Rapid Reproductive Analysis and Length-Dependent Relationships of *Lutjanus biguttatus* (Perciformes: Lutjanidae) from Papua New Guinea[1]

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Abstract: We describe a simple, inexpensive method for field-based histological analysis of fish gonads, and we used this method to describe the reproductive biology of the small snapper *Lutjanus biguttatus* from a remote area in Papua New Guinea (i.e., where laboratory equipment is limited and electrical service is lacking). We estimate male L_{50} at 13 cm FL and female L_{50} at 17 cm FL. Sex ratio is not significantly different from 1 : 1. There is no evidence for hermaphroditism. Fork length is a linear function of total length, FL = –0.1823 + 0.9647(TL), and weight is a cubic function of fork length, $W = 0.0129(FL)^{3.0049}$. This information was generated during a 2-week period when the majority of our time was occupied by fish surveys. Our analyses were limited by availability of specimens (not processing time); therefore the methods we employed can help fill one of the information voids that preclude life-history-based management of coral-reef fishes.

A lack of basic life-history information is one of the biggest challenges to the management and conservation of exploited coral-reef fishes. The sheer diversity of coral-reef fishes and the purported cost associated with the reproductive analysis of each species are often cited as obstacles to obtaining this important information (Roberts and Polunin 1993, Johannes 1998). An additional challenge is the lack of basic infrastructure (e.g., electrical service needed to operate laboratory equipment) in many parts of the developing countries where most of the world's coral reefs are located. However, sound conservation decisions simply cannot be made without estimates of reproductive (and other life history) parameters.

Here we describe a method for rapid, low-cost, histology-based reproductive analysis suitable for use in remote locations (i.e., it does not require electrical service). We demonstrate the effectiveness of the method by describing the reproductive biology and length relationships of the twospot banded snapper, *Lutjanus biguttatus*, at Kamiali Wildlife Management Area, Papua New Guinea (7° 18′ S, 147° 10′ E). Approximately 600 villagers maintain traditional tenure over the wildlife management area and obtain the overwhelming majority of their dietary protein from fish caught in its 15,000 ha of marine habitat. *Lutjanus biguttatus* is small-bodied, attaining a total length of approximately 20 cm, and is an important market fish in some areas (Allen 1985). It is found from the central Indian Ocean to Melanesia, and its distributional limits appear to be the Maldive Islands to the west, northern Australia to the south, the Solomon Islands to the east, and the Philippines to the north (Allen and Talbot 1985).

Materials and Methods

We collected specimens with a pole spear, measured fork length (FL) and total length

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(TL) to the nearest millimeter, and estimated whole body weight with the smallest-possible of two hanging spring scales (100 or 1,000 g capacity, with 1 or 10 g increments, respectively). We made a midventral incision from the vent through the pelvic girdle, noted sex and reproductive status (based on gross examination), then removed and fixed the gonads in a modified Dietrich's solution (6 parts water, 2 parts 95% ethanol, 1 part 37% formaldehyde solution, and 1 part distilled white vinegar) for 24 hr. We cut an approximate 8 mm^3 section from each gonad, placed the sections in a 24-well tissue culture plate, and dehydrated them in a graded alcohol series (30 min in each of 50%, 75%, and 95% ethanol). Using plastic embedding medium (JB4, Electron Microscopy Sciences) and following kit instructions, we then infiltrated the specimens in two changes of infiltration solution, transferred the specimens into embedding capsules (BEEM, size 00), and embedded them. We used single-edge razor blades to cut tissue blocks from embedding capsules and trimmed excess medium from both ends (exposing tissue on the conical end of the tissue block). Because high humidity in our open-air "laboratory" often prevented tissue blocks from hardening completely, we dehydrated the blocks for 12 hr in a "desiccating chamber" (a diver's dry box containing silica gel packets and placed in full sunlight).

We obtained 10 tissue sections (approximately 7 μm thick), distributed evenly throughout each tissue block, using a microtome (MT1 Porter-Blum) outfitted with a glass knife. We floated the tissue sections on water droplets distributed on microscope slides and dried the slides on a "warmer" (in this study, a glass sheet elevated ~2 cm above dark sand exposed to direct sunlight; for subsequent projects we used a less-fragile cast-iron baking dish, inverted and placed in full sunlight). We stained the tissue sections (now affixed to the slides) in a 0.5% solution of Toluidine Blue in water (w/w) for 15 sec. Excess stain was removed with a gentle stream of water, and the slides were once again dried on the "warmer." Tissue sections were examined at 40× with a dissecting microscope for evidence of reproductive maturity. We classified ovaries according to Wallace and Sellman (1981) and testes according to Nagahama (1983). Table 1 provides descriptions of oocyte stages used to evaluate ovaries. We considered females mature with the onset of vitellogenesis or when postovulatory follicles were present, and males mature when the testes contained visible spermatozoa (sperm cells with tails). We estimate size at sexual maturity (L_{50}) as the size at which a regression (three-parameter, sigmoidal) of percentage mature individuals in each 1 cm size class versus fork length (the average length of individuals within a size class) indicates that 50% of individuals are mature.

We used χ^2 analysis to test whether population-level sex ratios differ from 1:1 and exploratory regression analysis to examine whether sex ratios vary predictably with size class. We used linear regression analysis to predict TL and total body weight (W) as a function of FL.

RESULTS AND DISCUSSION

We histologically examined gonads of 16 male and 20 female *Lutjanus biguttatus*. Because our results are based on a low number of specimens, the reproductive parameters presented here should be considered preliminary. Figure 1 shows examples of mature testes and ovaries, and demonstrates the quality of histological preparations we were able to produce in the field. The smallest male with spermiated testes was 12.6 cm FL. This individual was also in the size class containing our estimate of male L_{50} (12.5 cm FL) (Figure 2). All males ≥14.0 cm were mature. Ovaries contained stage III (vitellogenic) oocytes in females as small as 16.2 cm FL. Female L_{50} is estimated as 16.7 cm FL (Figure 2). All females ≥18.6 cm were mature.

We found no evidence for sequential hermaphroditism in *L. biguttatus*. A two-tailed *t*-test for a sex-based bimodal size distribution was not significant ($t = 0.0782$, $df = 34$, $P = .9382$). Nor did we see classic histological signs of sex change (see Sadovy and Shapiro 1987): testes lacked a lumen or brown bodies, and ovaries did not contain spermatogenic

TABLE 1

Characters Used to Identify Oocyte Stages (Stage Names from Wallace and Sellman 1981)

Stage Number (Name)	Description and **Diagnostic Features**	Approximate Size Range (Typical Size)
I (Primary Growth)	Ellipsoid to circular. Prominent (>30% cell diameter), circular nucleus with well-defined borders; possibly containing small nucleoli. **Cytoplasm stains darkly and uniformly.** Zona radiata absent.	20–80 μm (50 μm)
II (Cortical Vesicle)	Larger (2–4×) and more circular than stage I. Boundary between nucleus and ooplasm indistinct. **Cytoplasm stains darkly and contains many nonstaining (white) lipid vesicles.** Mid to late stages with conspicuous, lightly staining, acellular protein "shell" (zona radiata).	120–250 μm (170 μm)
III (Vitellogenesis)	Large (usually 2× stage II) and circular. Nucleus usually visible only during early stage III. **Cytoplasm a mixture of oil droplets (nonstaining) and brightly staining, round, uniform, vitellin (yolk protein) globules.** Zona radiata prominent, stains brightly.	180–570 μm (350 μm)
IVa (Maturation)	Larger but often less circular than stage III. **Yolk protein coalescing into larger globules;** eventually forming an irregular mass at oocyte center. Oil droplets (nonstaining) also more variably sized and coalescing.	350–700 μm (550 μm)
IVb (Hydration)	Almost 2× larger than stage III, very irregularly shaped. **Cytoplasm a large mass of vitellin, usually staining lighter than previous stage.** Oil droplets (nonstaining) may be visible at periphery of vitellin mass or may have coalesced into a single, large droplet. Zona radiata conspicuously thinner and more irregular than in Stage III.	500–1,100 μm (850 μm)
POF (Post Ovulatory Follicle)	Small, collapsed band of follicular cells remaining in the ovary after ovulation. New POFs usually one-half the diameter of stage III oocytes and contain a conspicuous lumen. POFs degenerate quickly; their presence may indicate recent spawning.	

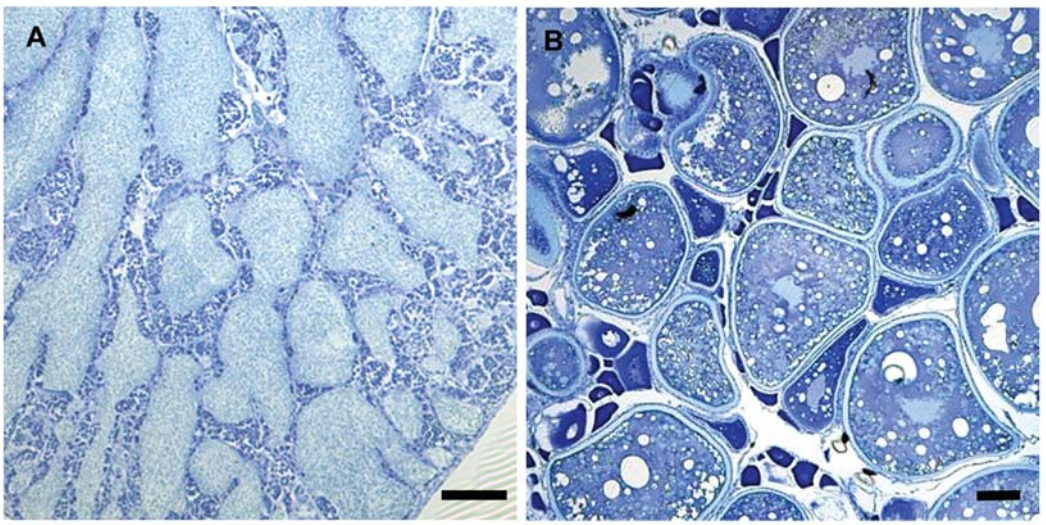

FIGURE 1. Histological preparations of gonads: (*A*) mature male, 140 mm FL; (*B*) mature female, 162 mm FL. Scale bars = 100 μm.

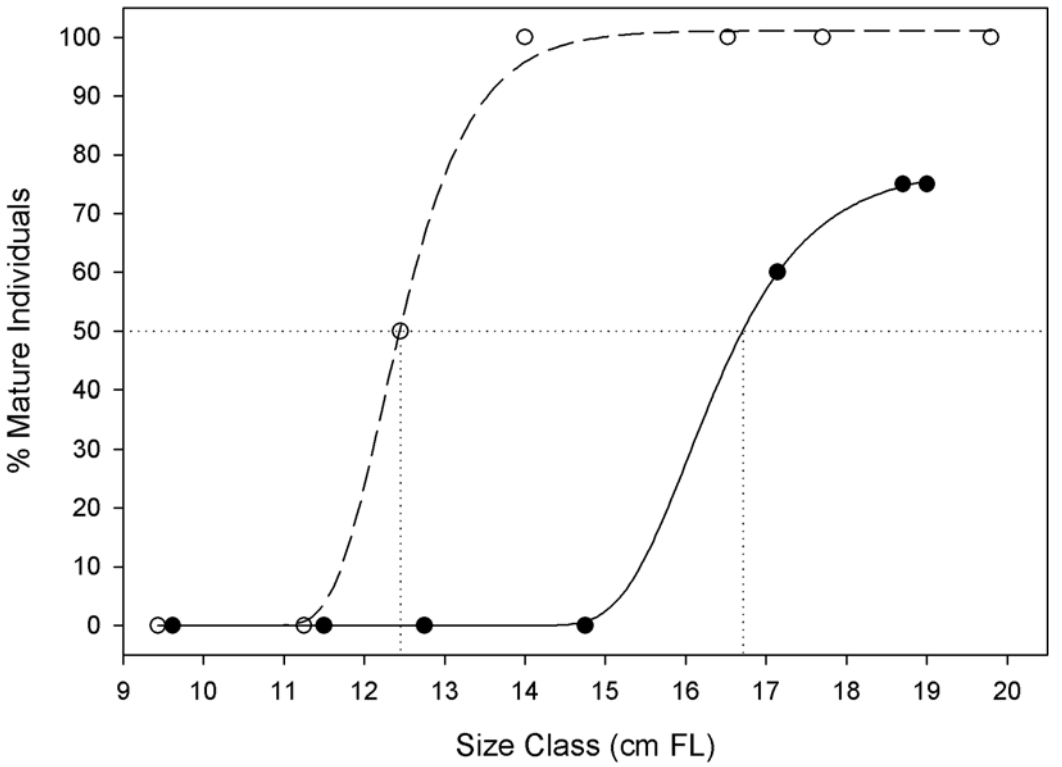

FIGURE 2. L_{50} for *Lutjanus biguttatus:* 50% of males (open circles, dashed line) are mature at 125 mm FL, 50% of females (closed circles, solid line) are mature at 167 mm.

tissue. *Lutjanus biguttatus* is apparently a gonochore.

Sex ratios can have profound impacts when predicting population-level reproductive output. Sex-ratio patterns are variable within the lutjanids; some species occur in a 1 : 1 ratio independent of size (Kritzer 2004, Russell and McDougall 2008), whereas the sex ratio of other species varies predictably with size (our interpretation of Davis and West [1992] and Heupel et al. [2009]). Overall, sex ratio in this *L. biguttatus* population, from the size class at male maturity (12 cm) through maximum observed size (19.8 cm), is 1 : 1.2 male : female. However, χ^2 analysis indicates that the observed ratio among these 22 individuals is not significantly different from 1 : 1 ($\chi^2 = 0.1818$, df = 1, $P = .6698$). Further, sex ratio does not appear to vary predictably with size. The latter analysis is based on a small number of individuals, with no more than five individuals present in any one size class. However, when present, size-specific sex ratios are striking; we have detected variable ratios with similarly small-sized samples in small-bodied Hawaiian fishes (Longenecker and Langston [2008] and unpubl. data).

Fork length is a linear function of total length (each in centimeters): FL = –0.1823 + 0.9647(TL); $r^2 = 0.998$, $n = 33$, $P = <.0001$. Total body weight (W) in grams is a cubic function of fork length in centimeters: $W = 0.0129(FL)^{3.0049}$; $r^2 = 0.993$, $n = 37$, $P = <.0001$.

Our results demonstrate that histology-based reproductive studies can be performed in remote field locations. This capability has profound implications for fishery management because macroscopic gonad classification systems are known to introduce excessive

error when describing reproductive parameters (Vitale et al. 2006). For instance, during this study we evaluated our accuracy in determining the sex and reproductive status of individuals based on macroscopic examination of several species. We misclassified reproductive status and/or sex in 47% of specimens examined (could not determine sex, 23%; incorrect sex, 1.5%; incorrect status, 18%; incorrect sex and status, 4.5%). Importantly, macroscopic examination led to overestimates of the number of mature females and underestimates of the number of mature males.

Histology-based reproductive studies can be done relatively quickly, and the low-cost method we employed eliminates one of the arguments against broad-scale reproductive analysis. We estimate the per-specimen cost of consumable supplies at US$1.45. We suggest that this cost is low enough that developing countries with limited resources could use the method we describe to assist with fisheries management decisions.

This study was accomplished over a 2-week period during which the majority of our time was dedicated to field surveys. Histological processing and evaluation of gonad sections took approximately 0.5 hr per specimen. We found our work to be limited by number of available specimens, not the time necessary to process them (during a subsequent field trip with the same time constraints, we processed and evaluated >400 specimens representing four other species in less time per specimen). Suitably accurate life-history parameters can be generated concurrently with expeditionary research. Field-based histology offers two advantages: (1) reproductive information can be generated when government or airline regulations make exporting or transporting biological specimens difficult, and (2) collecting efforts can be fine-tuned such that important size classes can be obtained while researchers are in the field (i.e., still have the opportunity to collect specimens).

Researchers wishing to use the method described here should find the following suggestions useful. Many of them are geared toward reducing the volume of supplies transported into the field and, by extension, decreasing purchase and shipping costs.

(1) Consumables we use, but that are not listed elsewhere in this article, are latex gloves, disposable plastic droppers, and absorbent tissue paper (for cleaning).

(2) Keeping track of specimens throughout the process is crucial. We precut 20 × 30 mm rectangles from heavy cotton paper and form a tear-away tab by making a 15 mm cut 3 mm from a long edge. We write the specimen number on the main body of the label and duplicate the number on the tear-away tab. The whole label is placed into the container (in our case, plastic sample bags) in which whole gonad specimens are fixed. When a gonad is sampled for histological processing, the tear-away tab is removed and accompanies the sample through the dehydration, infiltration, embedding, and sectioning process (the main body of the label can remain with the bulk of the gonad tissue). Conveniently, the tear-away tabs can be formfitted around the inside curvature near the open end of the embedding capsules; the resiliency of the heavy cotton paper will hold the label in place (away from the tissue sample) during the embedding process.

(3) We find that processing lots of 50 samples uses the plastic infiltration and embedding medium efficiently, and that we can complete the embedding process before the plastic medium polymerizes.

(4) An accurate balance is needed to prepare the infiltration solution. We use a battery-powered jeweler's scale with 50 g capacity and 0.001 g accuracy. This is also useful for other reproductive analyses such as calculating gonosomatic indices and estimating fecundity.

(5) Freestanding 50 ml centrifuge tubes are convenient for mixing infiltration and embedding solutions. Plastic medium kit instructions call for a magnetic stirrer to mix the infiltration solution; we mix the solution by capping the centrifuge tube and inverting until components are mixed.

(6) Plastic medium is the most costly consumable in the method we describe here. The quantity used can be reduced during the infiltration process; save the second change of infiltration solution from one lot of samples, and use it as the first change of infiltration

medium in the next lot of samples. Also, be sure that gonad samples are small (~8 mm^3). Large samples may require an additional change of infiltration solution.

(7) Plastic medium kit instructions call for infiltration to be performed on a shaker table or while inverting the samples several times during the process. Instead, we gently shake by hand the tissue culture plates in which we perform dehydration and infiltration.

(8) Commercially available embedding capsule holders are convenient; however we find that corrugated cardboard punched with properly sized holes works just as well for holding capsules during the embedding process.

(9) 1 ml syringes are useful for measuring polymerization accelerator when preparing the embedding solution. These should be used one time only.

(10) Plastic medium kit instructions suggest that embedding be done in an ice bath or refrigerator. Instead, we began this process in the evening so that polymerization would occur when ambient temperatures were lowest.

(11) Ethanol adequately cleans the work area after infiltration and embedding with plastic. An alcohol rinse also allows reuse of tissue culture plates and centrifuge tubes.

(12) Oxygen should be excluded from the embedding solution during the polymerization process. We placed filled, closed embedding capsules, along with desiccant packets, in a diver's dry box (i.e., the desiccating chamber described in the methods) to reduce oxygen exposure.

(13) An insulin syringe with fitted needle is convenient for distributing water drops to float tissue sections on microscope slides.

(14) The plastic embedding medium we use is water soluble. High humidity, such as will undoubtedly be experienced in an open-air laboratory in the coastal tropics, will make tissue blocks gummy and tissue sections hard to manipulate. Tissue sectioning must be done quickly (within 5 min) after tissue blocks are removed from the "desiccating chamber." If blocks become gummy to the point that tissue sections are hard to manipulate, the embedded specimen can be returned to the "desiccating chamber" to reharden. Tissue blocks ready for sectioning should not feel tacky; rather a tap on their side with forceps should not leave a mark.

(15) In high-humidity conditions, thicker tissue sections (up to 12 μm) are easier to make and manipulate.

(16) Glass knives dull quickly. We prepare one knife for each seven specimens we plan to process.

(17) Single-edge razor blades are indispensable. We use them to cut gonads, trim labels, remove tissue blocks from embedding capsules, and trim tissue blocks. We plan to use at least one blade for each 25 specimens processed.

(18) Battery-powered headlamps are handy light sources for microscope work.

(19) We stained only with toluidine because it is a dry stain (easy to transport) and easy to reconstitute in the field; however it stains nuclear material and cytoplasm with similar affinity. Those new to histology may want to employ a counterstain to more easily identify vitellogenic oocytes (which contain granules that stain brightly with eosin or similar stains). Commercial gram stain and Diff-Quik kits (omit first stain) work well, although the stain solvent (alcohol or acetone) will cause tissue-section crinkling.

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