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# Sex Determination of Sea Cucumber *Acaudina rosettis* from Madura Straits, Indonesia

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## ABSTRACT

*Acaudina rosettis* is one of the commercial sea cucumber species that is widely consumed on the east coast of Surabaya, East Java, Indonesia. Although its abundance in nature has begun to show signs of decreasing, studies on this species are still very limited. This study aimed to examine indicators that can be used to determine the sex of *A. rosettis* individuals. *Acaudina rosettis* samples were collected from Madura strait in the mid of Hijri month for three months. The sea cucumber samples were then dissected. Body wall without viscera and gonads was weighed. The gonads were then characterized macroscopically and microscopically, prepared into histological slides with hematoxylin-eosin staining. The results showed that A. rosettis samples could be classified into three sex groups based on the type of gamete cells observed in the tubules; male (spermatogenic), female (oogenic), and undetermined sex (no gamete cells found). Gonads had three colors; yellow, pink, and orange and were in five stages of reproductive phase; gametogenesis (G), mature (M), spawning (S), and post-spawning (PS). Male samples had yellow and orange gonads, female samples were pink and orange, and all undetermined sex were orange. The G gonads were yellow and orange, M were yellow and pink, while S and PS were orange. Body wall and gonad weight at the same reproductive phase did not show any difference between males and females. Based on the results, gonad color can be used as an indicator of sex determination, but only in G and M reproductive phases.

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## INTRODUCTION

East Coast of Surabaya is one of most productive areas in Surabaya producing sea cucumbers. Sea cucumbers are found in the Madura Strait, located between the island of Java and Madura. There are two main species sea cucumbers traded commercially in this area; *Phyllophorus sp.* and *Acaudina rosettis*. *Acaudina rosettis* was first identified as a new species from the Bay of Johor, Malaysia (O'loughlin & Ong, 2015). Previously, the same species was identified as *Paracaudina australis* due to lack of records (Winarni *et al.*, 2014). Scientific studies showed that this species has high nutritional content; high of protein (20.22%) and low carbohydrates (0.86%), and contained linoleic acid (0.016 %), arachidonic acid (0.032%) and Eicosapentaenoic which was found in previous study to be able to help wound healing and tissue formation (Widianingsih *et al.*, 2016).

Results of previous study on East Coast of Surabaya showed that the distribution level of *A. rosettis* was in moderate category for 2 years of observation (5.78% and 6.57% of total sea cucumber sample) (Winarni *et al.*, 2014). This indicated that there is an effect of trading this species as commercial commodity to its population in the nature. On top of this, this species has not been listed both in the IUCN and CITES databases. Thus, the continuing survival of *A. rosettis* needs to be noticed. For this reason, various information is

needed to support conservation efforts to maintain *A. rosettis* population living in Madura Strait. One of them is from the aspect of reproductive biology.

The study of sea cucumber reproductive biology in Indonesian waters is currently very limited and not exceptional for sea cucumbers traded commercially. *Acaudina rosettis* is one of the sea cucumber species that does not show sex dimorphism. The sex of *A. rosettis* and its reproductive phase could only be determined based on the presence or absence of spermatozoa and ovum in the histologic section in previous study (Widianingsih *et al.* 2018), has also been done for other sea cucumber species. There is still no information regarding the relationship between the macroscopic and microscopic structures of the gonads of *A. rosettis* and the macroscopic indicators that can be used to determine the sex of the *A. rosettis* sea cucumber.

This study was conducted to complement the existing reproductive biology data of *A. rosettis*. By knowing clear indicators of sex determination, this information is expected to help implement marine resource management arrangements in the waters around Surabaya.

## MATERIALS AND METHODS

### Sample collection and morphology observation

Samples of *A. rosettis* were collected from the east coast of Surabaya, in the Madura strait with a geographic range between 07°12'48.71" to 07°1 5'54.53" south latitude, and between 112°48'16.21" to 112°52'34, 97' east longitude. Sampling period followed Qomariyah calendar system or Javanese calendar, which was between 15th-20th of Hijri month, while in AD calendar August 21st, September 21st, and October 20st respectively. Every month, 25 samples of *A. rosettis* were collected from Madura strait. Species was identified as *A. rosettis* based on O' loughin & Ong (2015) (Figure 1). The sea cucumbers were anesthetized in MgCl 7% before dissected, removed of its viscera, then body walls were weighed. Gonads were weighed fresh, while body walls were dried first using oven at 50°C to remove water content and weighed until dry weight obtained was constant. The color of the gonads was recorded and the gonads were then fixed in a 10% neutral buffered formalin solution.


**Figure 1**. Sample of fresh *Acaudina rosettis* collected from Madura strait.

### *Histology preparation*

Fixed gonadal tubules were then prepared into histological slides. The tubules were dehydrated, embedded in paraffin, and then cut into 4 µm section. The tubules were cut in serial sections at distance of 25 µm to observe the different parts of the tubule. The sections were stained with Harris hematoxylin and eosin counterstain then evaluated using light microscope.

*Data analysis*

Total of 20 sections were observed using light microscope to determine sex and reproductive phase of each *A. rosettis* sample. Determination of sex and maturity stage of everyone was based on Winarni *et al.* (2012), where gamete cells and inner epithelium components were identified under magnification up to 1000x. Data of gonad color was analyzed descriptively, while data of gonad weight, body wall weight, and their correlation to maturity stage was analyzed statistically using Spearman correlation test ($p = 0.05$).

## RESULTS AND DISCUSSION

### *Gonad histology*

The histological section of the male gonad is presented in Figure 2, while histological section of female gonad in Figure 3. The male gonads are indicated by the presence of spermatogenic cells in the tubules, while the female gonads are indicated by the presence of oogenic cells. Samples without either gamete cells are determined as undetermined sex.

The development of oogenic cells from the female gonadal tubules of *A. rosettis* is presented in Figure 4. Previtellogenic oocytes develop from tubular wall, which will become vitellogenic oocytes, followed by post-vitellogenic oocytes. Previtellogenic oocytes are indicated by a nucleus,

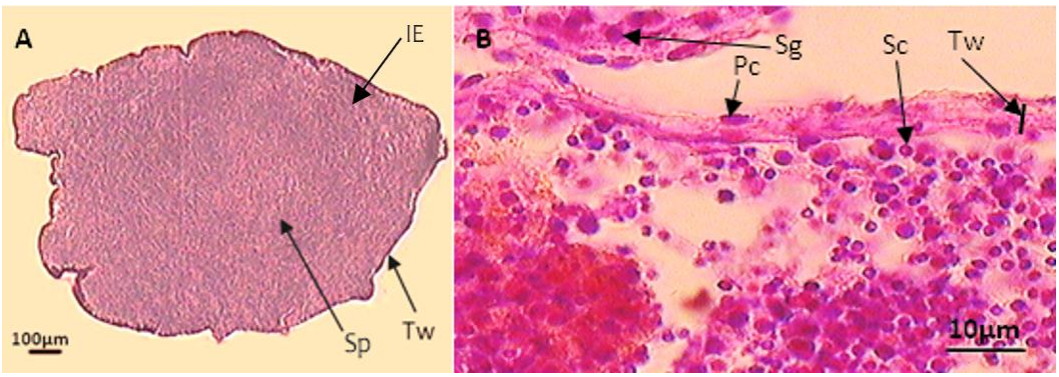


**Figure 2.** Histology section of male *Acaudina rosettis* gonads. A: cross-section of a tubule at mature phase; B: inner epithelium structure; IE: Inner epithelium; Sp: spermatogenic cells; Tw: tubule wall; Pc: Parietal cell; Sg: Spermatogonia; Sc: Spermatocyte.

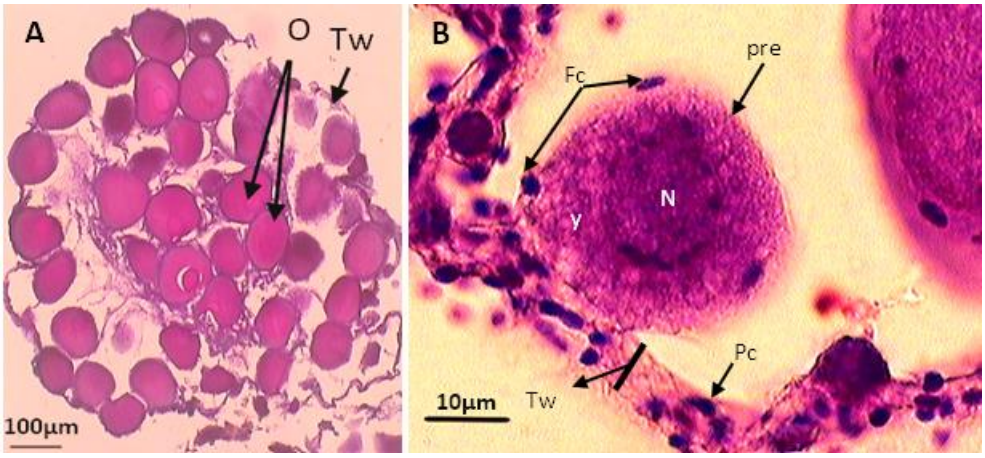


**Figure 3.** Histology of female *Acaudina rosettis* gonad. A: cross-section of tubule at mature phase; B: inner epithelium structure; O: oocyte; Tw: tubule wall; Fc: follicular cell; Pc: parietal cell; pro: previtellogenic oocyte; N: oocyte nucleus; y: yolk.

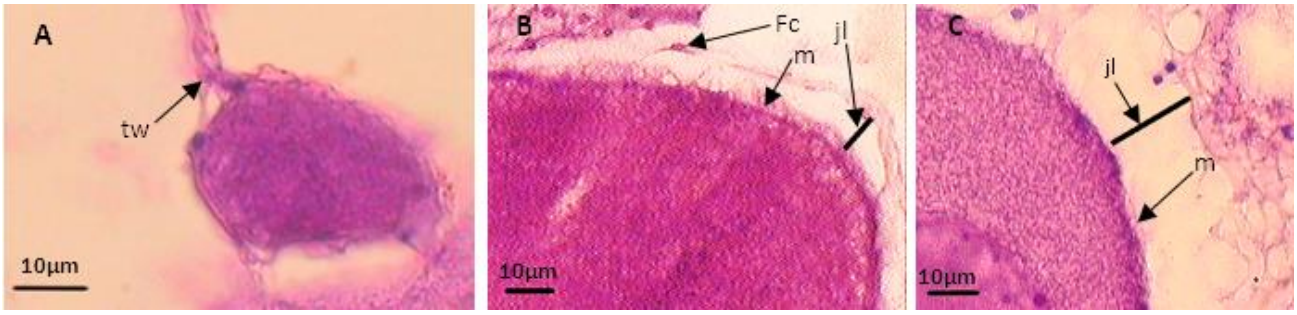


**Figure 4**. Oogenic cells in female *Acaudina rosettis* gonad. A: previtellogenic oocyte; B: vitellogenic oocyte; C: post-vitellogenic oocyte. tw: Tubule wall, Fc: follicular cell; m: microvilli, jl: jelly layer.


**Figure 5.** Color morphology of *A. rosettis* gonads. A: Male gonads colored creamy yellow, B: female gonads colored pink, C: undetermined gonads colored yellow.

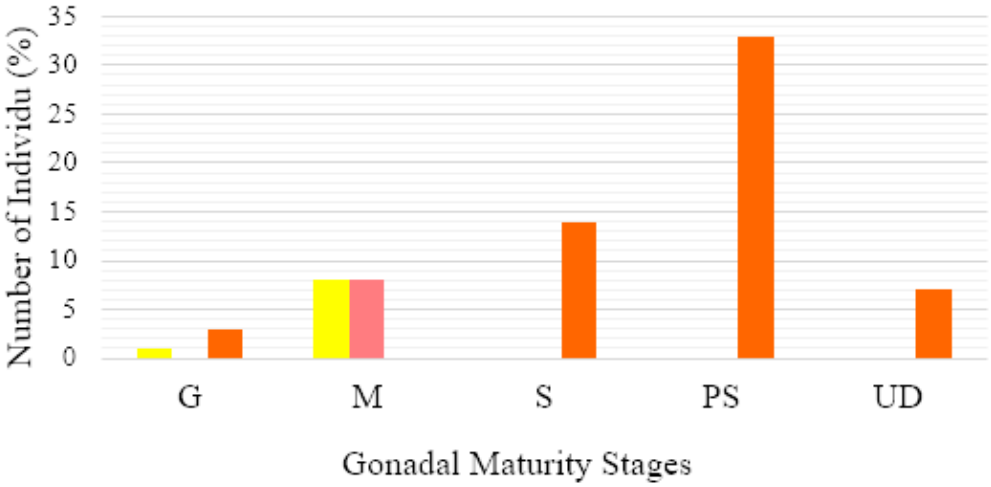


**Figure 6.** Percentage of distribution of individual gonad maturity stage to gonad color. G: gametogenesis; M: mature; S: spawn; PS: post spawning; UD; undetermined sex (n=74). Bar color represents gonad color (yellow, pink, orange).

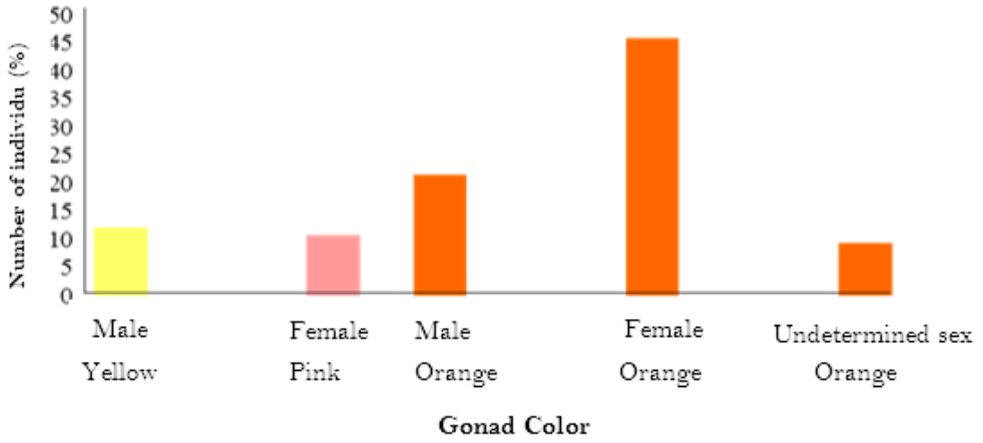


**Figure 7.** Distribution of the number of male and female individuals with a certain gonadal color (n = 74).

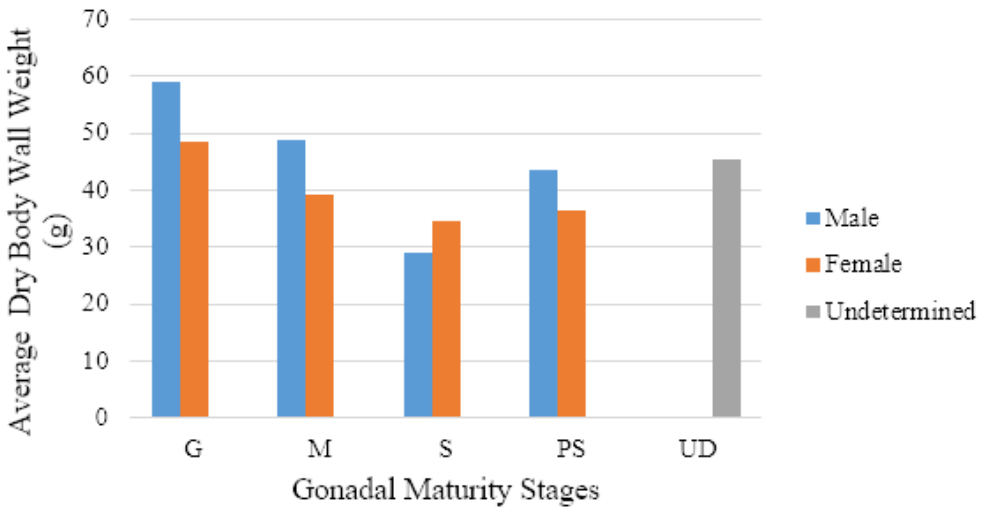


**Figure 8.** Average dry body wall weight of the sea cucumber *A. rosettis* at various stages of gonadal maturity. G: Gametogenesis; M: *Mature*; S: *Spawning*; PS: *Post-spawning*; UD: *Undetermined sex*.

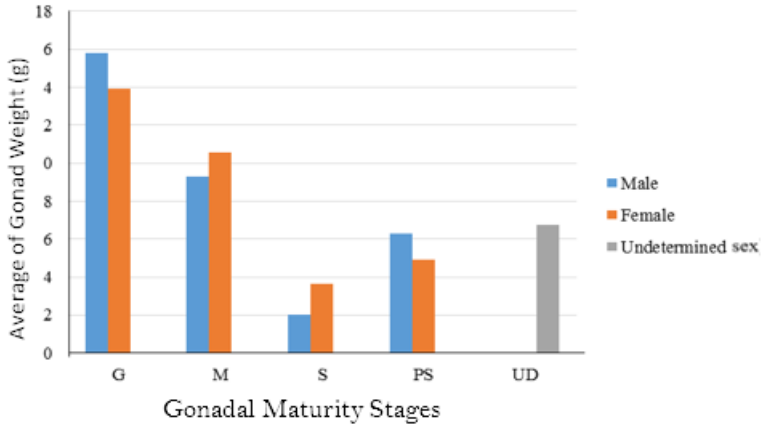


**Figure 9.** Average gonad weight of sea cucumber *A. rosettis* at various stages of gonadal maturity. G: Gametogenesis; M: *Mature*; S: *Spawning*; PS: *Post-spawningi*; UD: *Undetermined sex*

varying in diameter at each stage of gonadal maturation, located in the periphery of the gonadal tubules or in the longitudinal fold, surrounded by follicular cells (Figure 4A). Vitellogenic oocytes are characterized by larger size than previtellogenic oocytes, have separated from the tubular wall and longitudinal folds, have a centric nucleus, and have formed a jelly layer. The final stage is the post-vitellogenic oocyte. Most of the post-vitellogenic oocytes were observed in the lumen of the gonadal tubules, with thicker jelly layer compared to vitellogenic oocytes (Figure 4B).

Microvilli were found between follicular cells and oocytes, with thickness measured up to 5.028 µm. This was observed in vitellogenic oocytes (Figure 4B). In post-vitellogenic oocytes, the follicular cells were found to be relatively smaller in size, located outside microvilli. Microvilli in post-vitellogenic oocytes were fewer and shorter than microvilli in vitellogenic oocytes, but had a jelly layer that at mean of 17.334 µm thickness (Figure 4C). In male individuals, the presence of follicular cells accompanying spermatogenic cells could not be observed, but instead only parietal cells in the walls of the gonadal tubules was found

Inner epithelium of adult female gonads tubule *A. rosettis* was found to be composed of three types of cells; parietal somatic, follicular, and germ cells. The morphology of parietal somatic cells and follicular cells of male and female sample was almost the same, the difference was their position, in regard to oocyte, the follicular cells surrounded the oocyte, while parietal cells were in the inner side of tubule wall (Figure 3B).

### *Gonad Morphology*

Gonad morphology of *A. rosettis* is presented in Figure 5. From the individuals collected, there was a wide range of gonad coloration, ranging from creamy yellow, pinkish yellow, to orange. Results showed that of all gonads identified at gametogenesis stage, 25% were colored yellow, the rest were orange. In mature stage, 50% gonads were yellow and the remaining 50% pink, while the gonads at spawning, post-spawning, and undetermined sex stages were all orange (Figure 6).

Male individuals with orange gonads were found to be 21.62%, female individuals were 45.95% individuals, and undetermined sex individuals were 9.46%. The distribution of the number of male and female individuals related to the color of the gonads is presented in Figure 7.

### *Correlation of reproductive phase to gonad and body wall weight*

Individuals at a certain stage of maturity were found to have varying wall weights. At the gametogenesis stage, the body wall weight reaches 59.1 g, the mature stage has a body wall weight between 24.2-85.2 g, the spawning stage has a body wall weight between 28.8-29.3 g, the post-spawn stage weighs body wall between 28.6-57.5 g (Figure 8).

Based on the wall weight data for each gonad maturity of female *A. rosettis*, during the gametogenesis stage the body wall weight reaches 35.9-63.3 g, the mature stage has a body wall weight between 18.7-57.7 g, the spawning stage has body wall weight between 21.6-45.1 g, the post-spawning stage has a body wall weight between 25.7-56.4 g, while individuals with undetermined sex have a body wall weight between 32.7-62.1 g (Figure 9).

Based on the result of Spearman correlation test, the maturity stage of female *A. rosettis* correlated significantly with gonadal weight ($p <$

0.05), but not with gonad weight. Similar to it, in male *A. rosettis*, maturity stage was also found to be significantly related to gonadal weight ($p < 0.05$).

Sea cucumber *A. rosettis* has gonadal structure in long, branched tubules which open into a duct connected to outside environment. Similar structures were also found in various other sea cucumber species, such as *Holothuria scabra* (Nontunha et al., 2020), *Holothuria floridana* (Ramos-Miranda et al., 2017), and *Holothuria leucospilota* (Gaudron *et al*., 2008). As with other sea cucumber, *A. rosettis* did not show any apparent sexual dimorphism, so sex determination was carried out by microscopic methods to identify type of gamete cells present in the gonads.

Individuals used in this study was grouped into male, female, and undetermined, as also identified in previous study (Leite-Castro, *et al*., 2016). Male individuals are indicated by the presence of spermatogenic cells, while female individuals by oogenic cells at various vitellogenic stages. All stages of reproduction from gametogenesis to post-spawning could be found in both male and female sea cucumbers. Meanwhile, undetermined individuals are individuals who are not found to have certain gamete cells in their gonadal tubules (Leite-Castro, *et al*., 2016), presumably because they are in the post-spawning phase. The histological structure development of the tubules at various stages of maturity was found to be similar to the tubular structure of *Holothuria scabra* (Rasolofonirina *et al*., 2007) and *Stichopus herrmanni* (Balogh *et al*., 2019). The observed reproductive phase in the population of *A. rosettis* from the Madura strait tended to show a synchronous nature.

The male gamete cells found to compose the inner epithelium of *A. rosettis* included spermatogonia, spermatocytes, and spermatozoa. Spermatozoa ready to be spawned was indicated by round head structure with fairly long tail. This structure was found to be similar to spermatozoa from *Holothuria scabra* (Suphamungmee *et al*., 2018). The female gamete cells had a jelly layer that grows thicker as the oocyte matures, reaching up to 17.334 µm in the post-vitellogenic phase. This jelly layer can also be found in the oocyte of other species, such as *Holothuria forskali* (Laguerre *et al*., 2020).

From macroscopic observations, the gonad of *A. rosettis* were found to have colors ranging from creamy yellow, pinkish, to orange. Individuals with creamy yellow gonad color are in the reproductive phase of gametogenesis and maturation. Pinkish gonad color was found in individuals in the reproductive maturation phase, while orange color was found in some reproductive phases, including spawning, post-spawning, to gametogenesis, but not in mature phase. Based on gender, gonads with creamy yellow color were only found in male individuals, pinkish in female individuals, while orange color was found in both sexes and undetermined individuals. From these indications, orange gonad color could not be used to determine sex, but it was more likely to indicate the reproductive phase of *A. rosettis* individuals. As also found in *Australopithecus mollis*, color of gonads is associated with the maturity of the female gonads, which can also be used to estimate spawning time (Morgan, 2009).

Orange gonad color appeared in *A. rosettis* ranging from spawning, post-spawning, undetermined (spent gonad) up to gametogenesis stage, but did not appear during the maturation stage. Thus, orange color possibly indicated changes in gonadal tubules to prepare for the release of the gamete cells after they have matured inside the tubules, for example an increase in the amount of nutrients in the tubular wall. The color of the tubules then changes when they enter the gametogenesis stage to maturation. In male individuals, it was indicated by a creamy yellow color tendency, while in female individuals, it was indicated by a pinkish color. Gonad color in reproductive stage of gametogenesis until maturation can be used to determine sex.

Based on result of statistical test, both in female and male *A. rosettis* samples, maturity stage was correlated significantly to gonad weight, but not with body wall weight. As the gonadal weight changes through various maturity stages, it also indicates the development of gamete cells until they are ready to be spawned. On the other hand, the body wall of sea cucumbers contained various important nutrients used by sea cucumbers to survive, so they are often consumed as food or medicine (Zhong, *et al*., 2007; Gao, *et al*., 2011). As a source of energy for gametogenesis and reproductive cycles, nutrients from the body wall were channeled to the gonads, this would usually be indicated by decrease in body wall weight and color changes. Although not significant in the current study, in other study from *Cucumaria frandosa* showed that although the weight of body wall did not change, there was a decrease in energy levels in the body wall when there was an increase of energy

in the gonads as the reproductive cycle progresses (Hamel & Mercier, 1996).

## CONCLUSION

Based on the current study, sex determinants of *A. rosettis* that can be used were color of gonads in the reproductive phase of gametogenesis-maturation, where male individual was found to have creamy yellow color while female had a pinkish color. Orange color was found in male, female, and undetermined individuals during the spawning to gametogenesis reproductive phase, thus it could not be used as a differentiator but to indicate the spawning period. Gonad weight and body wall weight also did not differ between females and males, but their variations could also indicate *A. rosettis* reproductive phase.

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