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Oogenesis and spawn formation in the invasive lionfish, *Pterois miles* and *Pterois volitans*

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SUMMARY: The Indo-Pacific lionfish, *Pterois miles* and *P. volitans,* have recently invaded the U.S. east coast and the Caribbean and pose a significant threat to native reef fish communities. Few studies have documented reproduction in pteroines from the Indo-Pacific. This study provides a description of oogenesis and spawn formation in *P. miles* and *P. volitans* collected from offshore waters of North Carolina, U.S.A and the Bahamas. Using histological and laboratory observations, we found no differences in reproductive biology between *P. miles* and *P. volitans*. These lionfish spawn buoyant eggs that are encased in a hollow mass of mucus produced by specialized secretory cells of the ovarian wall complex. Oocytes develop on highly vascularized peduncles with all oocyte stages present in the ovary of spawning females and the most mature oocytes placed terminally, near the ovarian lumen. Given these ovarian characteristics, these lionfish are asynchronous, indeterminate batch spawners and are thus capable of sustained reproduction throughout the year when conditions are suitable. This mode of reproduction could have contributed to the recent and rapid establishment of these lionfish in the northwestern Atlantic and Caribbean.

Keywords: lionfish, *Pterois*, oogenesis, ovarian peduncle, oocyte, invasions.

RESUMEN: Ovogénesis y formación de la puesta de los peces invasores *Pterois miles* y *Pterois volitans*. – Los peces Indo-Pacíficos, *Pterois miles* y *P. volitans*, han invadido recientemente la costa este de los Estados Unidos y el Caribe y representan una significativa amenaza a las comunidades nativas de peces coralinos. Unos pocos estudios han documentado la reproducción en peces de la subfamilia Pteroinae del Indo-Pacífico. Este estudio presenta la descripción de la ovogénesis y la formación de puesta en *P. miles* y *P. volitans* recolectados desde aguas a mar abierto de Carolina del Norte, U.S.A, y las Bahamas. Mediante el uso de observaciones histológicas y de laboratorio, encontramos que no había diferencias en la biología reproductiva entre *P. miles* y *P. volitans*. Estas especies desovan huevos flotantes que están encerrados en una masa hueca de moco producida por células secretoras especializadas del complejo de la pared del ovario. Los ovocitos se desarrollan en pedúnculos altamente especializados, estando todos los estadios de los ovocitos presentes en el ovario de las hembras en puesta, y los ovocitos más maduros se localizan en la zona terminal, cerca del lumen del ovario. Dadas estas características del ovario, estas especies son asincrónicas, ponedores secuenciales indeterminados y son, por tanto, capaces de tener una reproducción sostenida a lo largo del año cuando las condiciones son adecuadas. Este modo de reproducción podría haber contribuido al rápido reciente establecimiento de estas especies en el noroeste del Atlántico y Caribe.

Palabras clave: lionfish, *Pterois*, ovogénesis, pedúnculo ovárico, ovocitos, invasiones.

INTRODUCTION

Two species of non-native lionfish, *Pterois miles* (Bennet, 1828) and *P. volitans* (Linnaeus, 1758), are now established along the southeast coast of the United States and in parts of the Caribbean (Morris *et al.,* 2009; Schofield 2009; Schofield *et al*., 2010). This is the second incidence of a pteroine invasion, the first being the establishment of *P. miles* in the Mediterranean Sea via the Suez Canal in 1991 (Golani and Sonin, 1992). The rapid establishment of these lionfish in both the Mediterranean Sea and the northwestern Atlantic raises many

questions regarding the reproductive biology, population growth, and ecological impacts (Morris and Akins, 2009; Morris and Whitfield, 2009) of these species.

P. miles and *P. volitans* are two of nine recognized species in the genus *Pterois* and can be distinguished from one another only by fin ray meristics (Schultz, 1986) or by analysis of mitochondrial DNA sequences (Hamner *et al.,* 2007; Morris and Freshwater, 2008). In the United States, lionfish are imported as an ornamental reef fish (Semmens *et al.,* 2004; Ruiz-Carus *et al.*, 2006) and they were most likely introduced into Atlantic waters from the Indo-Pacific by recreational or commercial aquarists (Hare and Whitfield, 2003; Ruiz-Carus *et al.,* 2006; Morris and Whitfield, 2009). Lionfish are dispersed as planktonic larvae by oceanographic currents (Ahrenholz and Morris, 2010) and densities are capable of reaching well over 400 lionfish per hectare in the offshore waters of North Carolina, U.S.A. (Whitfield *et al.,* 2002; 2007; Morris and Whitfield, 2009) and in the Bahamas (Green and Côté, 2008), with higher densities observed in the Atlantic than ever reported in their native range in the Indo-Pacific.

Lionfish are scorpaeniforms, which comprise a diverse order of fish encompassing a broad spectrum of reproductive strategies and adaptations (Kendall, 1991; Wourms, 1991). In general, the morphological and histological structure of the scorpaeniform ovary is poorly understood, and this has led to a lack of understanding of their reproductive evolution (Wourms, 1991). Some scorpaenids are known to spawn gelatinous, buoyant egg masses, including *P. lunulata* (Mito and Uchida, 1958), *Sebastolobus macrochir* (Masuda *et al.,* 1984), *Scorpaena guttata* (Orton, 1955), *Dendrochirus spp.* (Fishelson, 1975; Moyer and Zaiser, 1981), and *Helicolenus dactylopterus* (Krefft, 1961; Sanchez and Acha, 1988). Descriptions of ovarian structure have only been reported for *Dendrochirus brachypterus* (Fishelson, 1975; 1977; 1978), *H. dactylopterus* (White *et al.,* 1998; Muñoz *et al.,* 2002), and *S. alascanus* (Erickson and Pikitch, 1993). Detailed descriptions of ovarian morphology and cytology are unavailable for any pteroines.

The present study provides a description of the morphological and cytological structure of the ovary and of oogenesis in the lionfish *P. miles* and *P. volitans*. This description provides a foundation for understanding the reproductive biology of these non-native species and explains, in part, their rapid establishment and population expansion in their new ranges.

MATERIALS AND METHODS

Female lionfish (n=718) were collected by spearfishing or hand nets from the northwestern Atlantic (North Carolina and the Bahamas) throughout the calendar year and euthanized by excess anesthesia in a bath of tricaine methane sulfonate or by cervical transection. Ovaries were immediately removed and fixed in 10% neutral buffered formalin (NBF) for up to 30 days before being processed histologically. Ovaries less than 1 cm in length were preserved whole in 10% NBF. For ovaries exceeding 1 cm in length, tissue was excised from the mid-ovary and placed in 10% NBF. All fixed tissues were rinsed in phosphate buffered saline, dehydrated through a graded ethanol series, and embedded in paraffin using standard histological techniques**.** The paraffin blocks were sectioned at 5-6 μm and the sections were stained with a mixture of Mayer's/Harris hematoxylin and alcoholic Eosin Y (Sheehan and Hrapchak, 1980).

For scanning electron microscopy (SEM), one representative lionfish ovary was selected for imaging. Tissue samples were fixed in 10% NBF for 24 h and washed twice for approximately 15 min in 0.1 M phosphate buffer (pH 7.2-7.4) before dehydration in a graded ethanol series. After drying, the samples were attached to aluminum SEM stubs with carbon tape and then sputter-coated with approximately 20 nm of gold-palladium. SEM images were taken with a JEOL JSM-6360 LV scanning electron microscope operated at 5 kV accelerating voltage.

Photomicrographs of histological sections were taken of the ovaries of seven representative lionfish of a mean total length (mm) of 288.3±14.5 standard error of the mean. All photomicrographs were taken with a Leitz-Wetzlar Dialux 22 microscope equipped with a Leica DFC320 R2 digital camera and stage-specific maximum oocyte diameters (n=20 per oocyte stage) were measured using a calibrated ocular micrometer. Adult female lionfish were held in the laboratory and eggs (n=10) were taken from a recently released egg mass. The diameters of the eggs were measured under a Zeiss 475052-9901 dissecting stereomicroscope fitted with a calibrated ocular micrometer.

Ovarian morphology and oogenesis were compared between *P. miles* and *P. volitans* using five similar-sized sexually mature specimens of each species. The five fish (*P. miles* $\bar{x}$=280±20.8 mm total length and *P. volitans* $\bar{x}$=296.6±20.7 mm total length) were randomly selected from a pool of 21 *P. miles* and 381 *P. volitans*. Oocyte diameters (n=317 oocytes for *P. miles*; n=385 oocytes for *P. volitans*) were measured from the ovary mid-section using digital image analysis software (Image Pro Plus version 4.0). A viewing area with a width of 1.05 mm was assessed from the central stroma to the ovarian wall. Only oocytes exhibiting a distinct germinal vesicle (indicating that the oocyte was sectioned near the center plane) were measured. Oocytes in the late stages of final maturation (i.e. exhibiting no germinal vesicle) were also included. A Kolmogorov-Smirnov test for two independent samples was used to compare the relative frequency distributions of oocyte diameters between *P. miles* and *P. volitans*.

Species were identified with analyses of mitochondrial genes for cytochrome b (mtCytb). DNA was extracted from muscle tissue preserved in 90% ethanol with a PureGene kit (Gentra Systems, Minneapolis,

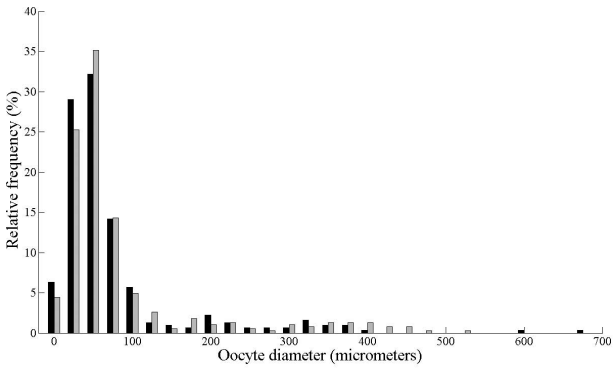

FIG. 1. – Relative frequency distribution of oocyte diameters of *P. miles* (gray bars) and *P. volitans* (black bars).

MN, USA) and used for amplification and sequencing of the mtCytb locus with the genotyping conducted as described in Hamner *et al*. (2007). Oogenesis was compared between species by assessing general ovarian and oocyte morphology based on the conventional understanding of oocyte growth in teleosts (Le Menn *et al*., 2007; Selman *et al*., 1989; 1993).

RESULTS

No differences in ovarian morphology or the relative frequency distributions of oocyte diameters were found between *P. miles* and *P. volitans* (p=0.9270) (Fig. 1). Given this finding, the term "lionfish" is used hereafter to describe both species. Lionfish ovaries are bilobed, fusiform organs located in the postero-dorsal region of the body cavity. The ovarian circulatory system comprises ovarian arteries and veins that enter anteriorly and extend centrally through each lobe. The central stroma of each lobe develops radially around this vascular system (Fig. 2) and is overlain by germinal epithelium. The oogonia are situated within the germinal epithelium. Immature oocytes are found near the central stroma and mature oocytes are positioned adjacent to the ovarian lumen, which lies beneath the peripheral ovarian wall (Fig. 2). The lumen of each lobe fuses caudally to form the gonoduct.

Four sequential oocyte stages categorize lionfish oogenesis: primary growth, cortical alveoli, vitellogenesis, and maturation. This characterization describes the cytological features of lionfish oocyte and follicle development, ovulation, and spawn formation using the key developmental stage-specific criteria summarized in Table 1.

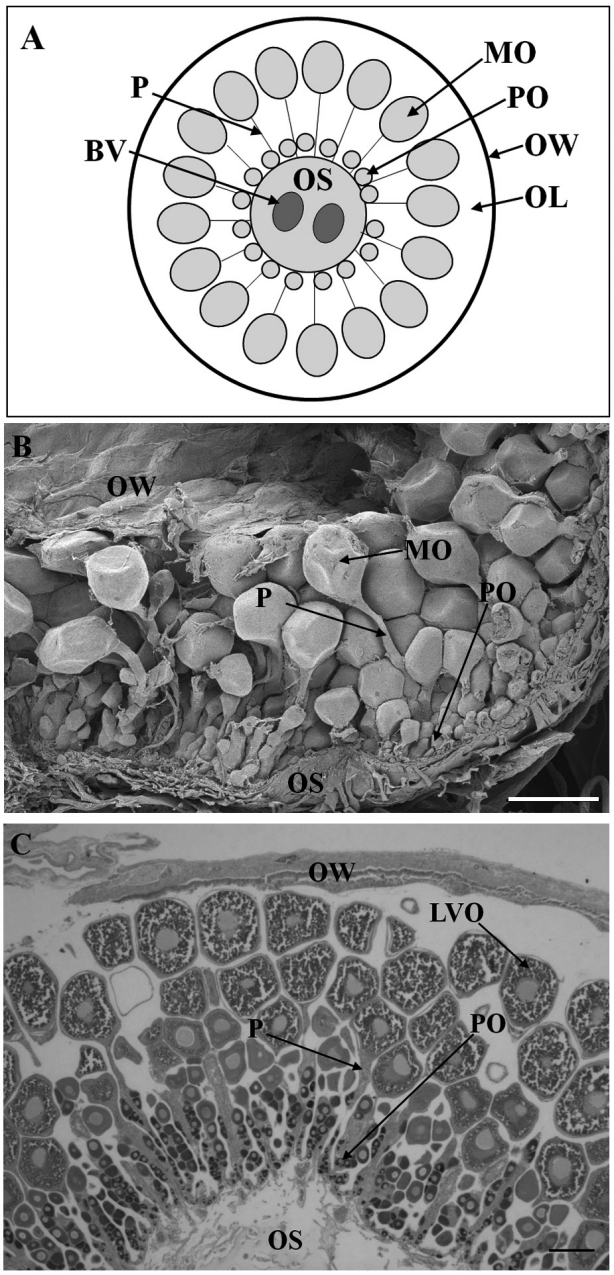

FIG. 2. – Transverse sections of lionfish ovaries depicting asynchronous oocyte production and cystovarian morphology. A) Line drawing, adapted from Koya and Muñoz (2007), B) electron micrograph (scale bar = 500 μm), and C) histological photomicrograph of an ovary whose most advanced oocytes are in the late vitellogenic stage (scale bar = 250 μm). BV, blood vessels; LVO, late vitellogenic oocyte; MO, maturing oocyte; OL, ovarian lumen; OS, ovarian stroma; OW, ovarian wall; P, peduncle; PO, primary growth oocyte.

Primary growth stage

Early oocytes in the primary growth-stage (20-60 μm in diameter) exhibit strongly basophilic ooplasm and a prominent germinal vesicle with vesicular nucleoplasm containing visible chromatin and single or several prominent basophilic nucleoli (Fig. 3). Oocytes are positioned near the central stroma with oogonia visible within the germinal epithelium (Fig. 3). As primary growth proceeds, oocyte diameter increases to 40 - 100 μm and multiple nucleoli become evident in the oocyte germinal vesicle (Fig. 3). Later, in the cortical alveoli stage, the nucleoli assume a peripheral position in concavities of the nuclear envelope (Fig. 4A). Oocytes in the late primary growth stage are positioned farther from the central stroma, towards the ovarian

TABLE 1. – Key cytological features characteristic of lionfish oocytes.

Oocyte stage (diameter)	Histological features
Primary growth (20-100 μm)	Basophilic ooplasm, prominent germinal vesicle, multiple nucleoli appearing during late stage
Cortical alveoli (80-165 μm)	Cortical alveoli appear in the ooplasm around the germinal vesicle, numerous nucleoli peripherally located around the germinal vesicle, ooplasm less basophilic, oocytes may become suspended on peduncles
Vitellogenic (130-500 μm)	Oocytes individually suspended on peduncles, germinal vesicle centrally located with multiple peripheral nucleoli, follicle elements thicker and more developed, yolk granules form a ring around the oocyte cortex and eventually occupy entire ooplasm
Maturation and ovulation (≥500 μm)	Germinal vesicle migrates peripherally and its membrane disintegrates, yolk granules coalesce, lipid droplets coalesce, egg detaches from peduncle and is ovulated from the follicle, gelatinous mucus produced by ovarian wall complex encompasses the ova
Atresia	Oocytes appear highly vacuolated, yolk disintegrates, lipid droplets coalesce into numerous larger oil globules, germinal vesicle disintegrates, apical segment of peduncle involutes and is reabsorbed

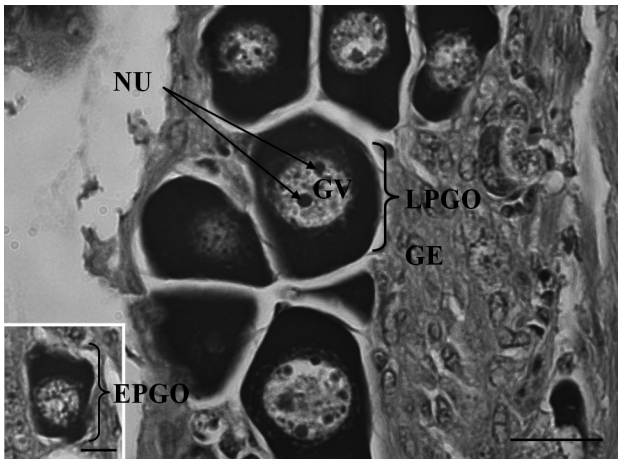

FIG. 3. – Early primary growth stage oocyte (inset) and late primary growth stage oocytes. EPGO, early primary growth stage oocyte, GE, germinal epithelium, GV, germinal vesicle, LPGO, late primary growth stage oocyte, NU, nucleoli. Scale bars: 50 μm and 15 μm (inset).

lumen. The ooplasm around the germinal vesicle becomes granulated.

Cortical alveoli stage

A well-developed follicular complex surrounding oocytes and the appearance of nascent cortical alveoli within the oocyte (80-165 μm diameter) characterize the cortical alveoli stage. The follicular complex consists of a zona radiata overlain by a monolayer of granulosa cells, a basement membrane, and a well-vascularized, multicellular outer layer of theca cells. The nucleoplasm of the germinal vesicle appears progressively more homogenous and the numerous nucleoli move to a peripheral position just under the nuclear membrane as the ooplasm becomes less basophilic (Fig. 4A). The cortical alveoli are distinguished from the ooplasm as opaque granules (Fig. 4B). The cortical alveoli appear initially in the ooplasm and later proliferate and are displaced peripherally as a dark granulated ring of ooplasm expands around the germinal vesicle.

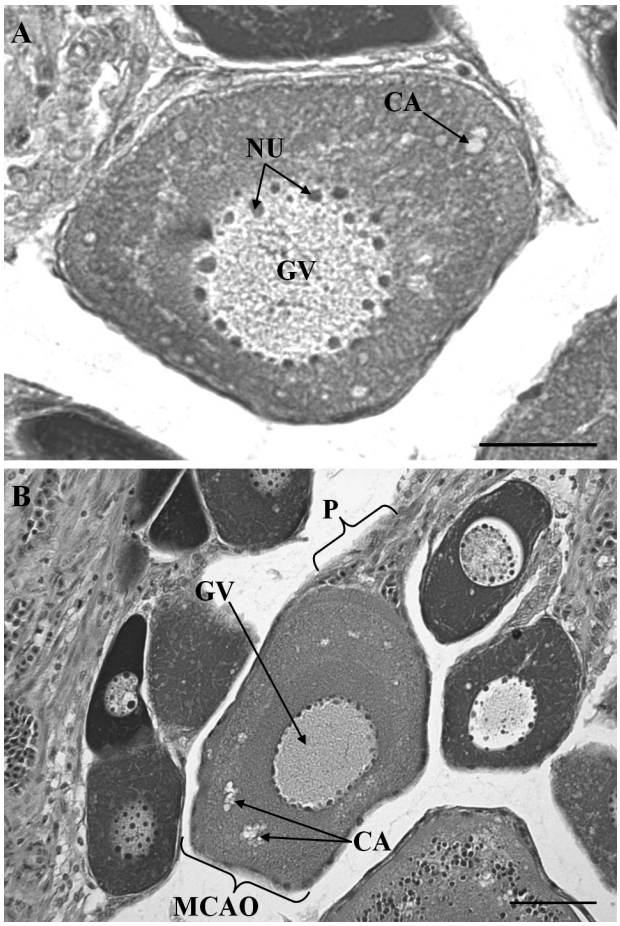

FIG. 4. – A, early cortical alveoli stage oocyte (Scale bar = 25 μm); and B, mid-cortical alveoli stage oocyte (Scale bar = 100 μm). CA, cortical alveoli; GV, germinal vesicle; MCAO, mid-cortical alveoli stage oocyte; NU, nucleoli; P, peduncle.

Towards the end of the cortical alveoli stage, single oocytes become suspended on individual peduncles, also termed pedicles, stems, branches, delle, or stalks (Erickson and Pikitch, 1993), that originate from the central ovarian stroma and extend towards the ovarian lumen (Fig. 4B).

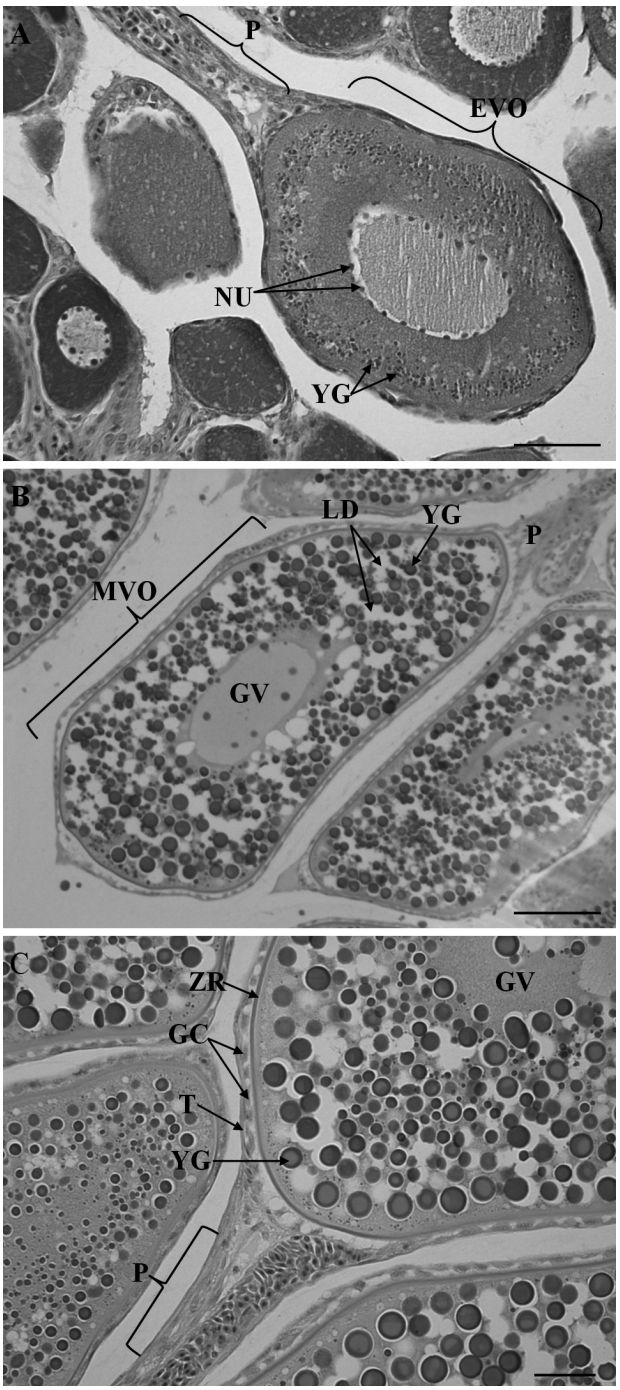

FIG. 5. – A, early vitellogenic stage oocyte (EVO) (scale bar: 50 μm); B, mid-vitellogenic stage oocyte (MVO) (scale bar: 100 μm); and C, follicular complex of vitellogenic stage oocyte (scale bar: 50 μm). GC, granulosa cells; GV, germinal vesicle; LD, lipid droplets; MVO, mid-vitellogenic stage oocyte; NU, nucleoli; P, peduncle; T, theca; YG, yolk granules; ZR, zona radiata.

Vitellogenic stage

The vitellogenic stage is characterized by single oocytes (130-500 μm diameter) suspended on peduncles with the follicular complex, including the zona radiata, granulosa cells, basal lamina, and theca cell layer, appearing thicker and more clearly differentiated than at earlier oocyte stages (Fig. 5). Multiple oocytes in the primary growth stage are visible along the base of the peduncles and are more concentrated closer to the stroma. The prominent germinal vesicle is centrally located with multiple peripheral nucleoli (Fig. 5A). Oocytes in the early vitellogenic stage have a granular, acidophilic yolk. The granules first appear in the peripheral ooplasm, and later form a ring around the oocyte cortex in the same region occupied by the cortical alveoli (Fig. 5A). As the vitellogenic stage progresses, the yolk granules increase in number and size until they are distributed throughout the ooplasm (Fig. 5B). As yolk granules accumulate within the oocyte, the cortical alveoli are displaced progressively toward the homogeneous and basophilic peripheral ooplasm.

Coincident with the deposition of yolk granules within the ooplasm is the deposition of lipid droplets. Lipids are eluted by histological processing and appear as empty spaces among the yolk granules in the oocyte sections (Fig. 5B, C). While considerable deposition of lipid droplets occurs as early as the cortical alveoli stage in some teleosts, most deposition of lipids into lionfish oocytes occurs during mid- to late-vitellogenesis (Fig. 5 and 6).

Maturation stage and ovulation

Rapid peripheral migration and disintegration of the germinal vesicle characterizes the oocyte maturation stage. As oocytes mature, the diameter increases to ≥500 μm and the large, distinct, and highly acidophilic yolk granules coalesce simultaneously with the accumulation of homogeneous yolk throughout the ooplasm (Fig. 6). This newly formed homogeneous yolk is less acidophilic and more translucent than the rings of homogeneous ooplasm formed earlier at the oocyte periphery and around the germinal vesicle.

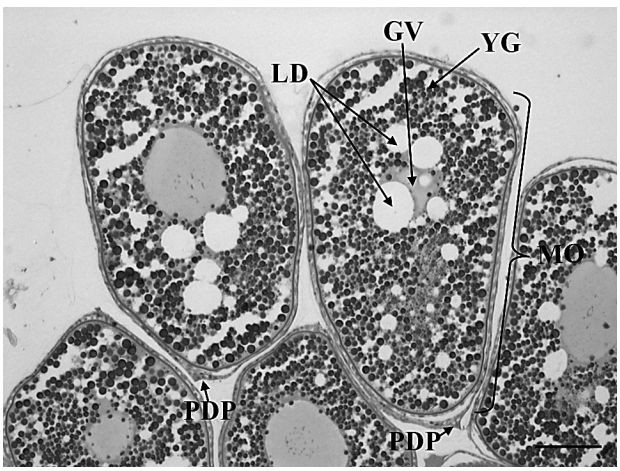

FIG. 6. – Early maturation stage oocyte exhibiting germinal vesicle migration, yolk granules, and lipid droplet coalescence (scale bar = 100 μm) GV = germinal vesicle, LD = lipid droplets, MO = maturation oocytes, PDP = peduncle detachment (ovulation) point, YG = yolk granules.

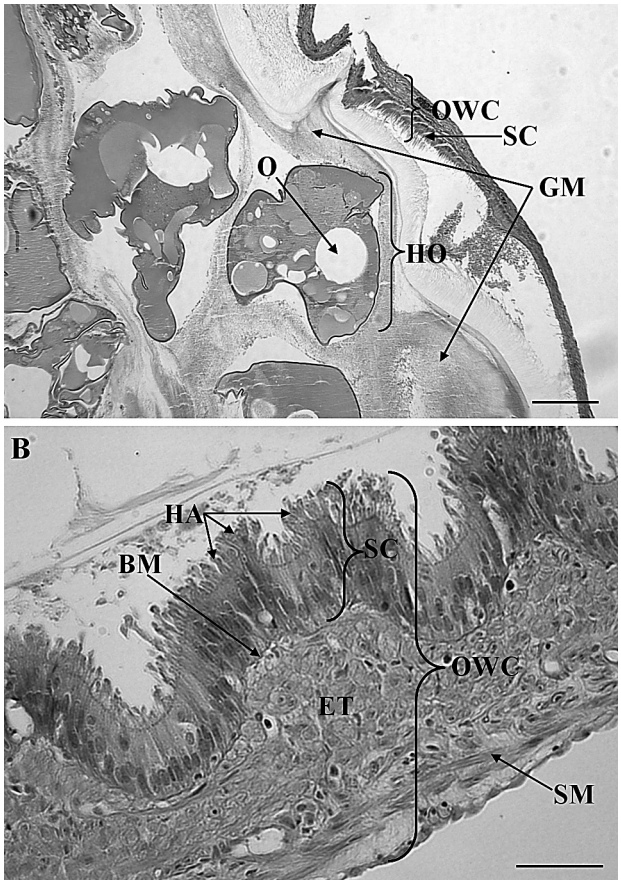

FIG. 7. – A) Ovulated eggs and ovarian wall depicting production of gelatinous matrix (scale bar = 150 μm) and B) ovarian wall complex (scale bar = 50 μm). BM, basement membrane; ET, endothelial tissue layer; GM, gelatinous material; HA, hair-like appendages; O, oil globule; OE, ovulated egg; OWC, ovarian wall complex; SC, secretory cells; SM, smooth muscle.

Lipid droplets coalesce to form progressively larger oil globules of various sizes within the ooplasm. The coalescence of lipid droplets in the central ooplasm precedes migration of the germinal vesicle toward the oocyte periphery (Fig. 6). The ooplasm eventually consists of one or a few large oil globules and several masses of homogeneous, translucent, and slightly acidophilic yolk, apparent against a background of more opaque yolk. Prior to ovulation, the stalk of the peduncle (bearing the maturing oocyte on its terminus) extends from its origin in the central ovarian stroma to the ovarian lumen. Maturing oocytes are sequestered near the opposing ovarian wall.

Mature oocytes detach from peduncles and are ovulated from their follicles at the point where the peduncular epithelium joins the follicular epithelium, thus leaving postovulatory follicles that thereafter consist of empty layers of thecal and granulosa cells. Simultaneously, a gelatinous matrix surrounds the new batch of ova (Fig. 7A). A single layer of specialized secretory cells located below the inner epithelium of the ovarian wall produces the encasing gelatinous matrix (Fig. 7B). These secretory cells are underlain by a basement membrane, an endothelial tissue layer, a layer of smooth muscle, and a fibrous layer of connective tissue, which collectively form the ovarian wall complex (Fig. 7B). During production of the gelatinous matrix, the secretory cells are columnar and spindle-like with hair-like appendages extending from their apical surface (Fig. 7B).

Spawn formation

Before release, the gelatinous egg masses slough off the ovigerous tissue from anterior to posterior and pass into the gonoduct leaving an opening at the anterior end of each gelatinous egg mass. Each ovarian lobe produces a single gelatinous egg mass, which is released separately during spawning (Fig. 8A). Ovulated eggs (n=10) extracted from egg masses shortly after spawning were slightly ovoid with a mean diameter of 804±25 μm. Each ovum contained one large oil globule, approximately 160 μm in diameter.

Atresia

After spawning, or when environmental conditions are not favorable for oogenesis and spawning, vitellogenic and maturation stage oocytes undergo preovulatory atresia. Atretic oocytes appear vacuolated

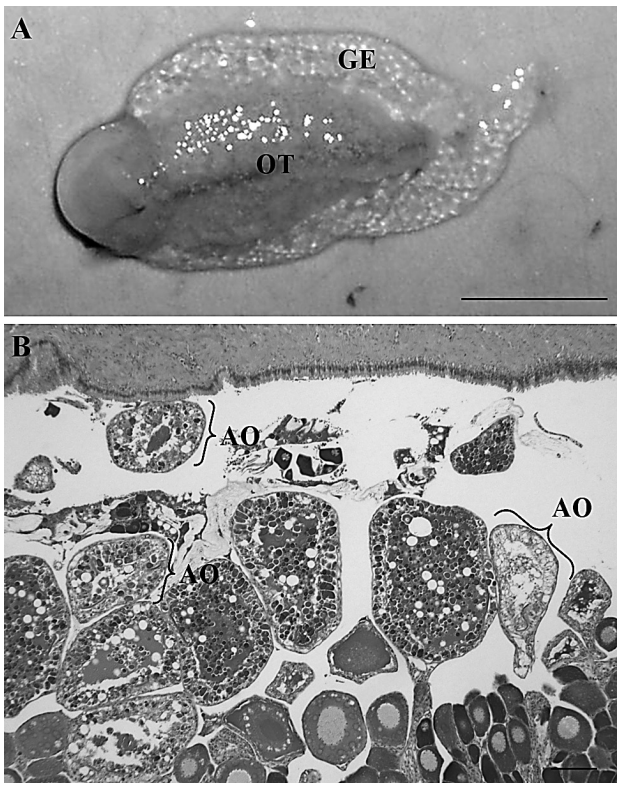

FIG. 8. – A, lionfish ovary with unreleased gelatinous egg mass (scale bar = 10 mm). Anterior end of the ovary is oriented to the left. B, oocytes in several stages of ovarian atresia. The most advanced-staged atretic oocytes are labeled as AO (scale bar = 100 μm). GE, gelatinous egg mass; OT, ovigerous tissue.

and are characterized by disintegrating yolk granules, diminishing homogeneous yolk, small lipid droplets that coalesce to form larger droplets, and in advanced atresia, the absence of a germinal vesicle. The apical segment of the peduncle that bore the oocyte is reabsorbed. Oocytes in the primary and cortical alveoli stage may remain in resting status until conditions are favorable for further development (Fig. 8B).

DISCUSSION

This is the first description of oocyte maturation, ovulation, atresia, and spawn formation in the lionfish, *P. miles* and *P. volitans*. All stages of oocyte growth and maturation were simultaneously observed in mature females, indicating that non-native lionfish in the Atlantic are asynchronous spawners (Murua and Saborido-Rey, 2003). This reproductive mode supports continuous production of eggs when environmental conditions are favorable.

This description of lionfish oogenesis will support future assessments of reproduction in the pteroines in both native and invaded ranges. For example, lionfish ovaries exhibiting only primary growth and cortical alveoli stage oocytes provide evidence that the local lionfish population is at a reproductively quiescent stage or is very early in its spawning season. The presence of oocytes at all stages of growth and maturation provides evidence that the local population is actively spawning. Conversely, the presence of highly atretic, vitellogenic and maturation stage oocytes in many individual female lionfish signals that the reproductive season is ending or has ended at that locale (Morris, 2009). This information is useful in forecasting the reproductive potential of invasive lionfish where seasonal changes in water temperature could influence reproductive output.

Lionfish ovarian morphology, while similar to that in some other scorpaenids, is uncommon among other teleosts. Lionfish ovaries are the most advanced of the cystovarian morphotypes (type II-3) described by Koya and Muñoz (2007). In lionfish ovaries, the vascular system is central, originates in the anterior end of the ovary, and runs longitudinally through the center of each lobe. The ovarian cavity is located between the ovarian wall and the central stroma. This ovary type is specialized for production of gelatinous secretions and utilizes specialized peduncular structures that support individual ovarian follicles during oocyte development.

Ovarian peduncles are seen in both viviparous (Hoar, 1969) and oviparous (Brummett *et al.,* 1982) fish, as well as some other vertebrates, including birds and reptiles (Franchi, 1962). Peduncles have been thought to enhance ovarian function by preventing oocyte crowding (Fishelson, 1975), by providing direct nutrient delivery to oocytes (Hoar, 1969), and by facilitating internal fertilization (Nagahama, 1983). Given that lionfish are asynchronous spawners capable of serial production of multiple batches of eggs, the highly vascularized peduncle of *P. miles* and *P. volitans* might enhance oocyte development via more direct oxygen and nutrient delivery to individual follicles. Additional ultra-structural and biochemical study of the ovarian peduncle could provide insights into its nutritive role.

The egg mass morphology of lionfish provides a potential mechanism for optimizing the fertilization rate. The eggs are embedded within a gelatinous matrix, which sloughs off from the ovarian lumen from anterior to posterior creating a hollow open-ended mass. As the courtship phase of reproduction ends, female lionfish ascend towards the water surface and release the hollow gelatinous egg masses, which are then fertilized externally by the male (Fishelson, 1975). We hypothesize that the hollow egg mass provides for sperm entrapment and concentration by inhibiting sperm dispersal, and thus facilitating fertilization. This adaptation might partly account for the relatively low batch fecundity observed in the lionfish (Morris, 2009).

The reproductive characteristics of *P. miles* and *P. volitans* – asynchronous mode, cystovarian morphology, vascularized peduncles, and the production of hollow buoyant gelatinous egg masses – might confer reproductive advantages that explain, in part, their rapid establishment in the Atlantic and Caribbean. These reproductive characteristics might also explain the successful invasion of *P. miles* into the eastern Mediterranean. The similarity of reproductive biology between these two closely related lionfish species is not surprising given their morphologic and genetic similarities (Schultz, 1986; Kochzius *et al*., 2003; Hamner *et al*., 2007), yet it may account, in part, for the observed invasiveness of both species. Given that the total reproductive output of the pteroines is largely undocumented, future studies of reproduction in *P. miles* and *P. volitans* should focus on assessments at the population level including estimates of batch fecundity and periodicity, spawning seasonality, and reproductive demographics including size at sexual maturity. This information will be critical for further elucidating the mechanisms of rapid establishment and expansion of these invaders into new habitats.

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