CRC REEF RESEARCH TECHNICAL MANUAL

DEFINING THE REPRODUCTIVE BIOLOGY OF A LARGE SERRANID *PLECTROPOMUS LEOPARDUS*

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FOREWORD

The authors have successfully produced a comprehensive guide to laboratory techniques for quantifying the gonadal development and fecundity of a large exploited grouper on the Great Barrier Reef.

The management of tropical fisheries is dependent on accurate estimates of demographic parameters. To quantify reproductive parameters such as fecundity and seasonal maturity schedules, scientists must apply rigorous and standardised laboratory methods. This technical manual pulls together methods and information from a wide range of sources to provide an important contribution to the establishment of such laboratory protocols.

The manual presents a useful guide for both students and practitioners of the reproductive biology of teleost fishes.

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1.0 Introduction

There are three main objectives to this manual. First, to provide a brief description of the ontogenetic and sexual development of gonadal tissue in the common coral trout, *Plectropomus leopardus*, as an introduction to the reproductive biology of a tropical protogynous hermaphrodite (section 2). Second, to provide details of the laboratory methods that we used to measure the reproductive stages and batch fecundity of *P. leopardus* (section 3). These were histology of gonad sections using haematoxylin and eosin stains, and counts of whole oocyctes, both standard procedures in reproductive studies. Third, to define the reproductive developmental stages in females and males, illustrated with colour plates, using detailed criteria to establish a standard for staging *P. leopardus* (section 4). We also discuss problems that we encountered in staging the gonads of *P. leopardus*, in section 5, to highlight some typical errors in histological interpretations, and provide some guidance in minimising these errors. Finally, in section 6, we provide brief concluding remarks. Much of this work is not new. We have relied heavily on previous reproductive work on *P. leopardus* by Ferreira (1995) and Adams (1996). Our aim is to synthesise information available from a variety of sources and provide a focussed laboratory guide for assessing the reproductive biology of a large exploited serranid on the Great Barrier Reef. Laboratory protocols developed for the CRC Reef's Effects of Line Fishing Project (Davies et al. 1998, Mapstone et al. 1998) have built on the work described in this manual.

We have structured the report to reflect the objectives described above. We have also included a glossary to assist with terminology. In section 2 we have referred to a number of developmental stages with which the reader may not be familiar. These terms are fully described in section 4 where we define the developmental stages in detail.

2.0 Gonad development in *Plectropomus leopardus*

2.1 Maturity

P. leopardus is a protogynous hermaphrodite (Goeden 1978, Ferreira 1995), therefore a complete ontogenetic series in sexual development from very young immature fish to older sexually mature individuals, is only found in females. Males do not pass through an immature phase because they develop from mature females. Coral trout are considered to be monandric

(Ferreira 1993, 1995), that is males arise from one pathway: through sex change in females (Reinboth 1967). Such males are termed secondary males.

Females can be classified as either immature (juveniles) or mature depending on the ontogenetic stage of development. Minimum size or age at first reproduction may be used to define the stage at which females reach ontogenetic maturity. This has been estimated at 32-36cm FL and 2-3 years for *P. leopardus* by Ferreira (1995), and at 28cm FL and 1-2 years by Adams (1996). Mature females may have either inactive (resting) or mature (ripe) ovaries depending on the seasonal schedule of reproduction. Developmentally, this can be clearly defined by the presence of vitellogenic oocytes.

Individuals that are changing sex are termed transitionals and can be identified by a number of developmental characteristics co-occurring in gonads comprised of ovarian tissue. Fish which have recently changed from female to male will have partially developed testes, though they are nevertheless functional males. These are classified as incomplete males based on the proportion of testicular tissue in the gonad. Males in which the testes are fully developed are classified as complete (Adams 1996). This classification is somewhat arbitrary, but the developmental pathways for testicular tissue (spermatogenesis and spermiogenesis) are poorly correlated with seasonal schedules. Therefore, unlike females, stages of gametogenesis in males are difficult to use for defining maturity.

Pre-maturational sex-change, where males develop from immature females, may also occur in *P. leopardus*. Ferreira (1993) reports one male developing directly from an immature female for the con-specific *Plectropomus maculatus* based on the observation that "no signs of previous spawning were present and the macroscopic appearance of the gonad was that of an immature ovary". Ferreira (1995) also reported pre-maturational sex change in *P. leopardus*, though numbers of individuals were not provided. Adams (1996) reports for *P. leopardus* "one male that changed sex prior to maturing as a female". Neither authors provide details of the criteria used to determine that the female phase was juvenile. Pre-maturational sex change has been reported in other protogynous hermaphrodites (Sadovy and Shapiro 1987) such as the emperor *Lethrinus nebulosus* (Ebisawa 1990), the gobiid *Coryphopterus personatus* (Cole 1983), and the scarid *Calotomus spinidens* (Robertson et al 1982).

The sexual maturity or developmental stage of a gonad is defined by the stages of oocytes or spermatocytes present. However, this is somewhat complicated by the fact that *P. leopardus* are multiple spawners (Goeden 1978, Ferreira 1995). This means that oocytes and spermatocytes develop asynchronously (Ebisawa 1990, West 1990, Nagahama 1983, Wallace and Selman 1981). Thus the gonads of sexually mature females contain oocytes in various stages of development, from pre-vitellogenic to vitellogenic oocytes (Ferreira 1995). Similarly, males may contain sperm cells at various stages of development, from spermatocytes to spermatozoa, though spermatozoa are present for large portions of the year.

2.2 Fecundity

Fecundity, or egg production, is usually expressed as annual fecundity: the number of eggs produced per year, by an individual, size or age class, population or stock. Counts of whole hydrated oocytes are used to measure fecundity. Annual fecundity is termed determinate in those species that produce all their eggs at one time such as haddock (*Melanogrammus aegelfinus*, Hislop et al 1978), whiting (*Merlangius merlangus*, Hislop 1975) and other species (Yamamoto 1956). In these species counts of hydrated oocytes at the time of spawning can provide a direct estimate of annual fecundity. In contrast, indeterminate fecundity is seen in fishes that continuously mature new batches of eggs throughout a typically protracted spawning season (Hunter et al 1985). This is recognised by the presence of oocytes in all stages of development in ripe females, as seen in *P. leopardus* (Goeden 1978, Ferreira 1995). Annual fecundity for species that spawn several batches within a season is less directly measured as a function of batch fecundity and the number of spawnings per year, or spawning frequency (Hunter and Macewicz 1985). Batch fecundity refers to the number of eggs produced in a single spawning batch (Hunter et al 1985).

Hydration represents the final vitellogenic stage prior to spawning. Hydrated oocytes are first seen within the ovarian lamellae. At ovulation they are released into the lumen. This should be considered when processing samples for oocyte counts. If ovulation has occurred oocytes may be lost from the lumen during tissue preparation (West 1990). It is therefore more accurate to measure batch fecundity of ovaries which are hydrated but have not yet ovulated. The latter is detected by the presence of free oocytes in suspension in preserved samples. With fresh samples (see below), this distinction is difficult and therefore batch fecundity measures will be based on hydrated oocytes that may be either within the lamellae or the lumen. A third

distinction is seen in samples obtained by "stripping" hydrated females. This technique, which involves applying firm hand pressure to the ventral surface of the fish (Rimmer et al 1994), is used to express eggs that have been released into the lumen (Figure 1). Counts from these samples will only give numbers of ovulated oocytes.

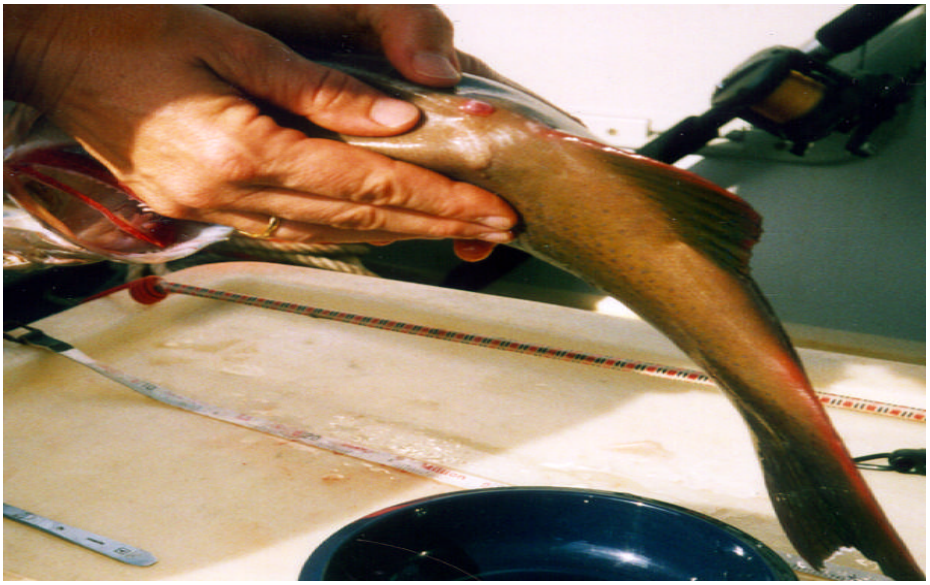

Figure 1. Expressing ("stripping") eggs from a ripe female *Plectropomus leopardus*.

3.0 Laboratory methods

3.1 Histology

Gonads were collected from Scott and Elford reefs on the northern Great Barrier Reef in 1992 and 1993 (Samoilys in prep.), and were preserved at sea in FAACC (Winsor 1984, 1991, 1994, see Appendix 8.2) within two hours of capture. For best results, samples should be transferred to 70% alcohol after one week of fixation. Here, histological processing of samples was conducted in the laboratory 6 - 18 months after capture without transferring samples to 70% alcohol. Although this is not recommended (Winsor pers. comm., Reilly pers. comm.) the appearance of the stained sections was clear, but there was a deterioration in the basophilic components of the tissue in some sections. This was due to the acid present in the FAACC. If samples are to be retained for bng periods without transferring to alcohol, 10% phosphate buffered formalin is recommended rather than FAACC.

3.1.1 Preparation of tissue blocks

All preserved gonads were weighed to 1.0g. Those used for whole oocyte counts (see below) were weighed to the nearest 0.1g.

Tissue sections were taken from the right lobe (looking down on the dorsal surface of the fish). If the right lobe was damaged (ie. spear damage during sampling, poor dissection), the left lobe was sectioned and the discrepancy noted for those samples. Histological assessment of both lobes of *P. leopardus* found no differences between the two (Ferreira 1995), therefore this study standardised to the right lobe. Three sections, 2-4 mm in thickness, were made: Proximal (P), Medial (M), and Distal (D). Proximal sections were cut as close to the junction between the two lobes as possible. Medial sections were taken from the middle portion of the lobe where the lobe showed a uniform thickness (Figure 2). Distal sections were cut approximately 2 - 4 mm from the end of the gonad.

Figure 2. Cutting the medial transverse section from a *Plectropomus leopardus* gonad.

Tissue sections were placed in tissue processing cassettes. All sections from the one gonad were placed in the same cassette. Large P, M and D sections that would not fit into the cassettes were separated either individually or as suitably sized pairs of tissue ie.[MD] sections together. Each cassette was marked with an 2B pencil ie. 523P, 546MD etc. The tissue cassette was then placed in 70% alcohol for a minimum of two hours.

Hydrated (see section 4) female gonads, including those that were stripped for oocyte counts (see below) were processed using agar moulds to hold the tissue together. The agar solution contained 3% Agar and 10% Formalin. The formalin preserves the agar (L.Winsor pers. comm.). Two – four millimetre sections (P,M,D) were cut for each gonad, as described above. Each section was then placed in melted agar in a metal mould. Having waited approximately 5 min for the agar to set, the agar block was removed from the metal mould and placed in a plastic cassette, labelled, and processed normally (see below).

Eggs that had been stripped from hydrated females (see below) were also processed for histological examination, to confirm that they were hydrated. Using a pipette, a sample of eggs was placed in the top of a syringe in a syringe unit (after Cook and Hotchkiss 1977, Figure 3). Excess formalin was removed with the pipette. Melted agar was then added and stirred gently to mix the eggs but keep them near the bottom of the syringe. Having waited approximately 5 mins for the agar to set the syringe was then ejected. The top half of the agar block was removed with a razor blade and discarded. The bottom 3-4mm of agar block was then placed in a plastic cassette with label and processed normally (see below).

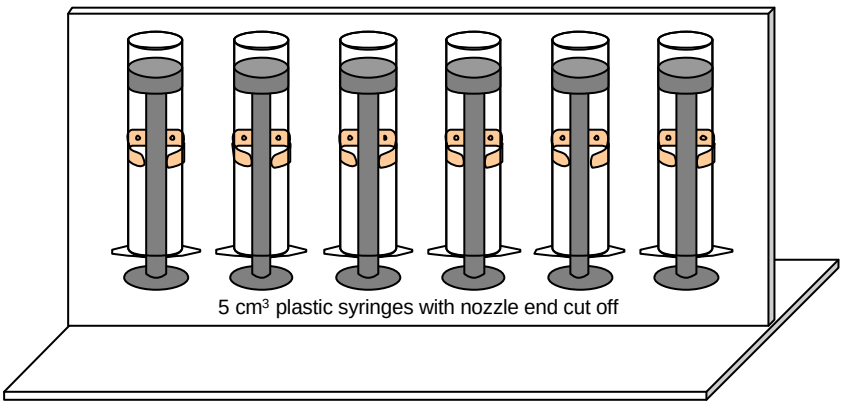

Figure 3. Diagram of a syringe unit (after Cook and Hotchkiss 1977) used for processing ovulated eggs histologically.

3.1.2 Processing of tissue

Gonads were routinely processed to paraffin wax using an automatic tissue processor (Winsor 1994). This machine dehydrates the samples, passing them through a graded series of ethyl alcohol; three changes of absolute ethyl alcohol; two changes of xylene; and finally impregnates them with paraffin wax. The cassettes were then placed in Labec Vacuum Setup for 30 minutes at 50 kPa, to complete the impregnation of the tissues with paraffin wax and remove any remaining air bubbles trapped within the tissue. The tissue samples were then embedded in paraffin wax using metal moulds, paying particular attention to the orientation of the individual samples (Figure 4). Tissue samples were then sectioned using a rotary microtome (American Optical (AO)). Sections were cut at 5µm (after Ferreira, 1993, 1995).

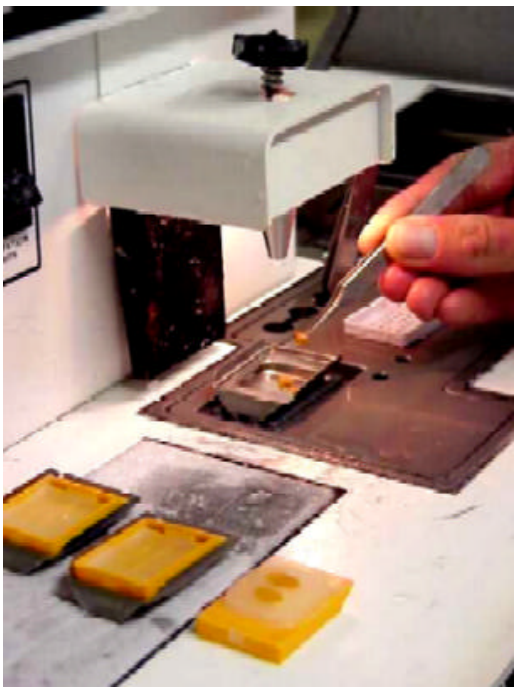

Figure 4. Embedding tissue sections in wax blocks at an embedding centre.

A ribbon of sections was cut from each block, and floated out on a waterbath set at 40°C and containing gelatine to aid adhesion of the section to the glass microslide. Two to three sections were then recovered onto microslides. Temperatures >40°C were found to be too hot for the very large ripe females. All microslides were cleaned in alcohol prior to mounting of the sections. The orientation of the proximal, medial and distal sections on the microslide (Figure 5) reflected the arrangement of tissue samples set during embedding (see above).

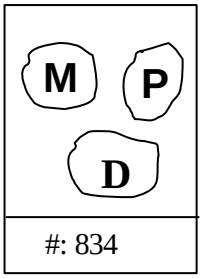

Figure 5. Example of position of different tissue sections on a glass microslide, to facilitate recognition.

Slides were placed in a jar containing approximately 5 mls of formalin and the jar placed in an oven (60°C) for 30 minutes. The heat causes formalin to form a vapour which aids adhesion of tissue sections to the microslides. Microslides were then removed from the formalin vapour jar, placed in a slide rack, and returned to the oven (60°C) to dry for a minimum of 1hr 30min (usually overnight) prior to staining.

Sections were routinely stained using Mayer's Haematoxylin and Young's Eosin-Erythrosin stains (Winsor 1991, 1994) until the desired cytological elements were clear in the tissue section. Haemalum is basophilic and stains nuclei blue. Eosin is acidophilic and stains cytoplasm pink-red. Staining involved a routine process where tissue sections are dewaxed and taken to water, stained, then dehydrated and cleared. Histoclear, which contains the transition solvent limonene (Winsor 1994), was used as the clearing agent. Stained slides were mounted with a coverglass using DePex mounting medium.

3.1.3 Reading histological sections - assigning stages

Slides were read in random order to avoid possible biases in interpretation associated with prior knowledge of collection time and fish size. Gonads were assigned to a developmental stage based on the most mature cell(s) present, regardless of how many there were (West 1990, Sadovy et al 1994). It is unusual for the most mature cells to be rare. For example, in the present study, in defining running ripe or hydrated females, 7 females out of a total of 117 running ripe females were found with only 1-2 hydrated oocytes.

The proximal, medial and distal sections were examined from 742 gonads. The sex structure of the population is shown in Table 1. Bisexuals were individuals which possessed both female and male cells capable of spawning at the same time (see section 4.2). Differences in development stage along the length of the gonad were not found by Adams (1996, n=30) who also examined longitudinal and saggital sections, and apparently were not examined by Ferreira (1993, 1995). To conform with Ferreira's (1993, 1995) and Adams (1996)'s work on coral trout reproduction, the medial section was taken as the standard, and different stages or cells in the proximal or distal sections were noted separately.

Table 1. Sex structure of population determined by reading all three sections (D,M,P) or by reading the medial section alone (M).

Sex	**D, M, P sections**	**M section only**
Female	440	445
Transitional	66	61
Male	243	243
Bisexual	3	3

Readings of proximal, medial and distal sections differed for 129 fish (17%, Table 2). These differences were analysed in terms of the error associated with reading the medial section only. The most accurate reading is that which detects the most advanced stage and any other cells used for defining reproductive status (e.g. post-ovulatory follicles).

Of the 129 fish that showed differential development along the length of the gonad, ten differed with respect to sex (1.3 % of the population) and these were all transitionals. Of the ten individuals, five would have been incorrectly sexed (classified as females) if only the medial section was read (Table 2). Thus, in terms of sexing a population, error rates are low (1.3%), but in terms of detecting transitionals within a population, the error associated with using medial sections only is relatively high (8.2%).

Table 2. Errors associated with reading medial sections alone when sexing and staging coral trout gonads. Number refer to the number of fish from a total sample of 742 individuals. Percentage error relates to the overall uncertainty or potential for error based on the number of gonads with a non-uniform development. * = based on using all three sections.

Category	**Total number in sample***	**Number with non-uniform development**		**Number incorrectly staged if medial section read**	
		Number	**%**	**Number**	**%**
All	742	129	17.4	N/A	N/A
Sex – all	742	10	1.3	5	0.7
Sex - Transitionals	66	10	16.3	5	8.2
Stage – Females	440	7	1.6	3	0.7
Stage – Males	243	0	0	0	0
Most mature cells – Female	440	14	3.2	6	1.4
Most mature cells – Transitionals	66	1	1.5	1	1.5
Most mature cells – Male	243	0	0	0	0
POFs	117	17	14.5	3	2.6

With respect to staging (e.g. Resting versus Ripe in females, see section 4.1 below), only females showed differences between medial and other sections (no stages were defined for transitionals, see section 4.2 below). Stages differed between sections for seven females (Table 2). Differences were seen between Running ripe and Ripe stages (n=5), for example hydrated oocytes not present in all sections, and between Resting and Ripe (n=2), for example yolk globule stage oocytes appearing in distal or proximal sections with only pre-vitellogenic cells in the medial section. Of these, three individuals (0.7% of all females) were less developed in the medial section compared with the proximal and/or distal sections, and would therefore have been incorrectly staged.

Within stages, the most mature cells were distributed unevenly in 14 females (excluding the 10 female/transitionals described above) and one transitional. Thirteen females differed within the ripe stage (e.g. yolk globule vs migratory nucleolus stages) and one differed within the resting

stage. In each case reading the medial section only would have classified 6 of these females incorrectly, i.e. the most mature cell would not have been detected (Table 2).

Other differences were minor and related to the non-uniform presence of cells such as atretic oocytes, post-ovulatory follicles, brown bodies and parasites. For example, of the 117 females with post-ovulatory follicles, only 3 would have been incorrectly classified as not having recently spawned if only the medial section was read (Table 2).

Despite these differences between sections, there was no consistent pattern in sexual development along the length of the gonad. For example, there was no gradation in development from proximal to medial to distal. On the basis of the low error rates between section readings it was concluded that taking the medial section as the standard reading was acceptable. Nevertheless, additional sections are recommended as a back- up because coral trout gonads are frequently infected with parasites which can engulf large sections of the gonad.

3.2 Whole oocyte counts for batch fecundity estimation

Counts of whole hydrated oocytes for measures of batch fecundity were done in volumetric or gravimetric sub-samples (Hunter et al 1985). Both techniques are described below. The gravimetric method was used on a subsample of hydrated ovaries collected and preserved in 1993 for maturity staging. The volumetric method was used for fresh ovaries (collected in 1994) and for samples of eggs that had been expressed (stripped) from freshly caught hydrated females in 1993. Fresh ovaries were collected and processed in 1994 because it was difficult to process the preserved samples from 1993 without damaging the oocytes. This problem was identified by Lowerre-Barbieri and Barbieri (1993), who developed a new technique for separating eggs prior to preservation. The method requires fresh ovaries, and therefore we obtained a small sample to trial this technique in 1994.

3.2.1 Gravimetric method

Twenty-six hydrated ovaries (preserved in FAACC) that had been collected during the new moon period in the afternoon or evening of the 1993 spawning season were selected for oocyte counts. Total gonad weight (to 0.1 g) was obtained prior to any sectioning (see above).

From each gonad three transverse tissue blocks approximately 2mm in width were cut as sub-samples. One block was taken from each of the proximal, medial and distal sections of the left lobe. Preferably, batch fecundity should be measured in both lobes to test for differences between lobes, but was not done here because the right lobe had already been dissected for histology (see above). Therefore, the estimates are relative estimates, standardised to the left lobe, and should be interpreted as such. Tissue samples were large enough (approximately 10mg) to contain 100 - 200 oocytes (Hunter et al 1985). Each section was weighed to 0.1g. The tissue sample was placed on a slide and covered with 33% glycerol for ~15 min. Oocytes were then teased apart and pipetted onto a raceway for counting under a dissecting microscope at 10-15X. Only the hydrated oocytes were counted. Hydrated oocytes are large, white and contain an oil droplet, characteristics seen in the hydrated oocytes obtained from the stripped females (see below). Oocyte counts per gram were extrapolated to the whole gonad weight, then averaged from the three blocks (sub-samples), to estimate the total number of hydrated oocytes per gonad.

3.2.2 Volumetric Method

The two samples that were processed volumetrically were prepared as follows:

Fresh ovaries

Ten fresh gonads were collected in 1994 and each whole ovary weight was measured to 0.1g. Gonads were then cut into right and left lobes and each lobe weighed to 0.1g. After separation of the oocytes from the ovary membrane (see below) the empty gonad sac was also weighed to 0.1 g. Any oddities in the tissue such as parasitism were noted.

Oocytes were separated from the left lobe while being held over a collection sieve. The sieve was made from a piece of nylon plankton net (250 ?m mesh) attached securely to the end of a 15 cm diameter PVC pipe, with a depth of 15 cm (Figure 6, after Lowerre-Barbieri and Barbieri, 1993). The width and depth of the pipe allowed manipulation of the gonad without spillage.

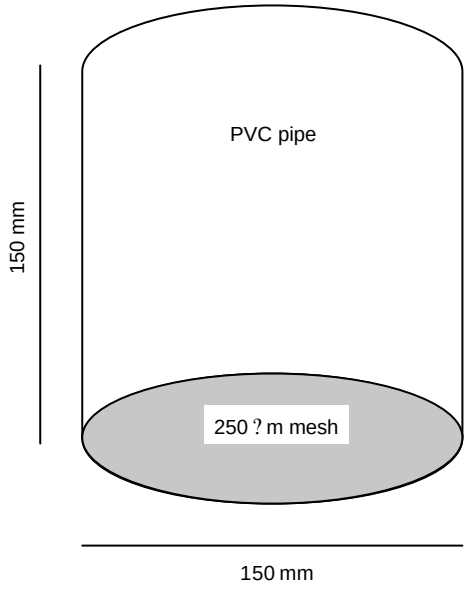

Figure 6. Diagram of the mesh sieve used to separate hydrated oocytes in fresh gonads.

Separating oocytes from the ovarian tissue first involved cutting a longitudinal slit with a scalpel down the length of the lobe. Holding the opened lobe over the 250 ?m mesh sieve, the oocytes were then dislodged by directing a strong, fine jet of water at the ovarian tissue. A length of 1 cm diameter soft plastic tubing attached to a standard laboratory faucet provided the jet of water. Typically, each lobe took between 10 and 15 minutes to be cleared of oocytes. Parasitised samples took a little longer to clear as connective tissue around the parasites tended to hold the oocytes together.

Once all the oocytes were removed from the ovary, they were then washed into a funnel using the 250 ?m mesh plankton net as a filter. The oocytes were then transferred to containers where they were preserved in 2% neutrally-buffered formalin.

Stripped eggs

Eight females were stripped in 1993 to express ovulated hydrated oocytes (Figure 1). The eggs were immediately placed in 10% formalin which turns them white and hard (more so than FAACC). The gonad was also fixed and preserved in 10% formalin for histological processing (see above). Traditionally Gilson's fluid has been used as a preservative for egg

counts because it hardens the oocytes and chemically separates them from the ovarian stromal tissue (Hunter et al 1985, Lowerre-Barbieri and Barbieri 1993). However it has substantial problems of oocyte shrinkage and destruction of hydrated oocytes, and it is also extremely toxic (Hunter et al 1985, Lowerre-Barbieri and Barbieri 1993). The destruction of hydrated oocytes makes it particularly inappropriate for fecundity estimation.

The freshly separated oocytes preserved in 2% buffered formalin (1994 samples) and the oocytes from the stripped females in 10% buffered formalin (1993 samples) were counted using the volumetric method described by Hunter et al (1985). The total volume of eggs was first measured to 1.0 ml in a graduated cylinder. Buffered formalin was added to round up the volume to a convenient number. To subsample, the oocytes were gently stirred to suspend them as evenly as possible in the formalin. 1.0 ml of the suspended oocytes was then extracted quickly with a Gilson pipette and placed in a glass raceway. Speed was important to obtain an evenly distributed subsample and to prevent blocking of the pipette. The total number of oocytes in the raceway was counted with a dissecting microscope at 10-15X. The oocytes were then replaced in the jar and the process repeated to obtain $\geq$3 replicate subsample counts from each ovary. As in the gravimetric method, oocyte counts per 1.0 ml were extrapolated to the whole volume, then averaged across sub-samples to estimate the total number of hydrated oocytes per gonad.

Note that the present study did not compare batch fecundities between right and left lobes and therefore, extrapolating egg counts from the left lobe to the total gonad weight or volume may be inaccurate. However, a paired *t*-test comparison between left and right lobe weights collected in 1994 revealed no significant differences between the lobes ($t = -1.181$, $df=9$, $p=0.268$).

4.0 Developmental stages of maturity defined

The maturity stages defined as a standard for histological interpretation of *P. leopardus* gonads are listed and described in the following tables. A description of female and male gametogenesis also follows. The definitions have been developed from a sample of 752 gonads collected from Scott and Elford Reefs in 1992-1993, with considerable reference to previous work on *P. leopardus* by Ferreira (1994,1995) and Adams (1996), and to other relevant texts

(Smith 1965, Yamamoto et al 1965, Yamamoto 1969, Nagahama 1983, Sadovy and Shapiro 1987, Ebisawa 1990, Sadovy and Colin 1995, Burton et al 1997). Assessment was made from the medial section of the gonad which gave 445 females (of which 35 were immature), 243 males, and 61 transitionals (see section 3.1.3, Table 1). The number of individuals of each maturity stage in the present study are also shown in the tables of definitions.

Since coral trout are multiple spawners the gonads contain oocytes and sperm cells at various stages of maturity. For females the stage of maturity is quite clearly defined by the most mature oocytes present (see section 3.0). Other criteria typical of each stage which assist in defining female maturity stage are also listed. For the transitionals and males, other criteria, such as features indicative of previous spawning as a female, and the development of the dorsal sperm sinus, are used to define sexual maturity, together with sperm cell stage. The diagnosis of male maturity schedules, however, is problematic because spermatozoa are present in male testes for long periods of the year (Samoilys in prep.).

4.1 Females

Female maturity stage can be clearly defined by whether the oocytes are vitellogenic or pre-vitellogenic (Table 3, section 4.1.2). Further definitions relate to hydration, the final stage of maturity prior to ovulation, and atresia, the process of degeneration (section 4.1.2). Final oocyte maturation and ovulation are not necessarily associated (Nagahama 1983). However, in *P. leopardus* it was extremely rare to find hydrated oocytes that had become atretic (residual). Most atretic oocytes were from the preceding yolk globule stage suggesting that once hydration occurs ovulation will follow.

Note that Ferreira (1993, 1995) did not differentiate ripe and running ripe into separate stages as defined here (Table 3). For *P. maculatus* Ferreira (1993), defined the ripe stage with either yolk vesicle, yolk globule or hydrated oocytes present. Ferreira (1995) varied this slightly for *P. leopardus* with yolk vesicle and early (primary) yolk globule oocytes defined as ripening and late (tertiary) yolk globule and hydrated oocytes defined as ripe.

The criteria for distinguishing between immature females and mature resting females are not infallible. This problem is discussed in section 5.

Table 3. Female gonadal development stages for *P. leopardus*, abbreviations refer to Plates. Defining characteristics are shown in bold italics. All oocyte cells that may occur are listed in developmental order (early stage first). Other criteria are useful aids in assigning gonads to a developmental stage. Numbers in parentheses refer to total number of individuals in sample.

Developmental Stage	Plate	Oocyte Stages	Other Criteria
Immature, **IM** **(35)**	**1**	***Pre-vitellogenic oocytes***: ?? oogonia, **oo** ?? chromatin nucleolus, **cns** ?? early perinucleolus, **eps** ?? late perinucleolus, **lps**	no sign of prior spawning: ?? ***no brown bodies*** ?? ***thin gonad wall*** ?? compact, lamellae well packed
Resting, **RE** **(134)**	**2**	***Pre-vitellogenic oocytes*** (as above)	***brown bodies (bb) common*** (102 fish) but not always present ***thick gonad wall*** lamellae not compact, often vaculated
Ripe, **RI** **(127)**	**3**	***Vitellogenic oocytes***: ?? yolk vesicle, **yv** ?? early and late yolk globule, **yg** ?? migratory nucleus stage, **mns**	may have atretic oocytes (**ao**), post-ovulatory follicles (**pof**) or brown bodies from previous spawning
Running ripe, **RR** **(117)**	**4**	***Hydrated oocytes***, **hy**	post-ovulatory follicles and atretic oocytes may be present
Spent, **SP** **(32)**	**5**	***Atretic vitellogenic oocytes***, **ao** ***Pre-vitellogenic oocytes***, **eps, lps**	lamellae disrupted and disorganised vascularised, vaculated, though not in early Spent stage brown-bodies generally present

4.1.1 Post - ovulatory follicles

When hydrated oocytes are released (ovulated) into the lumen they leave a ruptured follicle termed the post-ovulatory follicle (POF). These follicles have a brief life; they generally last around 24hr in the tropics because their rate of absorption is determined by water temperature (West 1990). In *P. leopardus* POFs were found to last around 24hr (Samoilys in prep.). The stages of disintegration of POFs can be distinguished, in other words POFs can be aged (Hunter and Macewicz 1985) to provide a more precise indication of time of spawning and to calculate spawning frequency. Based on the descriptions of *Engraulis mordax* (northern anchovy) POFs by Hunter and Macewicz (1985), four stages were recognised for *P. leopardus* (Table 4). Note that POFs breakdown completely after the Late stage and cannot be distinguished. The position of the nuclei are illustrated in Figure 7.

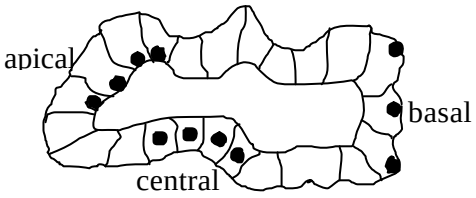

Figure 7. Position of nuclei in post-ovulatory follicles (see Table 4).

Table 4. Post - ovulatory follicle stages

STAGE	PLATE	DESCRIPTION		
		Folds	**Nuclei**	**Other**
Very early, 1	**6**	many and very convoluted	apical, clearly visible	2 follicle membranes: thecal layer + granulosa layer
Early, 2	**7**	many and convoluted	apical or central, clear	granulosa cells clearly defined
Mid, 3	**8**	fewer and not distinct	basal or pycnotic, but some may still be central or apical	granulosa cells less defined
Late, 4	**6**	none - compact	pycnotic or not visible	messy looking and contracted

4.1.2 Description of female gametogenesis

(abbreviations relate to Tables and Plates)

Oogenesis (pre-vitellogenic stage):-

*Oogonia (**oo**):-*

Small round cells, in nests, with separated chromosomes in nucleus (Stage 1, Ferreira 1994, Adams 1996).

*Chromatin nucleolus stage (**cns**):-*

Chromosomes no longer well separated, and form a network in nucleus (Stage 2 Ferreira 1994, Stage 1 Adams 1996).

*Early perinucleolus stage **(eps**):-*

Perinucleolus stains very darkly (Stage 3 Ferreira 1994, Stage 2 Adams 1996).

Late perinucleolus stage:-

Perinucleolus stains more faintly (Stage 4 Ferreira 1994, Stage 2 Adams 1996).

Vitellogenesis (vitellogenic stage = "ripening", or 2^{o} growth phase):-

*Early yolk vesicle stage (**yv**):-*

Yolk vesicles appear in the cytoplasm, dark lipid globules appear, granulosa cells' nuclei become differentiated.

*Late yolk vesicle stage (**yv**):-*

Lipid globules are more abundant and stain darkly; difficult to distinguish from early yolk vesicle stage, and were combined here (both vesicle stages: Stage 5 Ferreira 1994, Stage 3 Adams 1996).

*Yolk globule stages (**yg**):-*

There are primary, secondary and tertiary yolk globule stages and it is difficult to tell these apart. The stage depends on the position of the nucleus. In these stages the yolk vesicles are coalescing. The nucleus is not always seen in section, it depends on the position of the section (Stages 6 & 7 Ferreira 1994, Stage 4 Adams 1996).

Note: yolk globule stage oocytes will not necessarily proceed to full maturation (hydration) – see atretic stage below.

*Migratory nucleus stage (**mns**):-*

Yolk globules coalesce to form large empty spaces, typically 1-2, and the nucleus completes its migration to the cell membrane. This stage represents the start of hydration (Nagahama 1983, West 1990).

*Hydrated oocyte stage (**hy**):-*

The yolk globules have all coalesced and separated from the cell membrane. The centre of the cell appears all the same colour (pale pink) and the radiata is translucent and thinner (Stage 8 Ferreira 1994, Stage 5 Adams 1996).

Degenerating stage:-

There is a variety of degenerating cells present in female coral trout which aid in staging the gonads. These are described below. The Spent stage (Table 3) is defined by the presence of atretic oocytes

but only once the gonad has reverted to a pre-vitellogenic state. Atretic oocytes are often seen in ripe females, and therefore cannot be used alone to define the Spent stage.

Atresia :-

Atretic mature oocytes are collapsing un-ovulated oocytes. If the oocyte does not ovulate, the granulosa cells become hypertrophic and then phagocytic. They appear messy and the cell membrane is thin, disintegrating and the nucleus irregular. Atresia is not always a post-spawning phenomenon (Ebisawa 1990); it also occurs during sex change: mature eggs are phagocytosed first (Ferreira 1993, 1995). Similarly, mature oocytes may be unused for other reasons, these will also degenerate into atretic oocytes. Atretic oocytes are common in *Plectropomus leopardus*, suggesting that re-absorption of ripe oocytes is energetically preferable to ovulation if certain spawning conditions are not met.

Three stages, or ages, of atretic oocytes were recognised in *P. leopardus*, following the definitions of Hunter and Macewicz (1985). Two stages are illustrated in Plate 5.

*Post-ovulatory follicle (**pof**) stage:-*

When the hydrated oocyte is released the follicle remains, and looks collapsed. It consists of the granulosa layer of the oocyte cell membrane which can be clearly distinguished for a short time. The follicle then degenerates over approximately 24 hrs (Samoilys in prep.) during which the granulosa layer becomes less and less distinct.

Brown-bodies:-

Atretic oocytes give rise to brown bodies which appear as yellow/brown "blobs" with an oily appearance. Brown bodies can also be seen macroscopically on whole gonads (including preserved ones) as brown dots along their length. Brown bodies are present in spawning and post-spawning females and in both resting and post-spawning males. In the latter they may be hormonal structures that originated from atretic oocytes.

Fragmented (reabsorbed) previtellogenic oocytes:-

These are degenerating early oocytes. Ferreira (1993) reports they are commonly seen in male gonads, but they are difficult to distinguish from laboratory processing artifacts (Adams pers. comm.).

4.2 Transitionals

The key feature which identifies transitionals is the presence of spermatic tissue in a gonad consisting largely of ovarian tissue (Table 5). Sadovy and Shapiro (1987) describe the sperm tissue as proliferating and the ovarian tissue as degenerating. However, in early transitional *P. leopardus* the sperm crypts are small and few, and the ovarian tissue is not yet degenerating. In fact, male cells may occur only as spermatogonia which are difficult to detect. Thus, transitionals were defined here as gonads in which the male cells had at least completed spermatogenesis (see below), ie. spermatocytes had formed. The absence of spermatozoa in the dorsal sperm sinus (Hastings 1981) was used to distinguish transitionals from incomplete (early) males. Fragmenting and re-absorbing pre-vitellogenic oocytes are features listed by Ferreira (1993, 1995) and Adams (1996) for transitionals, but they are difficult to distinguish from artifacts caused by laboratory preparation (Adams pers. comm.). Other features listed in Table 5 draw on Ferreira (1993, 1994, 1995) and Adams (1996) and the present study.

Three bisexual fish were also identified in the present study. They were rare (0.4 % of the population) and have not been documented for *P. leopardus* before. These individuals were distinguished by the possession of both female and male cells capable of spawning at the same time. They contained hydrated oocytes and the dorsal sperm sinus was filled with spermatozoa (Table 5).

Table 5. Transitional and bisexual gonadal development stages. Criteria are separated into those that define the stage (Defining criteria) and into others (Other features) that are useful aids in assigning gonads to a developmental stage. Strongly defining characteristics are shown in bold italics. Numbers in parentheses refer to total number of individuals in sample. Abbreviations refer to Plates.

Developmental stage	Defining criteria	Other features
Transitional, **T (61)** **Plate 9**	***dorsal sperm sinus (dss) not fully formed; precursory dss may be present but contains no sperm cells*** ***gonad consists largely of ovarian tissue but sperm crypts are present***	fragmentation and reabsorption of pre-vitellogenic oocytes may occur brown bodies common (40 fish), but not always present yolk vesicle or yolk globule oocyte stages rare* (4 fish) atretic oocytes seen rarely (3 fish) sperm crypts typically at periphery of gonad wall sperm cells usually present: spermatogonia **sg**, spermatocytes **sc**, spermatids **st** spermatozoa **sz**, are rare and then only in crypts
Bisexual, B (3) **Plate 10**	***dorsal sperm sinus filled with spermatozoa*** ***hydrated oocytes***	gonad resembles a running ripe female with vitellogenic oocytes including hydrated oocytes (Table 3), except it contains a fully developed dorsal sperm sinus filled with spermatozoa

* In this context Ferreira (1993, 1995) refers to transitionals and females interchangeably.

4.3 Males

Both ontogenetic and sexual development must be considered when staging male coral trout gonads. Two ontogenetic stages were defined: incomplete (recently changed sex) and complete (Table 6), which give an approximation of how recently the male changed sex. Sexual stages (Table 7) are seen in both incomplete and complete males. Again, criteria listed in Tables 6 and 7 draw on Ferreira (1993, 1994, 1995) and Adams (1996), the present study and some standard texts (Wallace and Selman 1981, de Vlaming 1983, Nagahama 1983).

Table 6. Male ontogenetic stages of development. Criteria are separated into those that define the stage (Defining criteria) and into others (Other features) that are useful aids in assigning gonads to a developmental stage. Strongly defining characteristics are shown in bold italics. Numbers in parentheses refer to total number of individuals in sample. Abbreviations refer to Plates.

Developmental stage	Plate	Defining criteria	Other features
Incomplete ripe male, I (43)	**11**	***dorsal sperm sinus,* dss*, formed and filled with spermatozoa* sz** ***gonad dominated (>50%) by pre-vitellogenic ovarian tissue*** spermatozoa	lamellae not yet lobular- typical of complete male atretic oocytes may still be present spermatogonia **sg**, spermatocytes **sc**, spermatids **st**, may be present
Complete ripe male, C (187)	**12**	***gonad dominated (>50%) by spermatogenic tissue dorsal (*dss*) and central (*css*) sperm sinuses filled with sperm cells*** spermatozoa	sperm crypts join to form large intra-lobular sinuses, **css** pre-vitellogenic oocytes may still be present spermatogonia, spermatocytes, spermatids may be present

Table 7. Male sexual stages of gonadal development. Criteria are separated into those that define the stage (Defining criteria) and into others (Other features) that are useful aids in assigning gonads to a developmental stage. Strongly defining characteristics are shown in bold italics. Numbers in parentheses refer to total number of individuals in sample. Abbreviations refer to Plates. Note Spent and resting males may be incomplete or complete.

Developmental stage	Plate	Defining criteria	Other features
Ripe , R (230)	**11, 12**	***dorsal sperm sinus,* dss*, formed and filled with spermatozoa,* sz** ***central (*css*) sperm sinuses filled with spermatozoa***	atretic and pre-vitellogenic oocytes may still be present spermatogonia **sg**, spermatocytes **sc**, spermatids **st**, may be present
Spent, S (4)	**13**	***spermatozoa rare and only in crypts*** ***vascularised,* vs*, and well developed stromal tissue,* sr** ***disorganised***	other (early stage) sperm cells uncommon dorsal sperm sinus, **dss**, empty or collapsed with some spermatozoa brown bodies **bb**, common/abundant
Resting male, R1 (post-spawn) (7)	**14**	***spermatozoa not present or rare*** ***vascularised and well developed stromal tissue*** ***compact***	dorsal sperm sinus empty or collapsed with some spermatozoa brown bodies common/abundant other (early stage) sperm cells common
Resting male, R2 (pre-spawn) (2)	**15**	***spermatozoa rare and only in crypts*** ***little or no vascularisation*** ***little or no stromal tissue***	other (early) sperm cells dominate dorsal sperm sinus empty brown bodies common/abundant

4.3.1 Description of male gametogenesis

***Spermatogenesis*:-** (spermatogonia ✍ spermatocytes)

Spermatogonia:-

These are the largest male cells and they occur in a crypt. They resemble oogonia, but they do not develop a chromatin nucleus. The lack of clear, distinctive morphological features between oogonia and spermatogonia is well known (Reinboth 1980).

1^o spermatocyte stage:-

Large nucleus, small amount of cytoplasm.

2^o spermatocyte stage:-

Nuclei well separated and stain darkly.

Spermiogenesis:- (spermatocytes ✍ spermatozoa)

Spermatid stage:-

Spermatids have small nuclei, are close together, occur in crypts, and they stain dark red (with haematoxylin/eosin).

Spermatozoa:-

Spermatozoa are spermatids with tails which stain blue (haematoxylin); the tails stain pink (eosin) though these can be difficult to see. Spermatozoa in sexually mature males occur in crypts which have joined to form sinuses.

5.0 Problems in defining stages

5.1 Distinguishing between immature females and mature resting females

One of the most critical problems in staging *Plectropomus leopardus* gonads was the difficulty in distinguishing between immature females and mature resting females. Clearly, this is critical for establishing minimum size at first maturity, a vital population characteristic in exploited species used to determine minimum legal size in fisheries management (Hill 1992). There is a minimum legal size for the *P. leopardus* fisheries on the Great Barrier Reef, which has been in place at least since 1984 (Samoilys pers. obs.) though no records exist as to when it was introduced (Hancock 1992).

Gonad wall thickness (Sadovy and Colin 1995, Burton et al 1997) is a useful guide (Table 3) but it is not completely reliable because wall thickness can vary in different sized females, through poorly treated samples, or through differences in sectioning. Similarly the presence of brown bodies is indicative of a resting mature female, however not all resting mature females will have brown bodies, although the majority do (Table 3). Ferreira (1994) proposed that the relative proportion of early and late pre-vitellogenic stages may help in separating resting from immature females, but it seems unlikely that such differences could be distinguished from ontogenetic changes in oocyte development. Sadovy and Colin (1995) used the presence of prominent intralamellar muscle bundles in *Epinephelus striatus* as evidence of prior spawning. Unfortunately, such bundles were not apparent in *P. leopardus*. Preliminary work on histological stains for detecting lipofuscin which is present in mature females suggests such techniques may provide a more conclusive tool for distinguishing between mature resting females and immature females (Adams 1996).

In the present study the difficulty in detecting immature females with certainty is illustrated as follows. Of the 35 Immature females identified in a total sample of 169 pre-vitellogenic females, 14 were difficult to define with complete certainty, using the characteristics of gonad wall thickness and the presence/absence of brown bodies. Four of these fish were taken during September – October and therefore if they were mature females they were unlikely (66-100%, Samoilys in prep.) to have been in a pre-vitellogenic state. A further four were taken in November when the likelihood of being vitellogenic drops to 58-61% (Samoilys in prep.). Based on these likelihoods these 8 females were retained as Immatures. Note, than an

additional individual of 48.5 cm FL originally defined as immature was unlikely to be so at that size, and was therefore re-classified as Resting. It was also vaculated which is characteristic of resting females. In summary, the remaining six (17%) of the 35 (or 3% of the pre-vitellogenic females) were classified as Immature, but with no great certainty.

5.2 Defining male stages

In contrast to females, development stages in male coral trout gonads were much harder to define and were not closely linked to the spawning season. Ripe males were found throughout the year, including March and July which are outside the spawning season (Samoilys 1997), though sampling was incomplete (Samoilys in prep.). Resting or spent males were uncommon (n = 13 out of 243). The definitions proposed here for spent and resting (both pre and post-spawning) male coral trout differ from those of Ferreira (1993, 1995) and Adams (1996) and are an attempt to provide unambiguous definitions. The definitions described here are put forward as a proposal and require further testing. Discussions with Samantha Adams greatly facilitated the proposed definitions. The primary criteria we have used are the presence of spermatozoa, the presence of stromal tissue or vascularisation, and the appearance of the gonad.

If spermatozoa are present the male is considered to be capable of spawning and is therefore ripe unless the spermatozoa only occur in crypts and then rarely (1-2 crypts). In this case the male is considered to be either resting or spent (Transitionals also fall into this category). To distinguish between resting and spent males we have used the presence of stromal and vascular tissue and the appearance of the gonad. Spent and post-spawning resting males are the most difficult to distinguish because they both have well developed stromal and vascular tissue. However, spent gonads are disorganised, whereas resting gonads are compact (post-spawning) or organised (pre-spawning). Two types of resting males were apparent: pre-spawning resting males were characterised by gonads which were not vascularised and did not contain stromal tisssue and were not compact. They only differed from ripe gonads by their lack of spermatozoa.

The phenomenon of pre-maturational sex change, or fish that have become male prior to maturing as a female, has received little attention in this report (see section 2), because criteria were not established to define these individuals. This requires further research.

6.0 Conclusions

Assigning discrete stages to a continuous variable is always problematic. However, the ability to classify gonads into maturity stages provides a basis for quantitative evaluation of reproductive state. This is particularly useful for comparing the reproductive biology of populations through, for example, time and space, or in relation to impacts such as fishing. It is perhaps less useful for quantitative comparisons between species where differences in functional morphology of gonads are probably more interesting and revealing.

The usefulness of clearly defined developmental stages for quantifying the precise timing of spawning and spawning frequency is obvious, and has been demonstrated for female *P. leopardus* (Samoilys in prep.). What remains is to assess the usefulness of the male stages for examining spawning patterns in male *P. leopardus* in greater detail. Further, the application of the gonadal developmental stages proposed here to other Serranidae would also be an interesting avenue of study.

This report has highlighted some of the difficulties in assigning developmental stages to fish gonads. One of the most pressing needs is the development of reliable staining techniques for distinguishing between immature and mature resting females. This is vital if we are to accurately determine minimum size at first maturity in these important commercial species. In addition, the phenomenon of pre-maturational sex change in *P. leopardus* requires further investigation. There has been no research done on the juvenile developmental stages of coral trout gonads. This could provide interesting insights into the developmental pathways in coral trout reproduction, which would elucidate the proposal that diandric male *P. leopardus* may exist in the southern Great Barrier Reef (Adams et al in review).

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8.0 Appendix

8.1 Glossary of terms and features for describing Plectropomus leopardus gonads

8.1.1 Female structural terms

Lumen = cavity which collects ovulated eggs ready for emission

Lamellae = term used to describe the structure of ovarian tissue (see lobules below)

Nests = small round space containing oogonia

8.1.2 Male structural terms

Crypts = small, round space containing spermatogonia (early stages)

Lobules = term used to describe the structure of testicular tissue; lobules are small lobes, similar in form to the ovarian lamellae from which they have developed - this is a feature of monandric protogynous hermaphrodites (Sadovy & Shapiro 1987)

Sinus = long, elongate space containing spermatozoa (late stage). One main dorsal sinus runs along the length of the gonad. There are central sinuses also. Sinus walls stain dark

Vestigial lumen = remains of ovarian lumen, into which lobules project

8.1.3 General terms

Basophilic = "base loving" - stains acidic substances; the blue stain of Haematoxylin is an example - it stains spermatids, spermatozoa and nuclei

Cytoplasmic inclusions = organelles in the cytoplasm e.g. yolk vesicles etc.

Diandric = where there are two types of males in hermaphrodites: primary males, which develop directly from juveniles, and secondary males which develop from females (see monandric)

Eggs = oocytes that are at least ovulated (West 1990) - use for spawned gametes, otherwise the term oocyte is used

Functionality = as male or female

Gametogenesis = the development of gametes, sperm and ova

Gonad wall = wall of gonad; dorsal wall is usually thicker because it is attached to lateral body wall or mesenteries on dorsal side

Granulosa = outermost membrane of oocyte, see radiata

Hypertrophic = cells increase in size (a pathological term), e.g. granulosa cells of oocyte follicle

Monandric = one type of male in protogynous hermaphrodites, which develop from the female (see diandric)

Multiple stages of oocytes/sperm cells = Presence of multiple stages in the one gonad is logically an indication of multiple spawning

Ovulation = release of hydrated oocyte from its follicle

Perinucleus = membrane round egg nucleus; becomes less distinct with maturity

Proximate factors = as factors affecting gametogenesis e.g. temperature; see ultimate

Pycnotic = pycnosis is an autolytic process resulting in cell degeneration

Radiata = membrane of oocyte inside granulosa layer

Stromal tissue = supportive tissue in gonads e.g. between crypts - common in spent males

Ultimate factors = as factors affecting gametogenesis e.g. food availability; see proximate

Vacuolated = empty spaces or cavities

Vascularised = blood vessels developed in tissue

8.2 Chemicals and materials used

FORMALDEHYDE-ACETIC ACID-CALCIUM CHLORIDE FIXATIVE (FAACC)
(extract from Winsor 1991, 1994)

Chemical	**Measure**
formaldehyde (40%)	100 ml
acetic acid (glacial)	50 ml
calcium chloride (anhydrous) **OR** calcium chloride (dihydrate)	10 g 13 g
tap water	850 ml
pH	1.9-2.0

Specimens are fixed for a minimum of 24 hours at ambient temperature, but preferably a week (formaldehyde takes about a week to fix tissue components). No deleterious effects have been observed in tissues stored for about 2 months in the fixative.

FAACC fixative was designed by L. Windsor at JCU in 1981 specifically as a substitute for BOUIN'S PICRO-FORMAL-ACETIC ACID FIXATIVE, widely used as a fixative for fish gonads.

Bouin's (1897) PFAA fixative has several disadvantages which include cost, need to wash fixed specimens to remove picric acid prior to storage or processing (adding to cost), poor long term storage characteristics (loss of nuclear staining in stored tissues), hazards of picric acid

(explosive when dry; causes dermatitis), poor field fixative - containers invariably leak, invariably resulting in yellow-stained field notes and personal gear! Tissues fixed in Bouin's exhibit acidophilia (very eosinophilic in an H&E stain), though this can be overcome by reducing the formaldehyde to 4%. The cost of Bouin's fixative and subsequent ethanol washing is 5.6 times that of FAACC.

FAACC fixative has none of the disadvantages of Bouin's PFAA fixative. It is simple, relatively inexpensive, stable and non-staining. Tissues fixed in FAACC do not require washing in ethanol, and have good balanced staining characteristics. The appearance of stained FAACC fixed tissues closely approximates that of tissues fixed in Bouin's, and also that of gonads fixed in DAVIDSON'S FIXATIVE (used by some fish laboratories).

FAACC has been used for gonad fixation of a variety of fish species which include Coral trout, Red-throat emperor, Whiting, Anchovy, Spanish Mackerel, Black Marlin, Sail fish, Grunter, Threadfin and Parrot fish. It is also used as a gonad fixative for Giant clams, oysters, squid and cuttlefish.

In FAACC the formaldehyde is a non-coagulant cross-linking protein fixative. The acetic acid precipitates DNA and is included to off-set hardening due to formaldehyde, providing a reasonable texture for microtomy. Calcium (as calcium chloride) fixes certain lipids (eg. lecithin) and probably behaves as an additive fixative for some proteins (the role of calcium in fixatives is presently poorly understood).

10% PHOSPHATE BUFFERED FORMALIN

(Reilly pers. com.)

Chemical	**Measure**
Formaldehyde (37 – 40%)	100.00 ml
NaH2PO4.H2O **OR** NaH2PO4.2H2O	4.00 g 4.52 g
Na2HPO4 (Anhydrous)	6.5 g
Tap water	900.00 ml