ZOOLOGIA 38: e56529
ISSN 1984-4689 (online)
zoologia.pensoft.net

RESEARCH ARTICLE

Gonadal development and sexuality of *Larkinia grandis* (Arcida: Arcidae) inhabiting southeastern Gulf of California

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http://zoobank.org/85853685-58E7-4323-9EF8-6A9BCE1B8220

ABSTRACT. *Larkinia grandis* (Broderip & G.B. Sowerby I, 1829), an important fishing resource for Mexican communities, is an Arcidae clam. It is also considered a species with aquaculture potential. In this work we investigated the gonadal phases and sexuality in a population of *L. grandis* in the Gulf of California. Our findings support the hypothesis that there is one male per female in the population studied. It also documents that the shape, position and color of the gonads of *L. grandis* are consistent with observations in other Arcidae species. Additionally, five gonadal phases are differentiated and described in males and females (development, mature, spawning, post-spawning and resting), with a noticeable presence of brown cells during post-spawning and the onset of the resting phase, suggesting that those cells are involved in the reabsorption of remnants. Additionally, asynchronous gametogenesis in males, synchronic gametogenesis in females and batch spawning are defined. The results of this contribution can be used in the efforts to protect this bivalve.

KEY WORDS. Bivalve, estuary, gametogenesis, gonad, mangrove cockle, sex cells.

INTRODUCTION

Larkinia grandis (Broderip & G.B. Sowerby I, 1829) (Mollusca: Bivalvia: Arcida: Arcidae) is a clam distributed from the Ballena Lagoon (Baja California, Mexico) to Tumbes (Northern Peru) (Coan and Valentich-Scott 2012), where it is known by the common name of "mangrove cockle" (García-Domínguez et al. 2008) or "pianguas" (Lucero-Rincón et al. 2012), respectively. This bivalve is found near the coastline, living in close relationship with the roots of the *Rhizophora* spp. mangrove, buried in the muddy sediment, or very rarely, half-buried or exposed (Fischer et al. 1995). Several members of the family Arcidae, including *L. grandis*, are commercially exploited on the Pacific coast (Cruz and Palacios 1983, Cruz 1987a). The clam *L. grandis* is caught along with other species of the same genus [*Anadara mazatlanica* (Hertlein & A.M. Strong, 1943), *Anadara multicostata* (G.B. Sowerby I, 1833), *Anadara similis* (C.B. Adams, 1852), and *Anadara tuberculosa* (G.B. Sowerby I, 1833)] in Mexico (CONAPESCA 2018). Generally, the data provided by the Mexican government does not distinguish among mangrove cockle species and fishery management does not consider the biological characteristics of each species when exploiting them commercially in Mexico. Interest in these clams has grown in recent years due to their use in handicrafts and for direct human consumption. Additionally, mangrove cockles have aquaculture potential in Mexico (Sotelo-Gonzalez et al. 2019), following the example of other countries (Broom 1985, Galdámez-Castillo et al. 2007).

Studying the gonadal development and sexuality of wild mollusk populations of commercial importance helps to reveal their reproductive phases and to define their reproductive patterns (Bricelj et al. 2017). At the same time, it is a standard procedure to describe the changes in the tissues and cells of the gonad generated by the accumulation of energy and expulsion of gametes (Karray et al. 2015), and to explain the way in which organisms modulate the use of their reserves in relation to environmental variables (Boulais et al. 2017), under normal or anom-

alous conditions. Furthermore, research on the sexual behavior of bivalves helps to understand the interactions between them and their environment and can provide information for the development of selective breeding programs (Breton et al. 2018).

Previous studies on the gonadal development of *L. grandis* arrived at conflicting results. Four gonadal phases were identified in a population in Costa Rica (Cruz 1987a) and six phases in Nicaragua (Aguirre-Rubí 2017). Cruz (1987a) did not describe the resting phase and Aguirre-Rubí (2017) generalized the gonadal phases for three species [*L. grandis*, *A. tuberculosa* and *Polymesoda arctata* (Deshayes, 1855)]. In addition, Cruz (1987a, 1987b) stated that *L. grandis* is a gonochoristic (dioecious) species, while Aguirre-Rubí (2017) did not define the sexuality of the three species, but documented one hermaphrodite organism (female with intersex) among 40 analyzed individuals in which the male and female acini were separate.

Additional information for other species of Arcidae is mentioned below. Broom (1983) identified six gonadal phases and one hermaphrodite individual in *Anadara granosa* (Linnaeus, 1758) from the west coast of West Malaysia; Broom (1985) defined that in *A. granosa* the sexes are undoubtedly separate; the gonadal status of *A. granosa* and *Anadara antiquata* (Linnaeus, 1758) were studied in central Java (Afiati 2007a, 2007b) and one study suggests that both species could be protandrous sequential hermaphrodites (Afiati 2007b); Jahangir et al. (2014) published four gonadal phases for *A. antiquata* from Pakistan and only individuals with separate sexes were described. In Colombia, Manjarrés-Villamil et al. (2013) described the five gonadal phases and found 15 hermaphrodite individuals in *A. similis*, and mentioned that hermaphroditism in the species needs to be further studied. Ghribi et al. (2017) observed four phases in the gonads of 142 females and 42 males, and documented five cases of protandric hermaphroditism in *Arca noae* Linnaeus, 1758.

The gonadal status of *A. tuberculosa* has been studied in the Pacific Coast of Costa Rica (Cruz 1984), Mexico (Pérez-Medina 2005, García-Domínguez et al. 2008) and Colombia (Lucero-Rincón et al. 2013). These studies revealed differences in the number of gonadal phases and used different nomenclature. In Costa Rica, Cruz (1984) did not find evidence of sexual reversal in any specimen of *A. tuberculosa*. In Mexico, Pérez-Medina (2005) documented two hermaphroditic organisms of *A. tuberculosa* and defined that it is gonochoric, but with casual hermaphroditism. In contrast, Lucero-Rincón et al. (2013) determined that *A. tuberculosa* is a protandric hermaphrodite in Colombian Pacific.

This contribution describes the gonadal phases and sexuality in a wild population of *L. grandis* in the southeastern Gulf of California and provides additional observations about the sex ratio and the color and anatomy of the gonads.

MATERIAL AND METHODS

The clams were collected in the El Cohui estuary (25°26'–19°38'N; 105°48'–43°90'W) within the San Ignacio-Navachiste-Macapule lagoon system in the state of Sinaloa, Mexico (Fig. 1), where an important area for fishing of *L. grandis* is located. In total, 240 clams were collected by free divers, from August 2017 to July 2018. Each sample (n = 20 per month) was placed in a container with sea water and transported to the laboratory (30 minutes) for processing purposes.

First, the length (mm) and weight (g) of each clam were registered; next, the shells were opened, and the soft tissues were removed. The soft tissues were macroscopically analyzed to observe external alterations, the appearance of the gonads and their location within the visceral cavity (Álvarez-Dagnino et al. 2017).

The soft tissues were fixed with Davidson solution, rinsed with distilled water to remove excess fixative and placed in 70% alcohol until dehydration (Álvarez-Dagnino et al. 2017). Then, a longitudinal section of the gonad tissue was dehydrated and embedded in paraffin (Buesa and Peshkov 2009). Histological sections of 3 µm thickness were obtained and stained using the Hematoxylin-Eosin-Floxin (HEF) technique (Humason 1972).

The sex of individuals was identified from the histological sections. The sex ratio (number of males per female, n:1) was calculated dividing the number of males by the number of females (Álvarez-Dagnino et al. 2017). The Yates-corrected Chi-squared test was used to compared the observed sex ratio with the expected value 1:1, using χ^2 with n-2 degree of freedom and a significance level $\alpha = 0.05$ (Zar 1996).

The histological sections were analyzed qualitatively using an optical microscope (Zeiss model Axiostar 10x, 40x and 100x) to identify the gonadal phases (development, maturity, spawning, post-spawning and resting) based on cellular and tissue characteristics and stages of gametogenesis, considering the criteria of Aguirre-Rubí (2017) for *L. grandis*, Pérez-Medina (2005) and García-Domínguez et al. (2008) for *A. tuberculosa*, Manjarrés-Villamil et al. (2013) for *A. similis*, Broom (1983) for *A. granosa*, Afiati (2007a) for *A. granosa* and *A. antiquata*, Ghribi et al. (2017) for *A. noae*. Additionally we found sub-phases of gonadic resting and described them based on differences in the dispositions and thickness of the connective tissue and in the number of brown cells. The browns cells were identified according to Ghribi et al. (2017). These data were documented with a Nikon D5200 camera adapted to a microscope. The images were transferred and processed on a computer with the Sigma Scan Pro program (version 5.0 Systat Software, Inc., Richmon, CA, United States) (Álvarez-Dagnino et al. 2017) in order to measure the diameter of the oocytes (mean±standard deviation µm) of *L. grandis*. Only oocytes with a visible nucleus were measured, and due to their irregular shape, first, the area was measured and then the diameter (D) was calculated assuming a circular form of each oocyte. An amount of 1200 oocytes at different oogenesis stages were measured from 84 individuals.

RESULTS

All clams collected were adults. Their length and weight ranged from: 44.57 to 142 mm and 41.90 to 337 g, respectively. The male (n=95) and female (n=108) gonads were in different

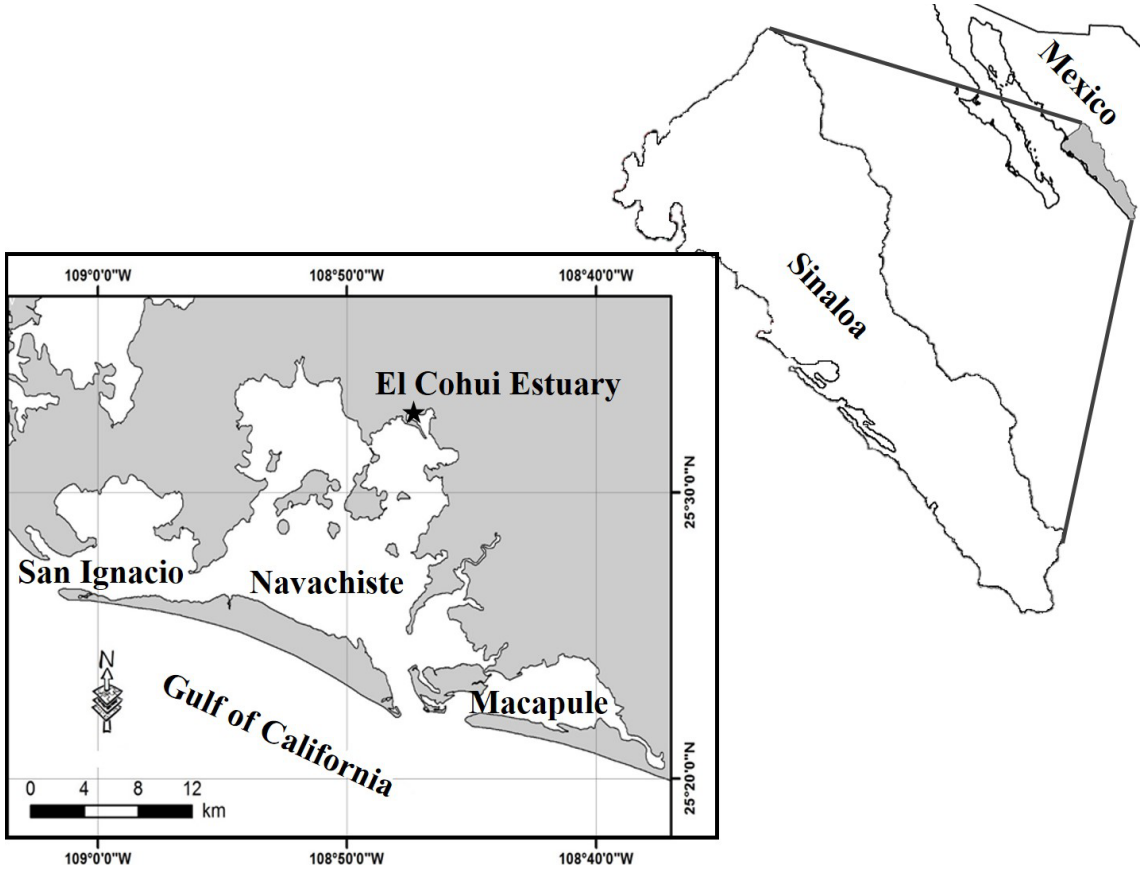


Figure 1. El Cohui estuary (★), San Ignacio-Navachiste-Macapule lagoon system, Sinaloa, southeastern Gulf of California, Mexico.

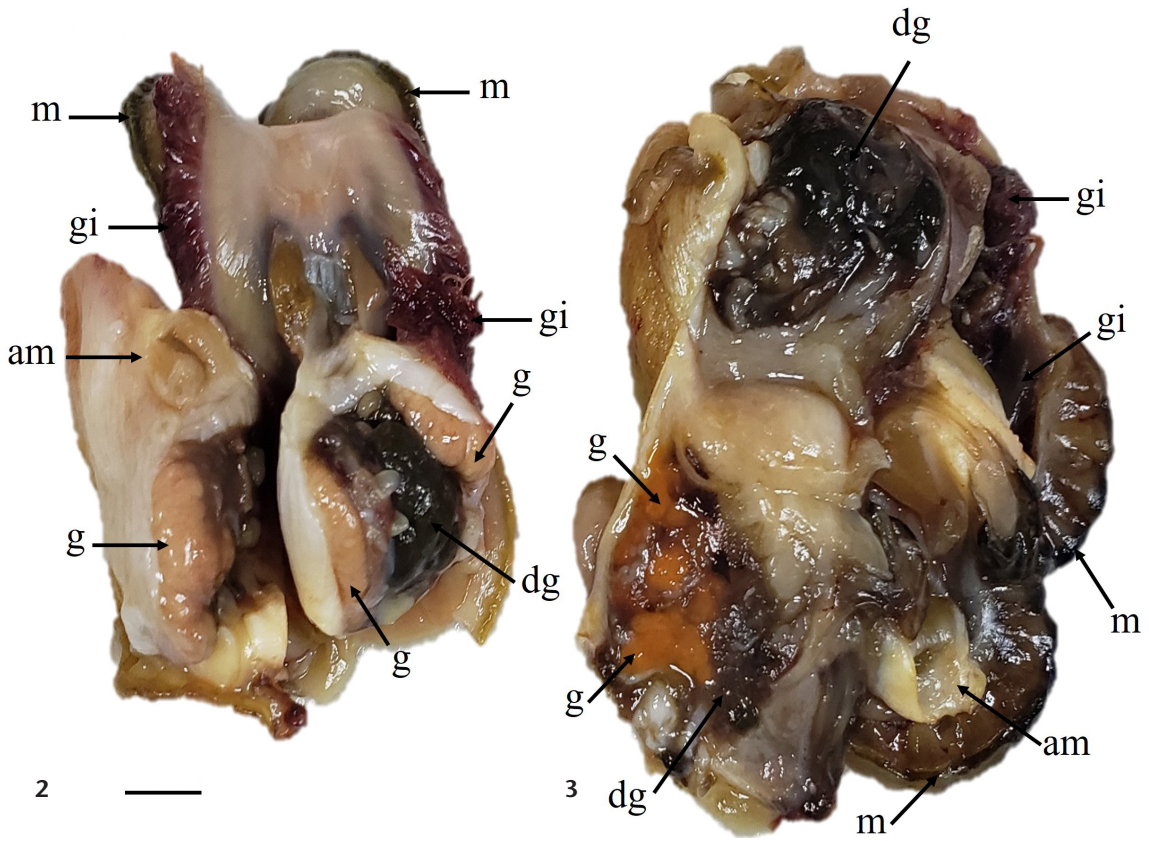
individuals in *L. grandis*, as in gonochoristic species, and the sex ratio was 0.88 males per female (0.88:1). This sexual proportion was not statistically different from the expected value 1:1 ($\chi^2_{203\text{-}2}$ = 12.07, p = 0.36), and neither were the sex ratios estimated for most months ($\chi^2_{\sim 20\text{-}2}$ = 0.07-2.57, p = 0.1-0.81), with the exception of April 2018, when there were more males than females (3:1; $\chi^2_{20\text{-}2}$ = 7.14, p = 0.02) (Fig. 4). Also, 37 indeterminate individuals (sexually) were identified. The external appearance of the gonads on *L. grandis* showed sexual dichromatism when gametogenesis occurs. This is more noticeable in their maturity phase. The gonad of males was cream-beige, and the gonad of females was orange-brown. The color tonality intensified as the gonad matured. The gonads were located within the visceral mass and occupied 20-40% of it (Figs 2, 3).

In females, the oogenesis cell stages distinguished were: oogonia (4.58 ± 0.80 µm D), previtellogenic oocyte (9.77±1.68 µm D), vitellogenic oocyte (17.65 ± 2.30 µm D) and yolked oocyte (33.08±2.83 µm D). In males, the spermatogenic cell stages observed were: spermatogonia, spermatocytes, spermatids, as well as spermatozoa. Females presented synchronic gametogenesis due to the presence of one or two gametogenic cell stages in a single acinus. While males presented an asynchronous gametogenesis because the simultaneous presence of all spermatogenic cell stages in a single acinus.

Five gonadal phases were differentiated for males and females: development, mature, spawning, post-spawning and resting. For the latter, three sub-phases were identified. Immature gonads (no previous ripening) were not defined. At the beginning of the progression of the gonadal phases, the acini are roughly round or elliptical, increase in size and their walls become thinner (development and mature phases), then the acini acquire irregular shape (after spawning phase) and finally the acini recover their roughly rounded or elliptical regular form (resting phase). Also the cell types and their quantity change due mainly to gametogenesis and reabsorption processes. The detailed description of gonadal phases for males and females of *L grandis* is in Table 1.

DISCUSSION

Among the bivalves studied, the majority were gonochoric (Gosling 2015). This is consistent with the findings for the population of *L. grandis* in southeastern Gulf of California, and also in the Pacific coast of Costa Rica, where hermaphrodite organisms were not found and the sexual proportion was



Figures 2–3. Internal morphology of *Larkinia grandis*: (2) male; (3) female. (g) Gonad, (dg) digestive gland, (m) mantle, (am) adductor muscle, (gi) gills. Scale bar: 1.0 cm.

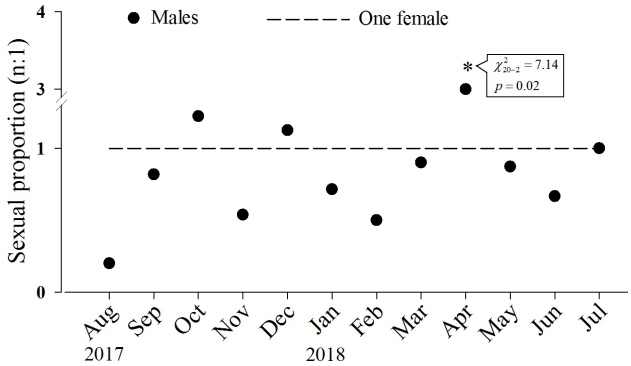
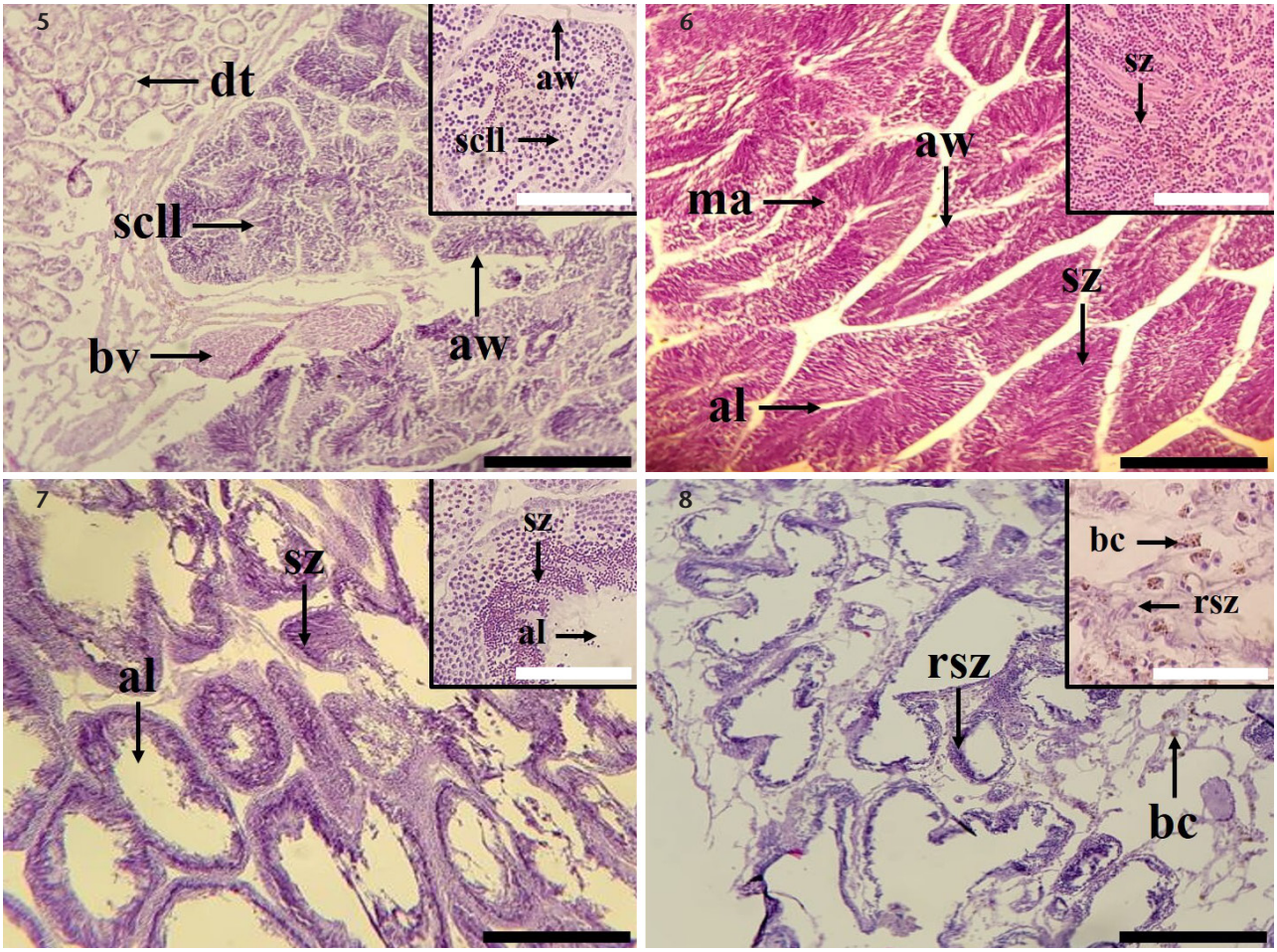


Figure 4. Monthly sexual proportion of *Larkinia grandis* during the 2017–2018 period. The dotted line marks one female as a reference for the estimated number of males by month. The asterisk (*) indicates that the sexual proportion is statistically different ($p < 0.05$) from the expected value (one male per one female, 1:1).

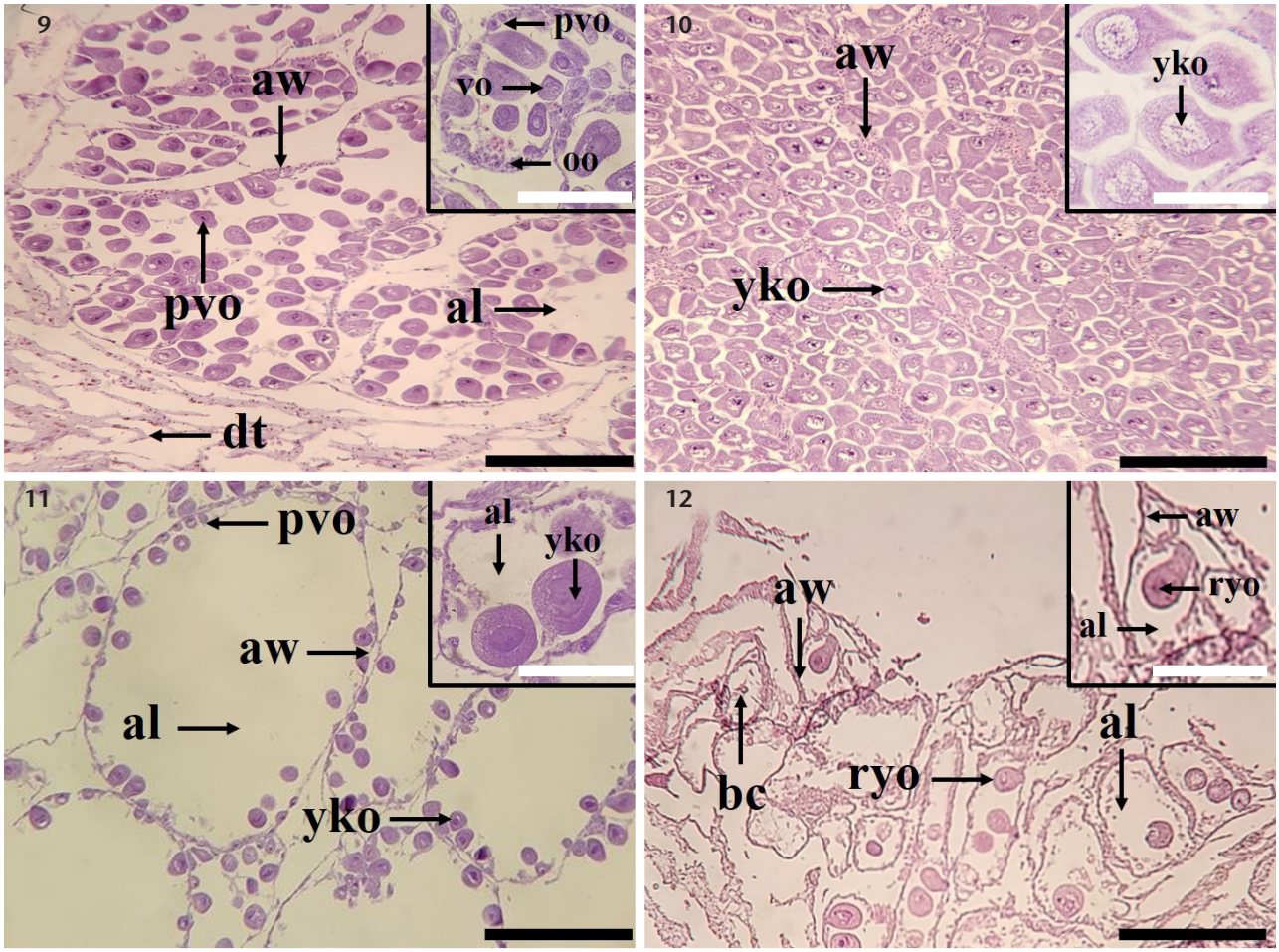
close to one male per female (1:1) (Cruz 1987a, 1987b). Only on April 2018, the studied population of *L. grandis* had more males than females, but the sex ratios estimated for the other months and the absence of hermaphrodites suggest that the deviation from the 1:1 sex ratio was casual. Aguirre-Rubí (2017) found one hermaphrodite specimen of *L. grandis* (2.5% of the total sample) on the Nicaraguan coast. In that publication, however, the sex ratio was not specified. Other Arcidae species have been defined as gonochoric, even though they have a low percentage of hermaphroditic organisms in the population, such as *A. granosa* (0.33%; Broom 1985) and *A. tuberculosa* (0.98%; Pérez-Medina 2005) because the percentage of hermaphroditism was considered low and the sexual proportion was close to 1:1. In these cases, hermaphroditism can be considered a casual phenomenon (Pérez-Medina 2005), and according with the observation of Aguirre-Rubí (2017), casual hermaphroditism could also occur in *L. grandis*. However, more research is needed on hermaphroditism in bivalves to improve our understanding of it (Breton et al. 2018). It is necessary to understand sexuality in species such as *A. tuberculosa*, which in different populations has been classified as gonochoric (Cruz 1984), gonochoric with casual hermaphroditism (Pérez-Medina 2005) and protandric hermaphroditism (Lucero-Rincón et al. 2013). These different sexualities have been defined according with different percentage of hermaphroditism, and some estimates of sex ratio and

Table 1. Description of the gonadal phases of males and females of *Larkinia grandis* in southeastern Gulf of California.

Phases	Males	Females
Development	Spermatogonia, spermatocytes, and free spermatozoa are attached to the acini wall or germinal epithelium. Connective and digestive tissues are reduced (Fig. 5).	Abundant previtellogenic or/and vitellogenic oocytes are observed in the acinus. Narrow empty spaces can be seen in the acinus, between oocytes. Oocytes diameter = 26.40 ± 2.46 µm (all stages) (Fig 9).
Mature	The gonadic tissue has reached its maximum development; acini are large and distended. The acini are large, completely filled and their lumens are barely appreciated. Spermatids and spermatozoa cells are located towards the lumen of the acinus (Fig. 6).	Acini completely filled of yolked oocytes, and these have granular cytoplasm. Yolked oocytes are rounded or polygonal. The acinus walls are very thin and there are no empty spaces in the acinus. Few immature oocytes are attached to the acini wall. Oocytes diameter = 32.79 ± 3.41 µm (all stages) (Fig. 10).
Spawning	Acini are almost empty. Spermatozoa in different development stage are dispersed within acinus. Abundant brown cells are observed (Fig. 7).	Few previtellogenic oocytes are attached to the acini's wall, suggesting that gonad would mature soon. The acini's lumens are partially or totally empty. Oocytes diameter (all stages) = 27.62 ± 4.26 µm (Fig. 11).
Post-spawning	Residual spermatozoa are in the acinus. Abundant brown cells are observed. The acinus walls are thicker (Fig. 8).	Residual yolked oocytes are reabsorbed and/or being phagocytized within the acini. Abundant brown cells are observed. The acinus walls are thicker. Oocytes diameter (all stages) = 28.85 ± 1.41 µm (Fig. 12).
Resting	The sex of *L. grandis* cannot be defined since differentiated sex cells are not observed. The acini are empty. The connective tissue that forms the acini's walls is abundant and noticeable. The acini are contracted and lumens are narrow. The differences in the dispositions and thicknesses of the connective tissue and in the number of brown cells allow to define three sub-phases of resting: Sub-phase 1: The acini are small and irregular in shape; a lot of brown cells are attached to the connective tissue (Fig. 13). Sub-phase 2: The acini are elongated, bigger and there is smaller number of brown cells (Fig. 14). Sub-phase 3: The acini return to their roughly rounded or elliptical regular form and brown cells are scarce (Fig. 15).	



Figures 5–8. Histological sections of *Larkinia grandis* male's gonads: (5) development; (6) maturity; (7) spawning; (8) post-spawning. (al) Acinus lumen, (aw) acinus wall, (bv) blood vessel, (bc) brown cells, (dt) digestive tissue, (ma) mature acinus, (rsz) residual spermatozoa, (scll) spermatogenic cells, (sz) spermatozoa. Black scale bars: 100 µm, white scale bars: 50 µm.

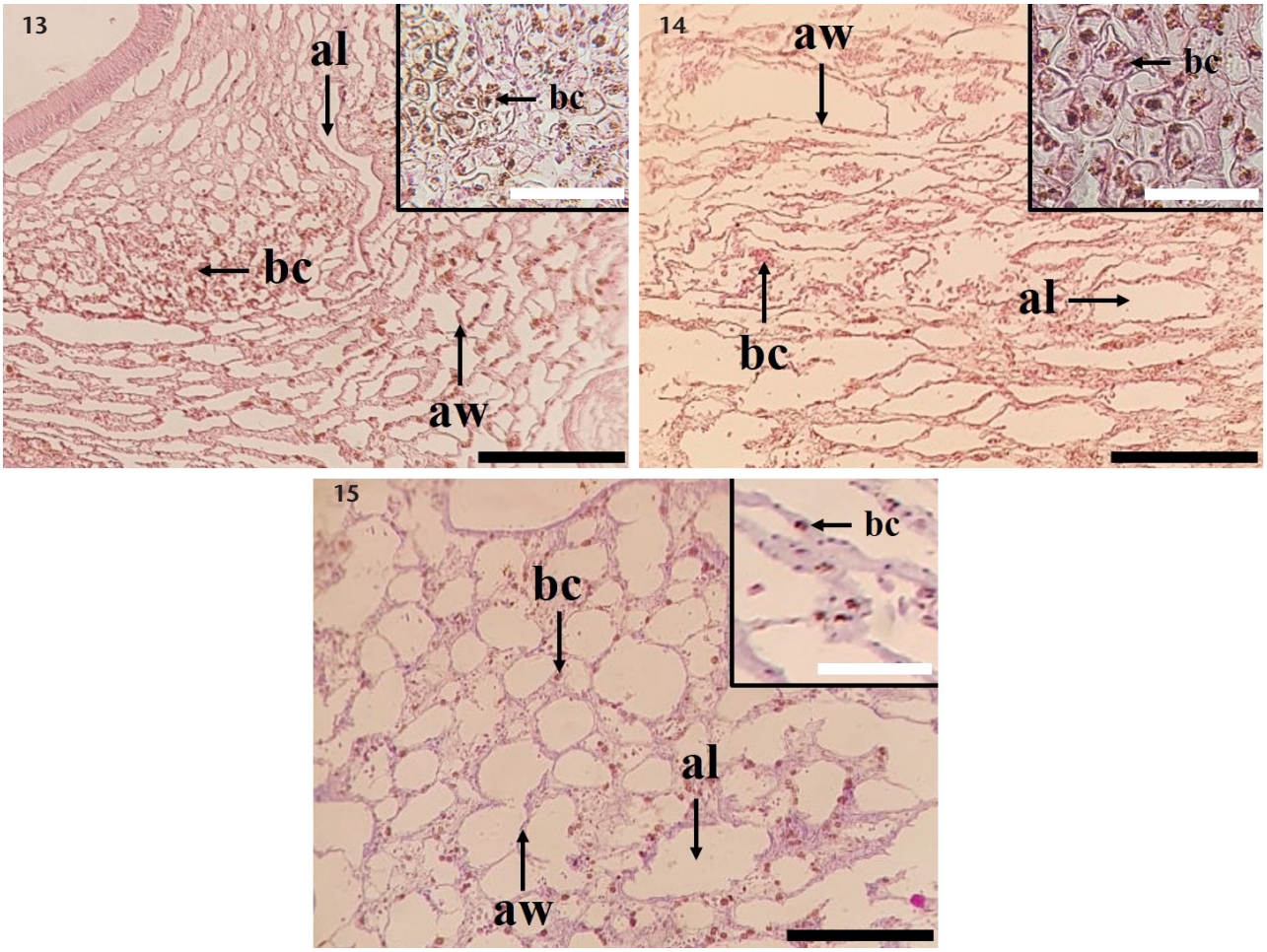


Figures 9–12. Histological sections of *Larkinia grandis* female's gonads: (9) development; (10) maturity; (11) spawning; (12) post-spawning. (al) acinus lumen, (aw) acinus wall, (bc) brown cells, (dt) digestive, (oo) oogonia, (pvo) previtellogenic, (ryo) residual yolked oocyte, (yko) yolked oocyte. Black scale bars: 100 µm, white scale bars: 50 µm.

size differences between males and females that suggest sex change. Some questions that remain to be answered are, what percentage of hermaphroditism should be in the population to rule out gonochoric sexuality? How could we define whether disparity in sex ratio and size between sexes is due to different longevity of females and males as suggested by Cruz (1987b)? The criteria to define sexuality need to be improved and clarified.

In this work, the shape and position of the gonads of *L. grandis* at the different gonadal phases are similar to those reported by Pérez-Medina (2005) and García-Domínguez et al. (2008) for *A. tuberculosa*, and by Manjarrés-Villamil et al. (2013) for *A. similis*. The gonad, regardless of sex, was located along the digestive gland. Karray et al. (2015) mentioned that gonad and digestive tissues in bivalve mollusks such as the sand clam *Cerastoderma glaucum* (Bruguière, 1789), are intertwined to facilitate the flow of nutrients according to the energy demand during the reproductive phases, while Menezes-Tunholi et al. (2016) highlighted the metabolic role of the gonad-digestive gland complex of the *Biomphalaria glabrata* (Say, 1818) gastropod. The tissue fusion described by both authors coincides with the conformation of the gonad-digestive gland complex in *L. grandis*.

In bivalve mollusks, the color of the gonad is considered a characteristic of sexual differentiation; frequently, female gonads are orange, while male gonads are creamy white (Mikhailov et al. 1995). However, there are species in which the gonads are of the same color. For instance, Meléndez-Galicia et al. (2015) and Álvarez-Dagnino et al. (2017) observed that in the rock oyster [*Crassostrea iridescens* (Hanley, 1854)] and the callista clam [*Megapitaria squalida* (G. B. Sowerby I, 1835)], respectively, gonads between sexes were of the same color throughout their reproductive cycle. In the present study, the gonads of *L. grandis* presented sexual dichromatism when gametogenesis occurs and the color tonality intensified as the gonad matured, similar to what has been observed in other bivalve species (Morriconi et



Figures 13–15. Histological sections of the resting sub-phases of *Larkinia grandis*: (13) sub-phase one; (14) sub-phase two; (15) sub-phase three. (al) acinus lumen, (aw) acinus wall, (bc) brown cells. Black scale bars: 100 µm, white scale bars: 50 µm.

al. 2002, Aragón-Noriega et al. 2007, Góngora-Gómez et al. 2016). The macroscopic dissection in *L. grandis* was not enough to distinguish their sex when the gonad is not mature, mainly due to the gonadal tissue being very small and difficult to examine with a naked eye. Histological analysis was necessary to adequately describe the sex of *L. grandis*, as well as in other clams (Cruz 1987b, Juhel et al. 2003, Lucero-Rincón et al. 2013), and the development of the gonads.

Five gonadal phases were differentiated in females and males of *L. grandis* (development, mature, spawning, post-spawning and resting). The resting phase was not previously described by Cruz (1987a), but in a complementary work, Cruz (1987b) mentioned one finding of an individual that could have been in the resting phase since it was different from the immature ones, but the tissue characteristics of both phases were not described. Meanwhile, Aguirre-Rubí (2017) grouped rest and spent gonads in one phase and described them as inactive and undifferentiated, but tissue details were not documented because his work was focused on other objectives. Immature individuals were not found in the sample of the present work. The smallest organism was 44.57 mm in length, and according with Cruz (1987b), the immature specimens are smaller than that, between 16.5 and 20 mm in length. Unlike the contributions mentioned above, this work describes the development process in only one phase, due to an overlap between the presence of previtellogenic and vitellogenic oocytes. And while Cruz (1987a) documented the maximum maturity phase and grouped attributes of the spawning and post-spawning process in one phase (spent), Aguirre-Rubí (2017) documented the mature, spawning and post-spawning phases, similar to the present work, but without describing the tissue details and only mentioning general features.

Despite the differences outlined above, the gonadal phases in *L. grandis* described by the present work are similar to the observations of Cruz (1987a) and Aguirre-Rubí (2017) for the species. The

difference is that the present work gives more detailed descriptions and uses slightly different nomenclature (Table 2). Additionally, the findings of this work are consistent with the descriptions of the gonadal phases of other species of Arcidae such as *A. tuberculosa* (Cruz 1984, Pérez-Medina 2005, García-Domínguez et al. 2008, Lucero-Rincón et al. 2013), *A. antiquata* and *A. granosa* (Jahangir et al. 2014), *A. similis* (Manjarrés-Villamil et al. 2013) and *A. noae* (Ghribi et al. 2017). It is important to note that there are different classifications of the gonadal phases even for the same species (Table 2), mainly due to the criteria applied by each researcher.

During the post-spawning phase and the onset of the resting phase of *L. grandis* (sub-phase 1), the presence of brown cells was noticeable, and their amount was reduced towards the end of the resting phase (sub-phases 2 and 3). Also, brown cells have been observed in the acini of ripe and partially spawned gonads of *A. noae* (Ghribi et al. 2017), in ripe and spawning gonads of *A. tuberculosa* (Lucero-Rincón et al. 2013) and in spawning and post-spawning gonads of *A. similis* (Manjarrés-Villamil et al. 2013). These cells were named as brown secretion granules in *A. tuberculosa* and *A. similis* (Lucero-Rincón et al. 2013, Manjarrés-Villamil et al. 2013). In mollusks, brown cells (also known as rhogocytes) are characterized by phagocytosis (endocytosis), role in metal ion metabolism, transport or storage of nutrients, synthesis or breakdown of respiratory pigments, supportive cell, selective reabsorption, and others (reviewed by Haszprunar 1996). In *L. grandis*, their presence and abundance after spawning suggests that brown cells are involved in the reabsorption of remnants during gonad recovery (post-spawning phase), and after the gonad is recovered, the abundance of brown cells decreases (resting phase).

Different stages of sex cells were distinguished in the same acini of male gonads of *L. grandis* (spermatogonia, spermatocytes, spermatids and spermatozoa), as reported by Ma et al. (2017) for the scallop *Chlamys farreri* (Johnes & Preston, 1904) and by Chatchavalvanich et al. (2006) for the freshwater mussel *Hyriopsis bialatus* Simpson, 1900. This asynchronous gametogenesis in males of *L. grandis* occurs because different cohorts of gametes that are forming occur simultaneously, and this indicates that spermatozoa production is continuous during the development and maturity of the gonadal phases in males. In contrast, female gonads presented synchronic gametogenesis due to the presence of one or two gametogenic cell stages in a single acinus, suggesting that oocyte production is discrete, and therefore it takes time for the next cohort to be ready to be released.

Since partially or totally empty acinus during the spawning phase of *L. grandis* were observed, and in some cases gamete-filled acinus, we suggest that this clam spawns in batches, a condition also reported by Pérez-Medina (2005), Manjarrés-Villamil et al. (2013) and Hernández-Hernández (2014) for *A. tuberculosa*, *A. similis* and *A. multicostata*, respectively.

ACKNOWLEDGEMENTS

The authors thank Instituto Politécnico Nacional for the logistical and economic support provided for the development of this research through the projects: "Morfometría y fisiología de la almeja venus *Chione undatella* de Bahía La Paz, y el puerto minero de Santa Rosalía, Baja California Sur, México (IPNSIP 20195442) and "Incidencia estacional de nematodos y *Perkinsus marinus* de la almeja venus *Chione undatella* de Bahía La Paz y del puerto minero de Santa Rosalía, Baja California Sur, México (IPNSIP 20190071). Also, we are thankful for the support of project PRODEP "Forzamiento ambiental en especies clave de los ecosistemas acuáticos" (Convenio DSA 511-6/17-7679 Folio UAS-PTC-131). Special acknowledgements for the laboratories and other support received from the Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional of the Instituto Politécnico Nacional and the Facultad de Ciencias del Mar, Universidad Autónoma de Sinaloa. M. I. Sotelo-Gonzalez thanks to CONACYT (Grant 714818). Special thanks to Jorge Manuel Elenes Lizarraga for the English language review.

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Table 2. Gonadal phases defined in different Arcidae species.

Species	Phases	Reference
Larkinia grandis	Gametogenesis, medium maturity, maximum maturity and spent.	Cruz (1987a)
	Undifferentiated, early development, late development, ripe, spawning and post spawning.	Aguirre-Rubí (2017)
Anadara tuberculosa	I. spawning, II. spawning and gametogenesis onset, III. ripe and IV. fully ripe.	Cruz (1984)
	In development, maturity, partial spawning/ejaculation and post-spawning/post-ejaculation.	Pérez-Medina (2005)
	Resting, developing, ripe, spawning and spent.	García-Domínguez et al. (2008)
	Undifferentiated or resting, development or vitellogenesis, maturity and spawning.	Lucero-Rincón et al. (2013)
Anadara antiquata	Developing, ripe, spawning, redeveloping and spent.	Jahangir et al. (2014)
Anadara granosa	Developing, ripe, spawning, redeveloping and spent.	Jahangir et al. (2014)
Anadara similis	Undifferentiated, development, mature, spawning and post-spawning.	Manjarrés-Villamil et al. (2013)
Arca noae	Developing, ripe, partial spawned and degeneration.	Ghribi et al. (2017)

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Submitted: July 15, 2020
Accepted: November 23, 2020
Available online: March 9, 2021
Editorial responsibility: Carlos Eduardo Belz

Author Contributions: All the authors contributed equally in the planning of the project and in the writing of the article. In addition, MISG, MGU, AMGG, CHS and JFAG did the samplings. JFAG and AMGG identified the species. MISG, CHS and RSC made the histological preparations, microphotographs and figures edition. MISG, AMGG, MGU and RSC did the diagnosis and histological descriptions. LASG performed the statistical analyzes and digital measurements.
Competing Interests: The authors have declared that no competing interests exist.