gonads. Also, some features of construction phases are difficult to see from images, although in fresh content they are easily differentiated. Workshops with fresh materials would also provide a better understanding of the agreement among maturity stagers. A suitable set-up for such a workshop is to use trial-discussion examinations, where gonads are presented and findings discussed and gonads again.

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