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Imposex Incidence, Morphological and Histological Description of Gonad in *Canarium urceus urceus* Linnaeus, 1758 (Mollusca: Gastropoda) in Caraga Region, Philippines

Fabio C. Ruaza Jr.*

College of Arts and Sciences, Surigao del Sur State University- Lianga Campus
*Corresponding author: fabioruaza@gmail.com

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Abstract The occurrence of imposex was detected in *Canarium urceus urceus* in Nasipit, Agusan del Norte, Caraga, Philippines. The imposex indices registered the Incidence of Imposex (IOI), Conversion of Potential Female Index (CPFI) and Relative Penis Length Index (RPLI) of 36%, 27%, and 30% respectively. The morphology of gonad of males was characterized by bright orange-red and whitish spot. A female gonad has a completely yellow in color. Imposex female's gonads has yellow or a mix of yellow and pale orange. The result of the histological analysis revealed that the male gonads were in the active and ripe stages. Out of the samples subjected for histological analysis, 70% undergo the ripe maturity stage and the rest of the samples were active. The female gonads were 80% active and the rest of the samples were ripe. For the imposex gastropods, 100% of the histological samples have the presence of ovarian spermatogenesis. It is recommended to conduct an analysis of Tributyltin (TBT) in the tissue of the gastropods and even on sediments.

Keywords: *Canarium urceus urceus, imposex, histology, gonad, Caraga Region*

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1. Introduction

The *C. urceus urceus* is an important commercial seafood in the Philippines and Indo Pacific Region. This species is abundant wherever it occurs, and is normally associated with sandy mud bottoms and seagrass beds [1,2]. It was the most abundant herbivorous gastropod within the study site. Since gastropods are indicator of pollution in marine environment [3], it might be that the *C. urceus urceus* in the area was already affected specially their reproductive conditions.

In the same area (Nasipit, Agusan del Norte) it was reported that there are occurrences of imposex in some gastropods species like the *Thais tuberosa* and *Cronia margariticola* [4]. Imposex or pseudohermaphrodism is a widespread phenomenon among prosobranch molluscs characterized by the development and superimposition of non-functional male accessory sex organs (i.e vas differences and/or a penis) on female or juvenile gastropods [5]. Aside, it was observed that the operculum of imposex is much wider compared to female and male. However, the male gastropod operculum is wider compared to a normal female [6].

The incidence of imposex in gastropods is a worldwide concern and has been studied in many gastropods [3,7,8,9]. This phenomenon is caused by exposure to Tributyltin compound and results in accumulation of testosterone in the gastropods [10,11]. Tributyltin is the most commonly used antifouling agent used in paints for the underwater hull of large boats [12]. It was used to kill the algae, mussels and barnacles found in the hulls of the vessels which are a historic problem that hinders the operation of marine vessels.

The use of imposex standard indices will be established to evaluate the incidence of imposex in other gastropod species in Nasipit, Agusan del Norte considering that it is an inexpensive method to establish occurrence, patterns and changes in TBT pollution in marine environment. Considering that there are no agencies in the Philippines cater the analysis of Tributyltin (TBT), the analysis of TBT was not included in the study. However, for the future studies, this analysis will be carried out.

This study is very essential in on the reproductive condition of *C. urceus urceus* in Caraga Region. There is no study conducted in this gastropod in the incidence of imposex, morphological and histological characterization of gonads. This study will be anchored on the aim of addressing marine pollution in the coastal areas and

to describe its reproductive aspects. Also, its significance on the perspective of sustainable marine and coastal management.

2. Materials and Methods

2.1. Study Site, Collection of Samples and Processing

The collection of samples was done in the months of November and December 2014. The collection was random based from the gleaners in all sampling sites. The collection sites were situated on the sea grass since this specimen is bound to rocks and in sea grasses further down the shoreline. Also, majority of the gleaners prefer to collect shells in the intertidal area. A Global Positioning System (GPS) was used in order to keep track the gleaners of the exact locations of the sampling sites.

2.2. Processing of Samples

The specimen was preliminarily narcotized with magnesium chloride solution 3.5 % [13] and preserved in a refrigerator. After 24 hours, the samples were placed in 10% formalin solution of seawater and kept frozen prior to analysis. The use of this technique is proved very practical [1]; slowly pull the soft body until the columella muscle snapped without the need to break the shell.

2.3. Recognition of Sexes

The *C. urceus urceus* was classified as female, imposex female and male using penis length and gonad color as diagnostic features. The females had no penis and gonads that were fully yellow in color. Males were recognized having penis and gonads that were orange-red and few whitish spot. Imposex females had small penis and gonads that was mix of pale orange and yellow. Also, imposex was examined through morphological deformities like the symmetry in tentacles and abnormal eye growth.

2.4. Histology of Gonads

A total of 30 individuals (10 males, 10 females and 10 imposex) were randomly selected for the histology of gonads. The color of the gonad is easily noticeable. However, the gonad is closely associated with the underlying digestive gland, which makes it difficult to dissect from the rest of the soma without damage and loss of gonadal tissue [14]. Therefore, this assemblage of tissues was digestive gland and gonad. The tissue was fixed with Bouins fluid containing Formalin, Glacial Acetic Acid, and Saturated Picric Acid in ratio 5:45: 75 for 24 hours at room temperature. Organs were rinsed in tap water and transferred into 70 % ETOH for storage until the color is soluble xylene. Gonads were submitted for histological preparations at histopathology laboratory of Butuan Doctors Hospital, Butuan City. The description of gonadal development and microscopic features of each maturity stage was adopted in *Bolinus brandaris [15]*.

Table 1. Gonad development and microscopic features of each maturity stage [15]

Maturity stage	Main features of gonadal condition
I. Resting	Gonads have little internal structure; few small and incipient primary germ cells (spermatogonia and oogonia) are dispersed in a large and loose matrix of connective tissue.
II. Active Gonads	Progressively increase in size; the connective tissue is condensed in favor of more developed and closely packed acini. in males, diverse spermatogenic stages are visible (spermatocytes, spermatids and a few spermatozoa that occur in the lumina of the acini); in females, vitellogenic oocytes proliferate and appear at the periphery of the acini.
III. Ripe Gonads	In females, The ripe gonad is very compacted, full of mature oocytes with vitellum and packed with yolk granules (although earlier oogenic stages might still occur near the wall). In males, gonads greatly increase in size; the connective tissue virtually disappears, the acini are more grouped and highly compressed; in males, the acini are distended and their lumina are full of mature spermatozoa.
VI. Spent Gonads	Gonads greatly reduce in size because most gametes were released; large empty acini with some gametogenic remains are scattered in loose matrix of connective tissue. Most acini are completely empty, although a few might still contain some mature spermatozoa or oocytes; the undischarged oocytes undergo atresia, degenerate and are reabsorbed.

2.5. Data Analysis

To determine the occurrence of imposex, the following indices for indirect biomonitoring were calculated: The Relative Penis Length Index (RPLI) is an index that quantifies the degree of imposex in the population and is obtained from the equation: (Mean length of female penis)/(Mean length of male penis) x 100 [16]. The Conversion of Potential Female Imposex Index (CPFII) is an index developed to calculate the potential females of a given area of population converted to imposex. Equation is obtained in (number of imposex animals)/(Number of Animals + Number of Imposex Animals) x 100 [17]. The Incidence of Imposex (IOI) was calculated by the Number of Imposex Animals)/(Total Numbers of Females) x 100 [18].

3. Results and Discussion

3.1. Occurrence of Imposex

A total of 169 of individuals of C. *urceus urceus* were collected from the Barangay Talisay, Nasipit Agusan del Norte. As shown in Figure 1, revealed that the IOI, CPFII and RPLI of 36 %, 27%, and 30% respectively in all sampling sites. The incidence of imposex (IOI) recorded 36 % of the population of the female has the occurrence of imposex. The extent of imposex is determined using the index of Conversion of Potential Female to Imposex Indices (CPFII). The study revealed that 27 % of the population of the potential females was converted into imposex. Moreover, a relatively high RPLI was observed, wherein a significant development of the penis among female gastropods developed 30 % of the actual male penis among the populations.

Figure 1. The imposex occurrences

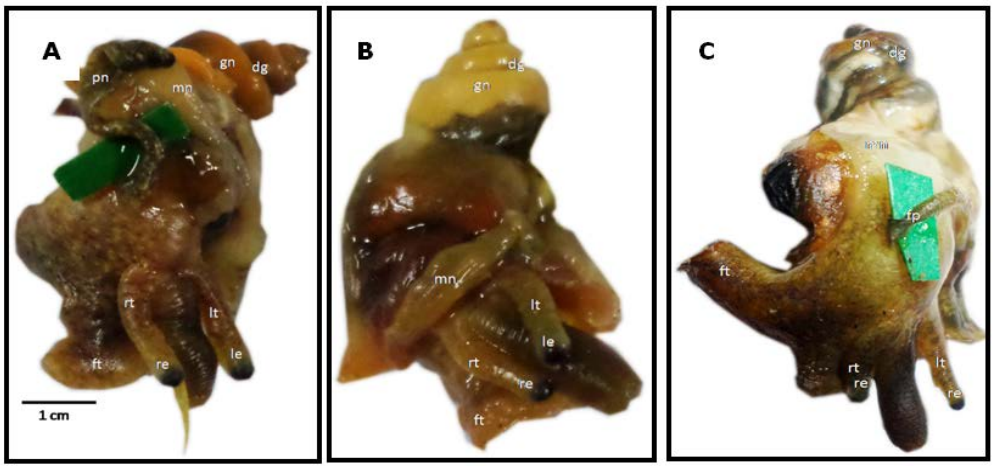

Figure 2. Internal structure of *C. urceus urceus* (A. male; B. female; C. imposex; mn: mantle; ft: foot; pn:penis; fp: female penis gn: gonad; dg: digestive gland; rt: right tentacles; lt: left tentacles; re: right eye; le: left eye)

3.2. Morphological Characterization

It has been observed that all imposex *C.urceus urceus* possess unequal length of tentacles (Figure 2). The right tentacle is shorter compared than the left tentacle. However, the male and normal female exhibit equal length of tentacles. The tentacles of the gastropods are their sensory organ [19]. They use them to feel around their environment. Also, they have a light-sensitive patch on the tip of the tentacles the eyes of the snail. There are also olfactory sense cells or sense of smell on the tentacles.The possibility of abnormal length of the tentacles of imposex gastropods may vary with the habitat they occupied. The presence of assumed organotin compound in the area may lead to the abnormal formation of the different parts of the gastropods. This result of the study was the same on the *Nucella lapillus* that there are morphological deformities from contaminated sites [3]. It was also reported in *Babylonia spirata* that there are morphological abnormalities in the growth of the tentacles and abnormal eye [20]. The male penis of *C. urceus urceus* consists of a long (1.13cm ± 0.21); and slender in the middle and stout tip penis. However, the female imposex are shorter (0.31cm ± 0.026), stout in middle to tip. In the male, the penis and the penis sheath are located together to the left of the extreme right margin of the mantle cavity. In imposex females, the arrangement of the penis was similar but differed in the distance between the penis sheath and the penis. For the female imposex the penis sheath was located at the edge of the ctenidium at the mantle skirt, at a distance from the penis. The penis lengths of the females are smaller compared to the male penis. The longest pseudopenis was 0.32 cm compared to the normal male of 1. 16 cm. For the morphological description of gonads using a dissecting microscope. It was observed that males were characterized by bright orange-red and whitish spot. A female gonad has a completely yellow in color and has a huge mass of oocytes, indicating a matured gastropod. Imposex females gonads has yellow or a mix of yellow and pale orange.

3.3. Histology of Gonads

Figure 3 shows the histological sections of the female gonad. It was observed that the female gonads collected during the sampling period were ripe (A-B) and active (C-D). Out of the samples subjected for histological analysis, 80 % undergo the active maturity stage and the rest of the samples were ripe. For the ripe gonad (Figure 3A-B), it was covered by the ventral space within the visceral area. There were no spaces inside and outside the follicle. Some oogonia and previtellogenic oocyte were found close to the follicles borders and germinal epithelium. It was also observed that the ripe female

gonad structure is compacted. For the active maturity stage (Figure 3C-D), the oocytes were scattered on the gonad surface. The follicles become larger with little amount of connective tissue between follicles.

The mature female, large eggs completely fill the lumen and a few oogonia are present along the germinal epithelium [14]. Mature eggs may appear polygonal in shape instead of being round or ovate, because they are so large and are competing for space. The collection of samples was done in the last week of November and the first week of December 2014. These suggest that the gonadal maturity on that month is their preparation for their spawning period. The oocytes size of *C. urceus urceus* in female and imposex. The average sizes of early and active oocytes were 1.4 ±SEM µm and 3.2 ±SEM µm respectively.

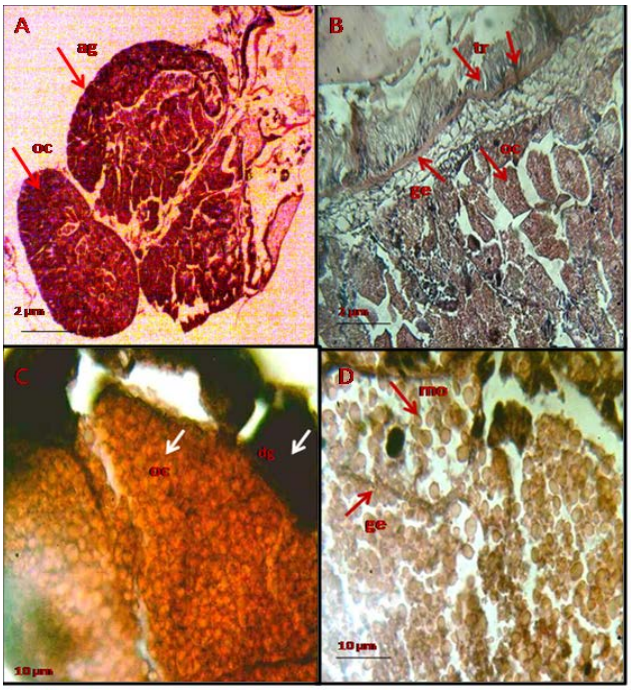

Figure 3. Histological sections of female gonads in *C. ureus ureus.* (A- B. ripe gonad; C- D. active gonad;; ge: germinal epithelium; oc: oocytes; mo: mature oocytes; tr: trabecula; dg: digestive gland; ag: alveolar gland)

Figure 4 shows the histological sections of male gonad. The male gonadal maturity was active (Figure 4B) and ripe (Figure 4A-C-D). Out of the samples subjected for histological analysis, 70 % undergo the ripe maturity stage and the rest of the samples were active. For the active male gonad (Figure 4B), the spermatogenic stages were visible, such as the spermatocytes, spermatids and a few spermatozoa that occur behind and clustered close to the germinal epithelium. The ripe male gonad (Figure 4A-C-D), has a highly compressed mature spermatozoa and a thin walls of gonadal follicles that were scattered in the follicles center. Also, the gonads increase in size and the connective tissue virtually disappears. The gonadal characteristics observed in female and male *C. urceus urceus* have the same gonodal maturity, the active and ripe gonad. The timing of testis and ovary development, and specifically the existence of mature males when females are not ripe, may be interpreted as a modality to increase the reproductive success [20].

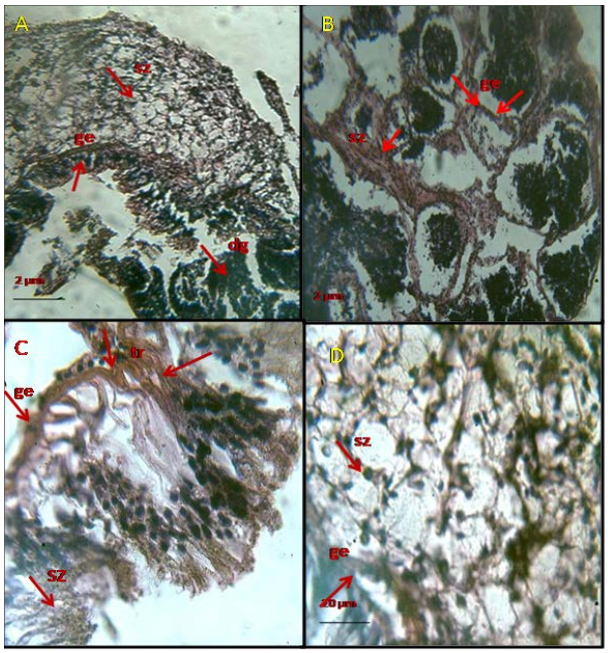

Figure 4. Histological sections of male gonads in C. urceus urceus (A- B. active gonad; C- D ripe gonad;; ge: germinal epithelium; sz: spermatozoa; tr: trabecula; sz: spermatids)

Figure 5 shows the histological sections of imposex gonads in *C. urceus urceus*. It shows that the imposex female has spermatozoa and oocytes (Figure 5A-B) and yolk granules that surround the gonadal follicles. All (100%) of the histological samples undergo this characteristics. Spermatid development and seminal vesicles were observed in the imposex female (Figure 5B). Several oocytes were observed that surround in the development of spermatid. However, the imposex oocytes, the average size is 3.8 ±SEM in which it is almost the same size in late active oocytes in normal female. Also, there are imposex oocytes that were observed with the average size of 1.3 µm that almost the same oocyte size in the early active oocytes in normal female. Nevertheless, the number of imposex oocytes is fewer compared to the normal female. This phenomena causes reproductive failure and a functional sterility because egg capsules can no longer be released, and accumulate in the oviduct and can be seen as an abortive black mass in the walls of the capsule gland [21]. The ripe gonad distinguished as compact oocytes in gonadal follicles. In the case of imposex, the oocytes were scattered in the gonadal follicles in which that is the characterization of an active gonad. The active gonad characterized as oocytes were scattered on the gonad surface. Also, it was noted that the presence of degenerated ova and yolk granules (Figure 5CD) by large amount in all samples of imposex. The gonad follicles has the spermatogonia, spermatocytes and few spermatids. The presence of yolk granules indicate the when the lumen is emptied of mature eggs during the copulation stage, the central lumen contains only a few scattered yolk granules [14]. Oogonia and small oocytes remain attached to the germinal epithelium. In the case of imposex the possibility that the wolfian duct designed to become a vagina has been blocked and disrupted with the influence of estrogen thus imposition of male organ occurred. The development in the gonadal tissue of imposex is called ovarian speramatogenesis.

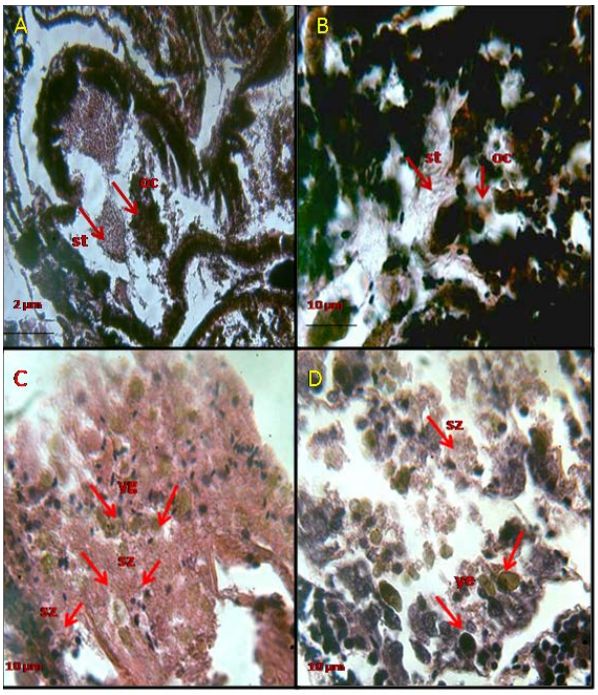

Figure 5. Histological sections of imposex gonads in *C. ureus ureus*. (A-B. imposex female showing spermatozoa and oocytes; C- D imposex female showing spermatozoa and yolk granules; ge: germinal epithelium; sz: spermatozoa; tr: trabecula; sz: spermatids; oc:oocytes; yg: yolk granules)

The histological section of imposexed female of *Babylonia spirata* is the formation of ovo-testes or ovarian spermatogenesis was observed [21]. This development causes reproductive failure and a functional sterility because egg capsules can no longer be released so that they accumulate in the oviduct and can be seen as an abortive black mass in imposex through the walls of the capsule gland. Finally, the oviduct ruptures, and as a consequence of female sterility and increased mortality, populations decline and the sex ratio shift in favor of males [22]. This result was consistent with the previous studies conducted in Nasipit, Philippines, that imposex was observed in murcideae species such as *Cronia margariticola* and *Thais tuberosa [4]*. However, *C. urceus urceus* exhibit less imposex compared to the other species of gastropods. In addition, the another factor that can influence the expression of imposex is age [23]. The species that are long lived or slower growing may be more likely to have high levels of TBT and thus exhibit imposex.

4. Conclusion

The occurence of imposex in Nasipit, Agusan del Norte of the *C. urceus urceus* was detected high using imposex indices, thus, suggesting abnormalities in the population. This gastropod is very sensitive to marine pollutants, hence, exhibiting imposex. The analysis of Tributyltin (TBT) content should be conducted in the tissue of the gastropods and even on sediments.

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