4.8 Sampling for maturity

Knowledge on the reproductive patterns of large pelagics, as well as the characteristics of growth and mortality, will largely define the regenerative capacity of a population. Hence they are extremely important for management and conservation, and the construction of reliable models for effective stock assessment.

Large pelagics are generally repetitive broadcast spawners, and hence require very high lifetime fecundities to ensure reproductive success. This is achieved through various degrees of protracted spawning, along with a combination of frequent spawning and relatively high batch fecundities (Cayré et Farrugio, 1986; Schaefer, 2003). This biological feature increases the complexity of maturity studies.

The main approach for assessing maturity patterns of tunas and billfishes is based on collecting gonads for detailed histological examination under a microscope. While this has proven to be an accurate method, providing valuable information, it is labour-intensive, expensive and lethal for the fish. In addition, gonad samples are often unavailable since tuna may be sold intact at the auction block and swordfish are gutted at sea. More recently, chemical approaches to determine maturity have been developed. These techniques are discussed.

4.8.1 Sampling for sex and maturity

The location and timing of sampling will be defined by the purpose of the programme. By obtaining samples throughout the year, temporal patterns in maturity can be ascertained. More detailed temporal coverage may ascertain daily cycles in spawning. For example, studies have indicated that spawning may be synchronised to occur at dusk in some species. Wide spatial coverage may determine spawning locations. These factors should be considered when sampling for maturity data, and prior knowledge used to appropriately focus sampling campaigns. Sex determination and collection of ovary material generally relies on access to carcasses that have not been eviscerated. Access may be restricted when dealing with high value species.

The gonads can be found in the ventral part of the body cavity. In sexually mature fish, both male and female gonads can frequently fill the area available in the body cavity. Ovaries are usually tubular, pink/red, and granular while testes are flat, white/grey and their ventral edges frequently have a wave-like outline. In billfish, male gonads have a relatively uneven appearance and irregular shape, with many nodules present on the external surface. Cross sections of male gonads have a characteristic rectangular shape, and when sexually ripe, milt is easily seen. This can be contrasted with females, which generally have a smooth external appearance and in cross section the goad is oval in shape and occasionally has a lumen (hole) in the middle. The weight of the fish is another factor in determining the sex of individual billfish, since the males of most species are considerably smaller (on average) compared to females. For example, Atlantic blue marlin males rarely grow over 100kg (220 pounds), while can grow over 400kg (880 pounds) and are common over 125 kg (275 pounds). In theory, therefore, it is unlikely to find male Atlantic blue marlin over 125 kg dressed weight!

For estimation of length-at-maturity and fecundity, a length-stratified sampling approach is appropriate. Length ranges comparable to the expected length-at-age distributions, or smaller length ranges, can be used. The sampling approach may need to be modified within the limitations of funding, given the requirement to sample different seasons, areas and years. Additional stratification may be required to compensate for fish behaviour, including spawning aggregation or spawning migrations.

On collection of samples (see below), the following details should be noted:

Date, vessel, species, length, weight, sex, weight of gonads, gonad sub-sample weight, location of capture (latitude and longitude), tuna school type and school association, and a unique sample number referring to the fish, sample from that fish, and including the region of the gonad sampled.

To estimate proportions of sexually mature individuals, precise criteria for the classification of maturity must be defined. This is discussed over the following sections.

4.8.2 Maturity stages

The visual assessment of ovaries to determine maturity stage is felt to be an imprecise indicator of reproductive condition (West, 1990). The use of histological and/or chemical methods is recommended. Scales are included here for completeness (**Table 4.8.1**).

Table 4.8.1 Maturity stages for visual examination of large pelagic gonads.

Stage	Criteria	
	Males	**Females**
I	Gonads small ribbon-like, not possible to determine sex by gross examination	Gonads small ribbon-like, not possible to determine sex by gross examination
1	**Immature**; testes extremely thin, flattened and ribbon-like, but sex determinable by gross examination	**Immature**; gonads elongated, slender, but sex determinable by gross examination
2	Enlarged testes, triangular in cross section, no milt in central canal	**Early maturing**; gonads enlarged but individual ova not visible to the naked eye
3	**Maturing**; milt flows freely if testes pinched or pressed	**Late maturing**; gonads enlarged, individual ova visible to the naked eye
4	**Ripe**; testes large, milt flows freely from testes	**Ripe**; ovary greatly enlarged, ova translucent, easily dislodged from follicles or loose in lumen of ovary
5	**Spent**; testes flabby, bloodshot, surface dull red, little or no milt in central canal	**Spawned**; includes recently spawned and post-spawning fish, mature ova remnants in various stages of resorption, and mature ova remnants about 1.0mm in diameter

4.8.3 Histological sampling and analysis

Histological sampling is the most common approach used to ascertain maturity stage for large pelagic species.

Either the whole gonad, or if too large gonad cross-sections (1 cm thick) taken across the central region of the ovary, should be taken immediately after the fish had been caught. These should be fixed in Bouin's solution, neutral 10% formalin, or 4% formalin in seawater, for return to the laboratory.

The samples should be dehydrated in increasing ethanol concentrations, clarified in Histolemon and embedded in paraffin wax. Sections (5-10 µm thick) can then be taken using a microtome. Sections can be stained with haematoxylineosin (Harris' hematoxylin followed by Eosin counter stain) alone, or supplemented with Mallory's trichrome and Periodic acidShiff (Pas) reaction, before viewing under the microscope. Magnification (eyepiece and objective) should be stated.

For females, the oocyte classification scheme developed by Hunter *et al*. (1986) is recommended (**Table 4.8.2**). This classification scheme covers both the spawning frequency and the likelihood that a female with continue to spawn (through the atretic state of the ovary). If alternative schemes are used (e.g. Corriero *et al*. (2003) for bluefin tuna) they should be fully referenced and the interpretations of immature and mature status detailed.

Table 4.8.2. Maturity stages from ovary histology examination

Stage	Maturity	Oocyte condition	Atresia	Comments
1	Immature	Majority of oocytes in late diplotene or early perinuleus stage	No atresia	Densely packed oocytes darkly stained with hematoxylin
2	Immature	Mix of early and late perinucleus stage oocytes. No yolk granules present	No atresia or minor atresia of unyolked oocytes	Early developing stage
3	Immature	Partially yolked	No atresia or minor atresia of unyolked oocytes	Red staining yolk granules or globules evident from cell periphery inward to within ¾ of distance to perinuclear zone
4	Mature	May be unyolked or partially yolked	Atresia of fully yolked oocytes evident	Considered to have reached a fully yolked and potentially reproductive state but regressed to a reproductively inactive state
5	Mature	Fully yolked oocytes present by no post ovulatory follicles observed	Zero or less than 50% atresia of fully yolked oocytes	A mature, potentially reproductive fish
6	Mature	Fully yolked oocytes present. Oocytes may be in migratory nucleus or hydrated condition and/or post ovulatory follicles present	Less than 50% atresia of fully yolked oocytes, generally zero or minor atresia	An actively swimming spawning fish with zero or minor atresia
7	Mature	Fully yolked oocytes present. Oocytes may be in migratory nucleus or hydrated condition and/or post ovulatory follicles present	Atresia of 50% or more of fully yolked oocytes	An actively spawning fish with significant atresia
8	Mature	Some fully yolked oocytes present but none in migratory nucleus or hydrated condition. No POFs present.	Atresia of 50% or more of fully yolked oocytes	A potentially reproductive fish with significant atresia
9	Mature	No fully yolked oocytes but atresia of fully yolked oocytes evident	100% atresia of fully yolked oocytes	A mature fish in non-spawning phase
10	Mature	No fully yolked oocytes present. Oocytes resemble Stage 1 or 2.	Advanced atresia of oocytes	A mature fish in advanced atretic, post-spawning phase

This classification scheme can be simplified into a maturity classification system (**Table 4.8.3**).

Table 4.8.3. Maturity classification system based upon Hunter *et al*. (1986)

Category	Stage	Fully yolked oocytes present	POF present	Comments
Immature	1, 2, 3	No	No	Oocytes have never reached fully yolked condition
Mature	4 to 10	Yes for St. 5-8	Yes for St. 6, 7	Having developed fully yolked oocytes
Reproductively active	5, 6, 7	Yes	Yes for St. 6, 7	Fully yolked oocytes present
Spawning	6, 7, 5*	Yes	Yes for St. 6, 7. No for St. 5	Histological evidence of recent or imminent spawning
Reproductively inactive/atretic/post spawning	4, 8, 9, 10	Yes for St. 8	No	Had developed fully yolked oocytes but now regressed to partially or completely inactive condition

* considered spawning only if oocytes observed in migratory nucleus or hydrated condition

The diameter of a set number of oocytes (e.g. 350-400 per section) should be measured in microns to obtain frequency distributions of selected stages of oocytes development.

Spawning time and location can be based on specimens with hydrated oocytes in the ovaries, which indicate imminent spawning.

For males, Abascal *et al*. (2004) and Schaefer (1996) have both developed keys for testes development in separate tuna species. That of Schaefer provides a guide for male spawning status, while the descriptions of Abascal *et al*. describe the microstructural and histological stages which may be found in tuna testis.

Schaefer's classification (**Table 4.8.4**), developed for *T. albacares* in the eastern Pacific, is based on the size of the sperm duct (vas deferens), thickness of the myoid tissue surrounding the duct, the amount of spermatozoa within the duct, degree to which the duct was convoluted, whether the tissue adjacent to the duct appeared to be heavily nucleated, and the staining characteristic of the tissue adjacent to the duct (under Haematoxylin-eosin stain).

Table 4.8.4. Development stages from testes microscopic examination

Stage	Vas deferens contents and structure	Van deferens epithelial stain
Pre-spawning	Devoid of sperm, extremely convoluted	Darkly stained
Spawning or recently spawned	Sperm filled, open duct smooth along its border	No conspicuous dark staining

Evidence of recent spawning in male *T. albacares* from the eastern Pacific was only found about 12 hours after the spawning event. Sampling must therefore be temporally focussed.

Abascal *et al*. (2004) noted two distinct zones in the cross-section of *T. tynnus* testes. In the outer region, seminiferous lobules have a think wall formed by the germinal epithelium, where germ cells develop in association with Sertoli cells. The lumina of the lobules are filled with spermatozoa that have been released after completion of the spermiogenetic process. The release of mature sperm from spermatocycts into the lobule lumina results in the germinal epithelium becoming discontinuous. In the central region of the testis, testicular lobules lose the germinal epithelium and become ducts where lobule function has shifted from sperm production to sperm storage. Only mature spermatozoa are found in this region, which fill the swollen lumina of the lobules. Gamete stages are listed in **Table 4.8.5**.

Table 4.8.5. Development stages of spermatozoa from histological and SEM examination

Stage	Description	Location
Primary spermatogonia	Large, ovoid cells, nucleus with diffuse chromatin and single central nucleolus. Large numbers of chromatid bodies in cytoplasm	Distributed along the germinal epithelium
Spermatogonia	Result from successive mitoses of primary spermatogonia. Found in small groups. Nucleus contains patchy chromatin	
Primary spermatocytes	Clusters, with cells interconnected by cytoplasmic bridges. Heterochromatic nucleus. Cytoplasm contains free ribosomes	
Secondary spermatocytes	Seldom found in histological samples. Cytoplasm reduced, nucleus shows diffuse chromatin forming moderately electron-dense patches	
Spermatids and spermatozoa	Found in groups with heads facing the lobule walls and bundles of flagella directed toward the seminiferous lobule lumen. Early spermatids have spherical nucleus with dense chromatin. Chromatin becomes more homogenous in mid spermatids. Chromatin condenses into a coarse granular pattern in late spermatids. The nucleus also assumes an ovoid shape, and forms a basal indentation over the proximal segment of the axoneme. Flagellum elongates, cytoplasmic mass reduces, and mitochondria coalesce around the proximal portion of the axoneme. Flagellum remains parallel to the base of the nucleus in spermatozoon.	Held within spermatocysts

4.8.4 Chemical approaches

In many fisheries, fish are either landed already gutted, or the value of the flesh prevents ventral opening and the determination of sex and sexual maturation. Absence of sexual dimorphisms also makes external identification difficult. In these cases, molecular endochrinological approaches allow the sex and maturation stage to be identified from blood and tissue samples. Through the collection of muscle samples from a broad size range of individuals from each management unit, maturity at size can be estimated.

Blood and muscle can be sampled for chemical analysis for reproductive hormones. Samples from mature individuals are obviously of greatest interest. A muscle biopsy punch has been developed to allow samples to be taken (Bridges *et al*., 2000), which samples approx 100-150mg of muscle without obvious damage to the fish. Blood and muscle samples should be frozen following extraction.

Blood should be centrifuged to collect plasma (e.g. 5000 *g* for 15 minutes). Resulting plasma samples can be analysed directly. Muscle samples need to be homogenised and steroids extracted with Diclormethane before measurement.

Assessment can be performed using standard Enzyme-Linked ImmunoSorbent Assay (ELISA) methods for sex hormones (e.g. 17β-estradiol (E_2), 17α-20β-dihydroxy-4-pregnen-3-one (17,20β P), 11-ketotestosterone (11-KT)) and the lipoprotein vitellogenin (Vtg), which is synthesised under the influence of E_2.

Changes in steroid hormones and vitellogenin can normally be correlated with gonadosomatic index (GSI) and oocytes diameter. 17,20β-P can be used to determine the pattern of both egg and sperm release. The relationship between testosterone and 11-KT may be used to identify sex, while various sex steroid rations can be used to define both the maturity and sex of a given fish (e.g. presence/absence of Estradiol, vitellogenin).

It should be noted that while steroids are stable at room temperature for several weeks, vitellogenin samples require storage at low temperatures.

4.8.5 Estimation of maturity-related features

The statistical procedure for deriving a maturity schedule involves fitting an appropriate weighted non-linear predictive regression model directly to the maturity data. The model can then be used to predict proportions sexually mature at specific lengths and/or ages. Also, statistical evaluations of spatial and temporal variation in

maturity functions can be conducted on the data. Maturity at length can be estimated through a logistic curve of the form:

$$\%mature = \frac{100}{1 + e^{-a(length+b)}}$$

or in the linear form:

$$\ln\left[\frac{p}{1-p}\right] = \alpha + \beta * length$$

where p is the probability that a tuna as mature, α and β are linear regression parameters of the model, and length is the fish length. 95% confidence intervals should be calculated. If length-stratified sampling has not been used, the model fit should be weighted by the number of samples at each length class, to ensure that limited sample sizes at the extremes of the sampled length range do not overly influence the fit of the model.

For billfish species, gonadal index (GI) can be calculated. This is the relationship between ovary weight (O_w) and length. Lower jaw fork length (LJFL) is generally used. GI is then:

$$GI_{LJFL} = \frac{Ow}{LJFL^3} * 10^4$$

(Albaret, 1977 ; Cayré and Laloé, 1986). Beyond a critical value of the GI, particular to each species, it is accepted that the individual studied is in a state of sexual maturity GI in swordfish greater than or equal to 2.09 is an *a priori* indication of females in an active reproductive stage (García Cortés and Mejuto, 2003). 95% confidence intervals should be calculated for GI where possible.

Gonadosomatic index (GSI) is the ratio between the gonadal weight and body weight, and can be indicative of the maturation state:

$$GSI = \frac{W_G}{W} * 100$$

where W_G is gonad weight, and W is the gonad-free weight of the individual. If a gonadosomatic index is calibrated, for example through the use of histology, it may be used to determine spatio-temporal spawning distributions. It is not sufficiently accurate for the classification of maturity or reproductive activity, however. This relies on the analysis of detailed histology data.

Estimation of the annual fecundity in tunas requires spawning frequency estimates by length classes, and corresponding estimates of batch fecundities over the length range of mature females. Knowledge of the appearance and longevity of postovulatory follicles in ovaries after spawning is necessary for estimation of spawning frequency. The frequency at which ovaries of mature females contain postovulatory follicles can then be used to estimate spawning frequency. Larger females appear capable of maintaining a higher spawning frequency.

Only at the final stages of oocyte maturation, beginning with the migratory-nucleus phase and followed by hydration, is there a distinct hiatus in the distribution of oocytes from which the batch fecundity estimates can be derived. Batch fecundity should only be estimated from ovaries in a hydrated but pre-ovulatory condition. Any loss of oocytes would bias fecundity estimates. Since many tuna are serial spawners (e.g. Corriero *et al*., 2003), fecundity estimates based on non-hydrated ovaries can significantly over-estimate batch fecundity as successive spawning batches cannot be clearly differentiated until the onset of hydration. Batch fecundity should be determined using the gravimetric method that counts the number of hydrated oocytes present in a weighted subsample of ovarian tissue (O_w). Sections of ovigerous lamellae should be taken from the anterior, middle and posterior region of the ovary. Each of these samples should be spread evenly over a microscope slide (longitudinal etching of the slide can aid counting), saturated with a glycerine solution and covered with a slide cover. The number of hydrated oocytes present in each sample should be counted three times, and the numeric mean of hydrated oocytes applied to calculate batch fecundity:

$$B_f = H_e * O_w$$

where B_f is batch fecundity, H_e is the number of hydrated oocytes per unit of weight in the tissue sample, and O_w is the ovary weight. Estimates from the anterior, middle and posterior sections of the ovary can then be averaged to yield an estimate representative of the entire sample. Multiple samples and individuals should be taken to obtain some measure of variability in batch fecundity estimates.

Batch fecundity at length is usually described by a power function of the form:

$$B_f = cL^b$$

where L is the length of the fish and c and b are estimated parameters. Batch fecundity at weight is usually described by a linear relationship of the form:

$$B_f = aW + b$$

where W is the weight of the fish.

Annual fecundity can then be estimated from batch fecundity estimates (number of oocytes released per spawning) and spawning frequency.

4.8.6 Further reading

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