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Date: 19. February 2016 at 11:01
To: Munawar Khalil khalil@unimal.ac.id

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The PDF for your manuscript, "Reproductive biology of blood cockle Anadara granosa (Bivalvia: Arcidae) in the northern region of the Straits of Malacca" is ready for viewing.

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From: **Editorial Office OSJ** em@editorialmanager.com
Subject: OSJO-D-16-00027 - Submission Confirmation
Date: 19. February 2016 at 11:02
To: Munawar Khalil khalil@unimal.ac.id

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Dear Mr Khalil,

Your submission entitled "Reproductive biology of blood cockle Anadara granosa (Bivalvia: Arcidae) in the northern region of the Straits of Malacca" has been received by Ocean Science Journal

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Thank you for submitting your work to our journal.

Kind regards,

Editorial Office
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From: **Jong Seong Khim** em@editorialmanager.com
Subject: OSJO: Your manuscript entitled Reproductive biology of blood cockle Anadara granosa (Bivalvia: Arcidae) in the northern region of the Straits of Malacca
Date: 30. March 2016 at 05:14
To: Munawar Khalil khalil@unimal.ac.id

Ref.: Ms. No. OSJO-D-16-00027
Reproductive biology of blood cockle Anadara granosa (Bivalvia: Arcidae) in the northern region of the Straits of Malacca
Ocean Science Journal

Dear Mr Khalil,

Reviewers have now commented on your paper. You will see that they are advising that you revise your manuscript. If you are prepared to undertake the work required, I would be pleased to reconsider my decision.

The reviewers' comments can be found at the end of this email or can be accessed by following the provided link.

This is your login information:
Your username is: khalil
Your password is: available at this link http://osjo.edmgr.com/Default.aspx?pg=accountFinder.aspx&firstname=Munawar&lastname=Khalil&email_address=khalil@unimal.ac.id

When revising your work, please submit a list of changes or a rebuttal against each point which is being raised when you submit the revised manuscript.

Please make sure to submit your editable source files (i. e. Word, TeX).

Your revision is due by 28 May 2016.

To submit a revision, go to http://osjo.edmgr.com/ and log in as an Author. You will see a menu item called 'Submissions Needing Revision'. You will find your submission record there.

Yours sincerely

Jong Seong Khim, Ph.D.
Editor-in-Chief
Ocean Science Journal

Reviewers' comments:

Reviewer #1: Overall, I think this work has value and is worth publishing, but the following polishing (number 1-9) and criticisms and suggestions (10-11) need for increase the value of manuscript.

1- Line 36-37: "The annual cockle production was reached 47,437 metric tonnes or equal to US$ 23.72 million" when? This sentence shows an increasing trend during a period. Please show when!

2- Line 41,42,43,43: Rephrase the following sentences: An understanding of the seasonal reproduction cycle of the blood cockle Anadara granosa in the northern region of the Straits of Malacca is essential before the culture for this commercially important species can be well managed especially important prerequisite for evaluating the regeneration capabilities of natural stocks and interpreting growth patterns.

3- Line 87, 88: Rephrase the following sentences: A total of 30 specimens from each sampling station were randomly for CI analyzed monthly.

4- Line 97, 98: Citation "Kim and Lee et al. (2008)" doesn't show in references

5- Line 104,105,106: Authors need to describe GI variations in results not in material method, please move these sentences to result of GI.

6- Line 196: Citation ""Hermann et al. (2009)" doesn't show in references

7- Line 214: citation "Ceballos" and references "Ceballoz". Names aren't the same, Please change it to a same form

8- Fig 4a: Ensure that both legends and figures are numbered and match up appropriately in text. 4a,4b,4c in legend and 4b,4c,4d in text

9- Figure 7: Please show legends according to what you have described in text (show 7a, 7b, 7c in figure legend

Discussion

10- As you mentioned in introduction, how does your data contribute to the advancement of this field (that can be highlighted) and what is recommended in future development?

11- Similarity studies have been done by some researchers in nearby locality. The authors need to compare the results of this study to support their findings

study to support their findings.

—

There is additional documentation related to this decision letter. To access the file(s), please click the link below. You may also login to the system and click the 'View Attachments' link in the Action column.

http://osjo.edmgr.com/l.asp?i=9561&l=IUDE42OK

Based on my studies in published articles about the Blood cockle, *Anadara granosa* is the most important commercial species in northern region of the Straits of Malacca, between Indonesia and Malaysia. This article describes the physiology and reproductive cycle of *Anadara granosa* through complete histological examination, fresh smear test, CI and GI of the gonadal tissue. Therefore, it allows scientists and aquaculturist to create a much more complete knowledge regarding reproductive cycle of cockles to select suitable period to culture and collect broodstock for artificial breeding and evaluating the regeneration capabilities of natural stocks. Therefore, I believe will be of interest to the readers of your journal in field of physiology, aquaculture and aquatic sciences and seems appropriate for Ocean Science Journal by doing some minor corrections.

Comments for authors:

Overall, I think this work has value and is worth publishing, but the following polishing (number 1-9) and criticisms and suggestions (10-11) need for increase the value of manuscript.

1- **Line 36-37:** "The annual cockle production was reached 47,437 metric tonnes or equal to US$ 23.72 million" when? This sentence shows an increasing trend during a period. Please show when!

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11- Similarity studies have been done by some researchers in nearby locality. The authors need to compare the results of this study to support their findings.

COVER LETTER FOR SUBMISSION OF REVISION MANUSCRIPT

Ocean Sciences Journal (OSJ)

COVER LETTER FOR SUBMISSION OF MANUSCRIPT
Date: April, 7th 2016

We appreciate the opportunity to revise our manuscript. With this cover letter, we will submit the revised manuscript (No. OSJO-D-16-00027) entitled, "Reproductive biology of blood cockle *Anadara granosa* (Bivalvia: Arcidae) in the northern region of the Straits of Malacca" for publication in OSJ. We carefully considered on the comments offered by the reviewers. We would like to thank referees for the careful and constructive reviews. Detailed corrections have listed below point by point and the major revised parts are highlighted in **red** color in revised manuscript. We want to extend our appreciation for taking the time and effort necessary to provide such insightful guidance.
Based the comments from the referees, we have made changes of the manuscript, which are detailed below.

Reply to the evaluation by the First Referee:
We would like to express our appreciation for your extremely thoughtful comments and constructive criticisms on our manuscript. As you will see below we have been able to revise and improve the paper as a result of your valuable feedback. Detailed corrections have listed point by point and the major revised parts are highlighted in **red** color in revised manuscript.

1- **Line 36-37: "The annual cockle production was reached 47,437 metric tonnes or equal to US$ 23.72 million" when? This sentence shows an increasing trend during a period. Please show when!**
Answer: We added the cockle production in Indonesia on 2009.

2- **Line 41,42,43,43: Rephrase the following sentences: An understanding of the seasonal reproduction cycles of the blood cockle Anadara granosa in the northern region of the Straits of Malacca is essential before the culture for this commercially important species can be well managed especially important prerequisite for evaluating the regeneration capabilities of natural stocks and interpreting growth patterns.**
Answer: Rephrase to "An understanding of the seasonal reproduction cycle of the blood cockle Anadara granosa is essential before the species culture. This bivalvia species can be well managed trough important prerequisite phase consisted by evaluating the regeneration capabilities of natural stocks and interpreting growth patterns".
3- **Line 87, 88: Rephrase the following sentences: A total of 30 specimens from each sampling station were randomly for CI analyzed monthly.**
Answer: Rephrase to "A total of 30 specimens (size range of 38–71 mm in length) from each sampling station were examined from June 2009 to September 2010.

4- **Line 97, 98: Citation "Kim and Lee et al. (2008)" doesn't show in references**
Answer: Added in references Kim TH, Lee KY (2008) Reproductive cycle and first sexual maturity of Sinonovacula constricca (Lamarck, 1818) (Bivalvia: Pharidae) in Western Korea. Korean Journal of Malacology, 24(2): 97-104.

5- **Line 104,105,106: Authors need to describe GI variations in results not in material method, please move these sentences to result of GI.**
Answer: We corrected it as suggested. We put this sentences into discussion part.

6- **Line 196: Citation ""Hermann et al. (2009)" doesn't show in references**
Answer: Added in references Herrmann M, Alfaya JEF, Lepore ML, Penchaszadeh PE, Laudien J (2009) Reproductive cycle and gonad development of the Northern Argentinean Mesodesma mactroides (Bivalvia: Mesodesmatidae). Helgoland Marine Research, 63(3): 207-218. doi: 10.1007/s10152-009-0150-2;

7- **Line 214: citation "Ceballos" and references "Ceballoz". Names aren't the same, Please change it to a same form**
Answer: We corrected it as suggested.

8- **Fig 4a: Ensure that both legends and figures are numbered and match up appropriately in text. 4a,4b,4c in legend and 4b,4c,4d in text**
Answer: We corrected it as suggested.

9- **Figure 7: Please show legends according to what you have described in text (show 7a, 7b, 7c in figure legend**
Answer: We corrected it as suggested.

Discussion

10- **As you mentioned in introduction, how does your data contribute to the advancement of this field (that can be highlighted) and what is recommended in future development?**
***Answer**: we added "The information of reproductive biology is essential for species managing and evolving sustainability policies of fisheries industry. These findings will be basic information for the blood cockle A. granosa stock management in the region" in abstract part and added in discussion part with line number: 294 to 302*

11- **Similarity studies have been done by some researchers in nearby locality. The authors need to compare the results of this study to support their findings.**
Answer: we added the sentence "Pathansali (1966), Narasimham (1988) and Broom (1983) were reported that A. granosa in Peninsular Malaysia and India has spawning season throughout the year with no apparent seasonal pattern. As the comparison, the spawning season of Archidae (genus Anadara) are presented in the Table 1". Table 1 was added in table part.

Abstract

A study on reproductive cycle of blood cockle *Anadara granosa* (Bivalvia: Arcidae) was conducted at three different areas in the northern region of Straits of Malacca. A total of 1,920 samples of adult *A. granosa* (38–71 mm of length) were collected from June 2009 until September 2010. The qualitative technique (gonadal microscopic fresh smear test and histology analysis) and quantitative technique (analysis of condition index and gonadal index) were used to predict monthly gonadal development stages on *A. granosa*. The gonadal index of *A. granosa* from Banda Aceh (Indonesia) (r=0.469, P>0.05) and Pulau Pinang (Malaysia) (r=0.123, P>0.05) did not show any correlation to their condition index, whereas gonadal index of *A. granosa* from Lhokseumawe (Indonesia) (r=0.609, P<0.05) showed moderate positive correlation to the condition index. During the 16 months sampling period, four reproductive cycles had been observed, one needs three to six months to complete. The process of releasing gametes in all populations are dribble spawning. The information of reproductive biology is essential for species managing and evolving sustainability policies of fisheries industry. These findings will be basic information for the blood cockle *A. granosa* stock management in the region.

Keywords: blood cockle, reproductive cycle, gametogenesis, gonadal index, condition index

1. Introduction

Anadara granosa is one of the 7500 of bivalve species in the family Arcidae, often called “blood arks” or “blood cockles” (Gosling, 2003; Arapov et. al., 2010). Their common name refers to the hemoglobin and hemocyanin pigments in their blood and tissue cells, giving their blood dark red colors (Ruppert and Barnes, 1994) which had allowed this species to live in oxygen-critical habitat (Broom, 1985; Terwilliger and Terwilliger, 1985; Cilenti et al., 2010). The species is indigenous to the intertidal mudflats bordering the coastal regions of many Southeast Asian countries particularly Indonesia, Malaysia and Thailand. *A. granosa* are mainly distributed in mangrove forest, mud vegetation or mixed areas. Intertidal species *A. granosa* was known as a keystone species at mangrove in several areas in the Northern Straits of Malacca. This species has also been one of the most important fisheries commodities in Southeast Asia for many years (Borrero, 1986; Broom, 1985; Suwanjarat et al., 2009).

The northern Straits of Malacca is an important nursery area for many intertidal organisms and a feeding area for migrating species. Being the most important species in terms of fisheries production, this cockle become the subject of extensive culture operation in West Malaysia (Broom, 1983). At the same time, the highest number of cockle wild stock harvesting activities in Sumatera and Java, Indonesia were established for meet the need of shellfish demand. In Malaysia, the annual production of blood cockle in 2009 exceeded 64,938.51 metric tonnes with valued at US$ 36.60 million (Jabatan Perikanan Malaysia (Malaysian Fisheries Department), 2010). The main blood cockle production areas in Malaysia is concentrated at Kedah (Merbok), Pulau Pinang (Juru), Perak (Kuala Gula, Kula Sangga-Matang, Kuala Trong, Sungai Jarum), Selangor (Kuala Selangor) and Johor (Muar). In Indonesia, this species can be found abundance in the coast of Wet Sumatera, Central and South Java, East and West of Kalimantan and other muddy bottoms in Sulawesi, Maluku and

Papua (Khalil et al., 2009). The annual cockle production in Indonesia was reached 47,437 metric tonnes or equal to US$ 23.72 million in 2009 (Kementerian Kelautan dan Perikanan Indonesia (Ministry of Marine Affairs and Fisheries Republic Indonesia), 2010). There is no available fresh data about cockle production in Indonesia after this publication.

The Northern Straits of Malacca is important areas for harvesting and culture of blood cockle *A. granosa* due to habitat suitability for spawning and growth (Mirzaei, 2015). However, the annual production statistics data shown the indication of decreasing in the number of stocks in decade. This situation may be due to inadequate the management aspects of the cockle. Species managing are needed for the evolving sustainability policies of fisheries industry. A through information of reproductive cycles is necessary for predicting annual recruitment, interpreting growth, mortality, and survival data in the marine culture of species (Shaw, 1965; Manzi et al., 1985; Sbrenna and Campioni, 1994). An understanding of the seasonal reproduction cycle of the blood cockle *Anadara granosa* is essential before the species culture. This bivalvia species can be well managed trough important prerequisite phase consisted by evaluating the regeneration capabilities of natural stocks and interpreting growth patterns. Detailed and comprehensive information of gonad development is also important for economic management for this species (Gribben et al., 2004; Peharda et al., 2006). This study aimed to investigate of the seasonal gonadal cycle of cockle *A. granosa* by using quantitative technique (gonadal index and condition index) through gonadal fresh smear test and gonad histology (qualitative technique) collected from the northern region of the Straits of Malacca.

2. Materials and Methods

2.1 Collecting of samples

Sampling of specimens was done with the purpose of analyzing and determining the reproduction cycle of *A. granosa* in the northern region of the Straits of Malacca. A total of 120 samples of adult *A. granosa* were collected monthly from June 2009 till September 2010 from the natural grounds in Banda Aceh (5°32'34.67"N-95°17'2.54"E), Lhokseumawe (05°09'35.3"N-097°08'29.4"E) in Aceh, Indonesia and Pulau Pinang (5°16'9.66"N-100°23'27.37"E) in Malaysia (Fig. 1). This sums up a total of 1,920 individuals, being the adult cockle with sizes ranging 38–71 mm of length. The sampling area was characterized by muddy bottoms which was surrounding by mangrove area, no wave action and exhibited high salinity. The specimen was collected from substrate with the depth 5-30 cm and salinity ranges from 10-33 ppt. Sampling activity on the field was done once a month over the specified time frame during low tide period. The live specimens were collected manually with the aid of harrow, running it through muddy area on the specified sampling location. After collecting, the specimens were stored in isotherm containers and immediately transported to the laboratory. The samples were fully removed from bio fouling and other adherences.

2.2 Qualitative technique

2.2.1 Gonadal microscopic fresh smear test

A total of 40 specimens per sampling site was randomly allocated for gonadal microscopic fresh smear test each month. All the specimens were dissected with the help of dissecting needle and pipette. Fresh smear procedure was adopted to observe the gonad content under light compound microscope (magnification = 100 x) to analyze the stages of the gonadal development. The sex and gametogenesis stages were identified using image analysis, which included 4 stages: (+1) indeterminate, (+2) developing, (+3) developed and (+4) Spawned (Rajagopal et al., 2006).

92

93 *2.2.2 Histology analysis*

94 A total of ten gonad specimen from each of three sampling site were allocated for this
95 analysis every month. Slides were prepared through the process of embedding paraffin wax
96 into the tissue. Haematoxyline and Eosin coloration were used for tissue coloring technique
97 (Howard et al., 1983). The initial process requires dehydration of the specimen tissue.
98 Dehydration was done through a series of steps of immersing sample into the different
99 concentration of alcohol solution. The sample would be embedded into a mold of wax block
100 as the next step and kept in refrigerator overnight before preparing for HE coloration. The
101 solution material for histology included bouins, alcohol (50%, 70%, 80%, 90%, 95% and
102 absolute alcohol), xylene, liquid wax, histosolve, HE solution and ammonia 1.5%. A
103 microtome was used to cut 5-7 µm thick tissue sections which are mounted on a glass
104 microscope slide. The light compound microscope was used to analyzing of the gonad
105 structure to recognize the sex and gametogenesis stages (divided to: (+1) indeterminate, (+2)
106 developing, (+3) developed and (+4) spawned).

107

108 *2.3 Quantitative method*

109 *2.3.1 Analysis of condition index (CI)*

110 Water displacement method was used to determine the condition index. A total of 30
111 specimens (size range of 38–71 mm in length) from each sampling station were examined
112 from June 2009 to September 2010. Each specimen was measured on the: dry flesh weight,
113 wet weight of shell in grams (g) and internal cavity volume (ml). Fresh cockle tissue
114 including its shell was weighted using digital balances. The flesh was dried at 105 °C for 72
115 hours to a constant weight. Volume of the shell internal cavity volume was calculated by

means of subtracting volume of shell (ml) from total wet volume (ml). These data were used to calculate the condition index using the formula described by Lawrence and Scott (1982):

Condition index = dry flesh weight (gram) x 100 / shell internal cavity volume (cm^3)

2.3.2 Analysis of gonadal index (GI)

Gonadal index calculated based on the formula proposed by Gosling (2003) and Kim and Lee et al. (2008): *Gonadal index = ∑ n individual from each stage level x gonad stage / n total specimen for each sampling batch.* The gonadal index (GI) was calculated for each sampling month through gonadal microscopic fresh smear test and histological analysis to estimate the proportion of the gonadal stages (indeterminate, developing, developed and spawned). The GI value was ranked to: 1 (all individuals gonad in the samples were in spawned stage), 2 (all individuals gonad in the samples were in indeterminate stage), 3 (all individuals gonad in the samples were in developing stage) and 4 (all individuals gonad in the samples were in developed stage).

2.4 Statistical Analysis

Raw data obtained was compiled and entered into Microsoft Office Excel 2011 (Macintosh version) for processing and analyzing of min, max, average, standard deviation and to generate illustrative graphical display. One-Way ANOVA statistical analysis and post hoc test was used to determine significance level ($P<0.05$ and $P<0.01$) in the values of each data cluster. Pearson correlation test was also utilized to determine and understand the relationship between differing variable (CI and GI). This all statistical analysis was applied using SPSS (*Statistical Package for Social Science*) release 20.0 for Macintosh.

3. Results

3.1 Gonadal structure of Anadara granosa

3.1.1. Gonadal microscopic fresh smear analysis

The description of gonad structure of *A. granosa* based on microscopic fresh smear analysis was shown below:

Stage 1 (indeterminate).

Male and female: determination of sex cannot possibly be determinate. Gonadal compound appeared to be empty and filled up only by network of connecting tissues. Unused residual of gametes can be found (Fig. 2)

Stage 2 (developing).

Male: the gonadal compound turned cream in color. Gametes have been very active and the testis was filled with spermatogonia and spermatid. Spermatozoa also found in limited numbers and sometimes found in tailed form and active swim (Fig 3a).

Female: the gonadal compound turned orange in color. Gametes in ovary begun to appear, which are previtellogenic oogonia, oocytes and limited number of oocyte vitellogenic. Oocytes were scattered and filled inside the follicle. Nucleus in oocytes vitellogenic have been started clearly visible. Oocytes have different uneven sizes (Fig 4a).

Stage 3 (developed).

Male: the gonadal compound turned more concentrated as a result of highly condensed developed spermatozoa. The spermatozoa already develop their own tail and swimming actively. Sometimes, spermatid can still be found in small numbers (Fig 3b).

Female: gonadal compound turned into intense orange and concentrated due to formation of highly condensed oocyte. Gametes were generally as mature oocytes. Oocytes has a similar form of polyhedral. The nucleus within the oocytes have matured and grown

bigger in size. The yolks were found in most of the mature oocytes. Previtellogenic oocytes can still be found in small amounts (Fig. 4b).

Stage 4 (spawned).

Male: gonadal compound reduced drastically. Spermatozoa has diminished. Unused residual spermatozoa can be found inside the lumen (Fig 3c).

Female: gonadal compound turned into bright orange due to lowest concentration of oocyte. Mature oocytes were found in small amount, but it's expected to be residue or absorbed as phagocytes. Most of the oocytes had no shape and nucleus appeared to shrink and disappear (Fig. 4c).

3.1.2. Gonadal histology analysis

Stage 1 (indeterminate).

Male and female: the stage is also called dormant stage, the sexes cannot be distinguished. Undeveloped gonads content during this stage was only consisting of connecting tissues and a handful of residual gamete leftover from the previous spawned stage (stage 4) (Fig. 5).

Stage 2 (developing).

Male: gonad was gradually filled up with spermatogonia, spermatocyte, and a small quantity of spermatozoa. The average diameter of the follicles at this stage was 117.77±19.58 µm. (Fig 6a).

Female: oocytes have diverse in range of size and generally were not on the same shape (irregular). Gonad was gradually filled up with oogonia as well as vitellogonia oocyte and vitellogenic oocyte, nucleus with uneven shapes. The average diameter of the follicles at this stage was 136.21±22.12 µm, whereas the average diameter of oocytes was 24.81±6.19 µm. (Fig 7a).

Stage 3 (developed).

Male: gonad was mainly dominated by spermatozoa content. Interfollicular space at this stage was seen to be experiencing constriction due to the growing of follicle size. Spematogonia still found in limited number and typically found on the side wall of the follicle. The average diameter of the follicles was 186.16±14.47 µm (Fig 6b).

Female: gonad was characterized by the dominance of vitellogenic oocytes with visibly large nucleus. Lumen space dominated by the polyhedral oocyte vitellogenic shape which was untouched or free from the follicle wall. The cytoplasm of mature oocytes had been filled by a number of yolk granule. The average diameter of follicles was 215.13±38.40 µm and oocytes were 30.01±6.80 µm (Fig. 7b).

Stage 4 (spawned).

Male: spermatozoa seemed to be reduced, as the follicle appeared almost empty. Spermatogonia totally did not found (Fig 6c).

Female: residual oocyte was present. The follicles wall seemed to damaged and unfilled. Phagocytes were found round the residue oocytes (Fig. 7c).

3.1.3. Gonadal development cycle

This section attempts to make a comparative study focusing into the gonad percentage (for each stage) for all three sampling locations, covering Banda Aceh (Indonesia), Lhokseumawe (Indonesia) and Pulau Pinang (Malaysia). Figures 8a, 9a, 10a, as well as 8b, 9b and 10b, depict the computation of gonad percentages per month for all the 4 phases discussed covering a span of 16 months, from June 2009 till September 2010, through gonadal microscopic fresh smear analysis and gonadal histology analysis, respectively. Figures 8c, 9c and 10c, as well as 8d, 9d and 10d, depict the monthly condition index (CI),

and monthly gonadal index (GI), respectively, covering a span of 16 months, from June 2009 till September 2010.

4. Discussion

4.1. Gonad development for Anadara granosa

The recorded CI values for the samples indicated significant varying values every month for samples of the same sampling location as well as those from different sampling locations. The difference in the trend of CI value indicated status of the population of blood cockle throughout the year. High CI value implies the gonad has already reached maturity. However, CI is not always linearly correlating to its breeding pattern. This can be shown from the comparison of the monthly CI vs GI values. The GI value is an assumed indication of the breeding status. Sudden drop in GI value signifies the occurrence of spawning activities. From analysis, there was no linear correlation between CI and GI values for samples from Banda Aceh and Penang. However, a linear correlation between these values can be noted for samples from Lhokseumawe. These were proven from Pearson correlation test, indicating CI values for samples from Banda Aceh ($r=0.469$ at $P>0.05$) and Penang ($r=0.123$ at $P>0.05$) have no significant correlation to their respective GI, but there is a mild correlation for samples from Lhokseumawe ($r=0.609$ at $P<0.05$). Negative correlation has also been reported from a few other sources. Hermann et al. (2009) reported negative correlation between CI and gametogenesis cycle for bivalvia *Amarilladesma mactroides* (Reeve, 1854). Mladineo et al. (2007) also reported zero correlation between CI and GI for bivalvia *Modiolus barbatus* (Linnaeus, 1758). The same applies to bivalvia *Mercenaria mercenaria* (Linnaeus, 1758) from gulf of Narragensett in the States, as reported by Marroquin-Mora and Rice (2008).

The GI values obtained throughout the year indicate high diversity among the three sampling locations. This is expected due to the differences in the habitat condition as well as the breeding season. Blood cockles for all three sampling locations indicate a rapid transition from gonad development to maturation phase. GI analysis shows spawning activity happened every month throughout the year with varying intensity. GI value will increase during gametogenesis and decreases after spawning. The high GI value corresponds to the highest maturation level, being level 4. The fast-paced in transition could have been one of the strategies for the blood cockles to increase the amount of gamete released, by means of shortening the breeding cycle whilst the surrounding factors permit. This behavior characterizes the usual pattern of reproduction in tropical regions. Species adopt opportunistic strategies to develop the gonadal matter from energy which has available from food rather than from energy stored inside somatic parts (Cárdenas and Aranda, 2000). Freites et al., (2010) was found that *Anadara notabilis* shown continuous reproductive cycle throughout the year. Environmental condition such as particulate organic matter, temperature and food availability were regulating factors of reproduction in *A. notabilis*.

The study shows GI for all three sampling locations (Banda Aceh, Lhokseumawe and Pulau Pinang) has a breeding cycle lasting to an average of 3~6 months. During the 16 months sampling period, four reproductive cycles had been observed. For the *A. granosa* population from Banda Aceh (Indonesia), cycle I occurred from June to October 2009, cycle II from November 2009 to January 2010, cycle III from February to April 2010, and cycle IV from April to September 2010. In Lhokseumawe (Indonesia), cycle I started from June to August 2009, cycle II from September 2009 to January 2010, cycle III from February to June 2010 and cycle IV from July to September 2010. For *A. granosa* population in Pulau Pinang (Malaysia), cycle I started from June to October 2009, cycle II from November 2009 to February 2010, cycle III from February 2010 to April 2010 and cycle IV from April 2010 to

September 2010. All the three populations started of the first cycle around June 2009 and ended the fourth cycle also around the same time, September 2010. Population from Lhokseumawe (Indonesia) showed tendency to spawn faster compared to the other two populations. However, during the third cycle, populations from Banda Aceh (Indonesia) and Penang (Malaysia) depicted a more rapid and shorter cycle lasting approximately 2~3 months, compared to Lhokseumawe (Indonesia) which took about 5 months.

4.2 Breeding Pattern of Anadara granosa

Generally, bivalvia breeding process characterizes a continual and seasonal pattern (Ceballos-Vazquez et al., 2000), as well as iteroparous in nature, continually and repeatedly breeds throughout its entire life span (Dame, 1996). Bivalvia gives birth to its young by means of gametogenesis. This process is then followed by the release of one or several gametes. The process of rearranging back empty gonad with new gametes for the next cycle signals the beginning of a new breeding cycle (Gosling, 2003). Random variation in the breeding trend amongst cockle populations of different geographical locations (Penang, Banda Aceh and Lhokseumawe) gives an unclear gonad development pattern. A well balanced distribution of male-female population for blood cockle is indeed supported by a sex ratio analysis done through this study. This shows gonad development and spawning period is parallel between the two opposing sexes, a scenario known as synchrony. According to Levitan (1993), synchrony in gonad development for bivalvia is crucial to further increase the possibility of effective mating. Extended spawning duration from one to two months is an indicator which characterizes a breeding strategy for the bivalvia species. Such strategy is essential to maintain sustainability of the cockle species within the habitat. Generally, sporadic gamete mating will happen concurrently under suitable surrounding conditions. Blood cockles for all three sampling locations, and in general, exhibits tendency to be

characterized as bivalvia brachidictics, which is capable of undergoing continual breeding cycle throughout the year, with varying spawning intensity every month. Pathansali (1966), Narasimham (1988) and Broom (1983) were reported that *A. granosa* in Peninsular Malaysia and India has spawning season throughout the year with no apparent seasonal pattern. As the comparison, the spawning season of Archidae (genus Anadara) are presented in the Table 1.

The information on the reproductive cycle of *A. granosa* provided in this research deliver a valuable knowledge into the reproductive biology of this edible species and are crucial for initiating its commercial aquaculture as well as for the sustainable management of wild stocks. Other, the data on spawning periodicity might be able to use for identification of trochophore or veliger larvae in the wild habitat and for seed collection activities. When bivalvia culture production depend on natural seed supply, the timing of seed collection is critical since potential brood stock are suitable for a short duration. Information presented in this research indicate that quantitative method (condition index and gonadal index) are precise indicator in *A. granosa* brood stock.

Acknowledgements

This project was supported by Universiti Sains Malaysia-Postgraduate Research Grant Scheme. The authors would like to thanks to Marine Sciences Laboratory Universiti Sains Malaysia, Aquaculture Department, Malikussaleh University Indonesia, Malaysia Quarantine and Inspection Services (MAQIS) Malaysia and Indonesia Fisheries Quarantine Service for their continuous support in making this project a success.

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434 **Captions for figures**

435

436 **Fig. 1.** Sampling location

437 (Insert after line 81, page 4)

438 **Fig. 2.** Gonadal structure of *Anadara granosa* based on microscopic fresh smear analysis at

439 indeterminate stage.

440 (Insert after line 148, page 7)

441 **Fig. 3.** Gonadal structure of male *Anadara granosa* based on microscopic fresh smear

442 analysis:

443 (a) Stage 2 (Developing)

444 (b) Stage 3 (Developed)

445 (c) Stage 4 (Spawned).

446 Spz: Spermatozoa; St: spermatid; VSz: vitellogenic spermatozoa.

447 (Insert after line 174, page 8)

448 **Fig. 4.** Gonadal structure of female *Anadara granosa* based on microscopic fresh smear

449 analysis:

450 (a) Stage 2 (Developing)

451 (b) Stage 3 (Developed)

452 (c) Stage 4 (Spawned).

453 EVO: early stage of vitellogenic oocyte; LVO: late stage of vitellogenic oocyte; Nl:

454 Nucleus; RO: residual oocyte; YG: yolk granule.

455 (Insert after line 174, page 8)

456 **Fig. 5.** Gonadal structure of *Anadara granosa* based on histology analysis at indeterminate

457 stage.

458 FW: follicle wall; Lu: Lumen; EL: empty lumen; Ct: connective tissue.

459 (Insert after line 181, page 8)

Fig. 6. Gonadal structure of male *Anadara granosa* based on histology analysis:
(a) Stage 2 (Developing)
(b) Stage 3 (Developed)
(c) Stage 4 (Spawned).
FW: follicle wall; Lu: lumen; Spz: spermatozoa; MS: mature spermatozoa; SD: sperm ductus; St: spermatid; DS: degenerative space; DSz: degenerative spermatozoa; FF: follicle fragment; EF: empty follicle; Ct: connective tissue.

(Insert after line 205, page 9)

Fig. 7. Gonadal structure of female *Anadara granosa* based on histology analysis:
(a) Stage 2 (Developing)
(b) Stage 3 (Developed)
(c) Stage 4 (Spawned).
FW: follicle wall; Lu: Lumen; EVO: early stage of vitellogenic oocyte; LVO: late stage of vitellogenic oocyte; MO: mature oocyte Nl: nucleus; FF: follicle fragment; EF: empty follicle; RO: residual oocyte; IS: interfolicular space; YG: yolk granule.

(Insert after line 205, page 9)

Fig. 8. *Anadara granosa* gonadal development pattern from Banda Aceh, Indonesia (June 2009-September 2010).

(Insert after line 216, page 10)

Fig. 9. *Anadara granosa* gonadal development pattern from Lhokseumawe, Indonesia (June 2009-September 2010).

(Insert after line 216, page 10)

Fig. 10. *Anadara granosa* gonadal development pattern from Pulau Pinang, Indonesia (June 2009-September 2010).

(Insert after line 216, page 10)

Captions for table

Table 1. Comparison of spawning period with the highest intensity of releasing gamete in genus Anadara.

(Insert after line 293, page 13)

From: **Editorial Office OSJ** em@editorialmanager.com
Subject: OSJO: Submission Confirmation for OSJO-D-16-00027R1
Date: 7. April 2016 at 13:53
To: Munawar Khalil khalil@unimal.ac.id

Ref.: Ms. No. OSJO-D-16-00027R1
Reproductive biology of blood cockle Anadara granosa (Bivalvia: Arcidae) in the northern region of the Straits of Malacca

Dear Mr Khalil,

Ocean Science Journal has received your revised submission.

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JK

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Ocean Science Journal

Dear Mr Khalil,

Reviewers have now commented on your paper. You will see that they are advising that you revise your manuscript. If you are prepared to undertake the work required, I would be pleased to reconsider my decision.

The reviewers' comments can be found at the end of this email or can be accessed by following the provided link.

This is your login information:
Your username is: Munawar Khalil
Your password is: available at this link http://osjo.edmgr.com/Default.aspx?pg=accountFinder.aspx&firstname=Munawar&lastname=Khalil&email_address=khalil@unimal.ac.id

When revising your work, please submit a list of changes or a rebuttal against each point which is being raised when you submit the revised manuscript.

Please make sure to submit your editable source files (i. e. Word, TeX).

Your revision is due by 05 Aug 2016.

To submit a revision, go to http://osjo.edmgr.com/ and log in as an Author. You will see a menu item called 'Submissions Needing Revision'. You will find your submission record there.

Yours sincerely

Jong Seong Khim, Ph.D.
Editor-in-Chief
Ocean Science Journal

Reviewers' comments:

Comments for the draft from the Reviewer#2

Title: Reproductive biology of blood cockle Anadara granosa (Bivalvia: Arcidae) in the northern region of the Straits of Malacca

The blood cockle Anadara granose has a wide distribution range, as is stated in the instruction, from tropical to temporate regions in Asia. Accordingly, annual gametogeneis of A. granosa distributed in different areas in Asia may vary due to different temperature and salinity regimes. For this reason, this paper can be accepted and published in Ocean Science Journal, although several major revisons must be proceeded prior to become accepted.

1. This study use both gonad smear and histology to evaluate the maturity of gonad. As is well known, all the bivalves go through several reproductive stages called 1) resting, 2)early development or gonial mitosis, 2) late developmet, initiation of vitellogenesis, 3) ripe and spawning, and 4) spent. Depending upon reproductive stage, size of gamete cells (i.e., egg and sperm) vary greatly. In early stage of gametogenesis, I don't believe it is possible to observe or distinguish the eggs or sperm using gonad smear. That is why almost all the studies in reproductive biology of bivalves or gastropod use histology. In this study, you have applied histology to evaluate the gonad maturity. Is there any specific reason that you must use gonadal smear to determine gonad stage of A. granosa? If it is not, then the gonad smear part must be dropped. Resolution of gonad cells in the Figures 2 and 3 are very poor and unacceptable.

2. Ocean Science Journal accepts studies on marine biology process including interaction of environmental conditions and marine animal reproduction. The present study reports annual gametogenesis of A. granosa from tropical areas, which is somewhat rare, since reproductive biological studies in marine bivalves have been reported mostly from temperate regions. However, the present study focuses only on the gametogenic cycle, and I don't see discussion of interaction between A. granosa annual gametogenesis and the environmental parameters at the study site. In the revison, this must be added and discussed with proper citations.

3. As this paper discussed, A. granos has a wide range of distribution and several studies have reported annual gametogenesis of A. granosa from other areas. In the revison, you need to include previous studies reported annual gametogenesis of A. granosa, and compare your observations with the findings of previous studies reported from elsewhere.

4. English used in this study does not meet standard of the journal. Accordingly, the English used in the revision must be improved.

—

COVER LETTER FOR SUBMISSION OF REVISION MANUSCRIPT

Ocean Sciences Journal (OSJ)

COVER LETTER FOR SUBMISSION OF MANUSCRIPT
Date: August, 4th 2016

We appreciate the opportunity to revise our manuscript. With this cover letter, we will submit the revised manuscript (No. OSJO-D-16-00027) entitled, "Reproductive biology of blood cockle *Anadara granosa* (Bivalvia: Arcidae) in the northern region of the Straits of Malacca" for publication in OSJ. We carefully considered on the comments offered by the reviewers. We would like to thank referees for the careful and constructive reviews. Detailed corrections have listed below point by point and the major revised parts are highlighted in **red** color in revised manuscript. We want to extend our appreciation for taking the time and effort necessary to provide such insightful guidance.
Based the comments from the referees, we have made changes of the manuscript, which are detailed below.

Reply to the evaluation by the Second Referee:
We would like to express our appreciation for your extremely thoughtful comments and constructive criticisms on our manuscript. As you will see below we have been able to revise and improve the paper as a result of your valuable feedback. Detailed corrections have listed point by point and the major revised parts are highlighted in **red** color in revised manuscript.

1. This study use both gonad smear and histology to evaluate the maturity of gonad. As is well known, all the bivalves go through several reproductive stages called 1) resting, 2)early development or gonial mitosis, 2) late developmet, initiation of vitellogenesis, 3) ripe and spawning, and 4) spent. Depending upon reproductive stage, size of gamete cells (i.e., egg and sperm) vary greatly. In early stage of gametogenesis, I don't believe it is possible to observe or distinguish the eggs or sperm using gonad smear. That is why almost all the studies in reproductive biology of bivalves or gastropod use histology. In this study, you have applied histology to evaluate the gonad maturity. Is there any specific reason that you must use gonadal smear to determine gonad stage of A. granosa? If it is not, then the gonad smear part must be dropped. Resolution of gonad cells in the Figures 2 and 3 are very poor and unacceptable.
   Answer: Many author using different method for determining stages on the bivalvia gonadal development. Commonly, they use 4-6 stages, but several malacologist using until 7 stages for determining gonadal stages on bivalvia. For the bivalvia in tropical areas, using 4 stage is the best way to determine the gonadal development due rapid changes in

gonadal compounds (sperm and egg). The author using (Rajagopal et al., 2006) as the reference for the method to determine gonadal development stages in bivalvia Anadara granosa.

This paper was combined between histology test and gonadal microscopic fresh smear test. The author using 2 methods in the same time to reduce the margin error. Microscopic fresh smear test is common using to determine the gonadal development in bivalvia. for ex:

"Mohite, S. A., Mohite, A. S., & Singh, H. (2008). On condition index and percentage edibiliy of the shortneck clam Paphia malabarica (Chemintz) from estuarine regions of Ratnagiri, west coast of India. Aquaculture Research, 40(1), 69-73."

"Pouvreau, S., Gangnery, A., Tiapari, J., Lagarde, F., Garnier, M., & Bodoy, A. (2000). Gametogenic cycle and reproductive effort of the tropical blacklip pearl oyster, Pinctada margaritifera (Bivalvia: Pteriidae), cultivated in Takapoto atoll (French Polynesia). Aquatic Living Resources, 13(1), 37-48."

"Thomas, S. (2013). Reproductive studies on the short neck clam Paphia malabarica (Chemnitz) from Dharmadom Estuary, Kerala, India. Indian Journal of Fisheries, 60(4), 47-50.

"Jayabal, R., & Kalyani, M. (1987). Reproductive cycle of the estuarine bivalve Meretrix meretrix (Linn) of the Vellar estuary. Indian Journal of Fisheries, 34(2), 229-232.

"etc"

Using microscopic fresh smear test are possible to produce due to time consuming. The gonadal compounds also able to identified clearly using high resolution of light microscope equipment. The number of sperm or eggs inside the gonad are base to identification the stages.

Figure 2 and figure 3 was corrected using image processing software for fulfill the journal requirement.

2. Ocean Science Journal accepts studies on marine biology process including interaction of environmental conditions and marine animal reproduction. The present study reports annual gametogenesis of A. granosa from tropical areas, which is somewhat rare, since reproductive biological studies in marine bivalves have been reported mostly from temperate regions. However, the present study focuses only on the gametogenic cycle, and I don't see discussion of interaction between A. granosa annual gametogenesis and the environmental parameters at the study site. In the revison, this must be added and discussed with proper citations.

   ***Answer**: the author was added sub chapter 4.3: Factors that affected reproduction cycle of Anadara granosa in the northern region of the Strait of Malacca*

3. As this paper discussed, A. granosa has a wide range of distribution and several studies have reported annual gametogenesis of A. granosa from other areas. In the revison, you need to include previous studies reported annual gametogenesis of A. granosa, and compare your observations with the findings of previous studies reported from elsewhere.

   ***Answer**: the author was added table 2: Comparison of spawning period with the highest intensity of releasing gamete in genus Anadara.*

4. English used in this study does not meet standard of the journal. Accordingly, the English used in the revision must be improved.

   ***Answer**: the manuscript was checked and corrected by professional English translator in biological sciences and native speakers.*

Abstract

A study on the reproductive cycle of the blood cockle *Anadara granosa* (Bivalvia: Arcidae) was conducted at three different areas in the northern region of the Strait of Malacca. A total of 1,920 samples of adult *A. granosa* (38–71 mm length) were collected from June 2009 until September 2010. Qualitative techniques (gonadal microscopic fresh smear test and histology analysis) as well as quantitative techniques (analysis of condition index and gonadal index) were used to predict monthly gonadal development stages of *A. granosa*. The gonadal index of *A. granosa* from Banda Aceh (Indonesia) (r=0.469, P>0.05) and Pulau Pinang (Malaysia) (r=0.123, P>0.05) did not show any correlation to their condition index, whereas gonadal index of *A. granosa* from Lhokseumawe (Indonesia) (r=0.609, P<0.05) showed moderate positive correlation to the condition index. During the 16 month sampling period, four reproductive cycles were observed: each from three to six months. The process of releasing gametes is dribble spawning, and is the same in all populations. Information on the reproductive biology of this species is essential for species management and to improve the sustainability practices of the fisheries industry. These findings provide basic information on the biology of the blood cockle *A. granosa* for stock management in the region.

Keywords: blood cockle, reproductive cycle, gametogenesis, gonadal index, condition index

1. Introduction

Anadara granosa is one of 7500 bivalve species in the family Arcidae, often called "blood arks" or "blood cockles" (Gosling, 2003; Arapov et. al., 2010). Their common name refers to the hemoglobin and hemocyanin pigments in their blood and tissue cells, giving their blood a dark red color (Ruppert and Barnes, 1994) which has allowed this species to live in oxygen-critical habitat (Broom, 1985; Terwilliger and Terwilliger, 1985; Cilenti et al., 2010). The species is indigenous to intertidal mudflats of many Southeast Asian countries, particularly Indonesia, Malaysia and Thailand. *Anadara granosa* are mainly distributed in mangrove forests, muddy vegetation and mixed areas. The intertidal species *A. granosa* is known as a keystone species in mangrove habitats in several areas in the northern region of the Strait of Malacca. This species has also been one of the most important fisheries commodities in Southeast Asia for many years (Borrero, 1986; Broom, 1985; Suwanjarat et al., 2009).

The northern Strait of Malacca is an important nursery area for many intertidal organisms and a feeding area for migrating species. Being the most important species in terms of fisheries production, this cockle has become the target of an extensive culturing operation in West Malaysia (Broom, 1983). At the same time, harvesting of wild stock of cockles in Sumatra and Java (Indonesia) is at an all-time high to meet the demand for shellfish. In Malaysia, the annual production of blood cockles in 2009 exceeded metric 65,000 tonnes, which is valued at US $36.60 million (Jabatan Perikanan Malaysia (Malaysian Fisheries Department), 2010). The main blood cockle production areas in Malaysia are concentrated in Kedah (Merbok), Pulau Pinang (Juru), Perak (Kuala Gula, Kula Sangga-Matang, Kuala Trong, Sungai Jarum), Selangor (Kuala Selangor) and Johor (Muar). In Indonesia, this species can be found in abundance on the coast of West Sumatra, Central and South Java, East and West Kalimantan and other muddy bottoms in Sulawesi, Maluku and

Papua (Khalil et al., 2009). The most recent data available on annual cockle production in Indonesia is from 2009 when it reached 47,437 metric tonnes, or equal to US$ 23.72 million (Kementerian Kelautan dan Perikanan Indonesia (Ministry of Marine Affairs and Fisheries Republic Indonesia), 2010).

The Northern Straits of Malacca is an important area for the harvesting and culture of the blood cockle *A. granosa* due to suitability of the habitats for spawning and growth (Mirzaei, 2015). However, annual production statistics indicate a decrease in stocks in the last decade. This situation may be due to inadequate management of wild cockle populations. Fisheries management is needed to improve policies for the sustainability of the fisheries industry. Thorough information on reproductive cycles is necessary for predicting annual recruitment, as well as interpreting growth, mortality, and survival data in the marine culture of species (Shaw, 1965; Manzi et al., 1985; Sbrenna and Campioni, 1994). This data is lacking for the blood cockle *Anadara granosa* but is essential to optimize aquaculture of this species. This bivalve species can be managed more effectively after evaluating the regeneration capabilities of natural stocks and interpreting growth patterns. Detailed and comprehensive information on gonadal development is also important for economic management of this species (Gribben et al., 2004; Peharda et al., 2006). This study aimed to investigate the seasonal gonadal cycle of the cockle *A. granosa* by using quantitative techniques (gonadal index and condition index) through gonadal fresh smear test and gonad histology (a qualitative technique) from specimens collected from the northern region of the Strait of Malacca.

2. Materials and Methods

2.1 Collecting of samples

A total of 120 samples of adult *A. granosa* were collected monthly from June 2009 till September 2010 from the natural habitat in Banda Aceh (5°32'34.67"N-95°17'2.54"E), Lhokseumawe (05°09'35.3"N-097°08'29.4"E) in Aceh, Indonesia and Pulau Pinang (5°16'9.66"N-100°23'27.37"E) in Malaysia (Fig. 1). The total number of specimens sampled was 1,920 and the adult cockle sizes ranged from 38–71 mm in length. The sampling area was characterized by muddy substrate which was surrounded by mangroves, no wave action and high salinity. The specimens were collected at a depth of 5-30 cm and salinity ranged from 10-33 ppt. Sampling activity in the field was conducted once a month over the specified time frame during low tides. The live specimens were collected manually with the aid of harrow, which was run through the muddy area to pull bivalves to the surface. After collecting, the specimens were stored in isotherm containers and immediately transported to the laboratory. The samples were cleaned to fully remove all fouling organisms and other adherences.

2.2 Qualitative technique

2.2.1 Gonadal microscopic fresh smear test

A total of 40 specimens per sampling site were randomly allocated for the gonadal microscopic fresh smear test each month. All the specimens were dissected with a dissecting needle and pipette. The fresh smear procedure was adopted to observe the gonad content under a compound light microscope (magnification = 100 x) to analyze the stages of the gonadal development. The sex and gametogenesis stages were identified using image analysis, which included 4 stages: (+1) indeterminate, (+2) developing, (+3) developed and (+4) spawned (Rajagopal et al., 2006).

2.2.2 Histology analysis

A total of ten gonad specimens from each of the three sampling sites were allocated for histological analysis each month. Slides were prepared through the process of embedding paraffin wax into the tissue. Haematoxyline and Eosin coloration were used for tissue coloring (Howard et al., 1983). The initial process requires dehydration of the specimen tissue. Dehydration was done through a series of steps of immersing the sample into varying concentrations of alcohol. The sample was embedded into a mold of wax next and kept in a refrigerator overnight before preparing it for HE coloration. The solutions used for histology included bouins, alcohol (50%, 70%, 80%, 90%, 95% and absolute alcohol), xylene, liquid wax, histosolve, HE solution and 1.5% ammonia. A microtome was used to cut 5-7 µm thick tissue sections which were mounted on a glass microscope slide. The light compound microscope was used to analyze the gonad structure to recognize the sex and gametogenesis stages (divided into: (+1) indeterminate, (+2) developing, (+3) developed and (+4) spawned).

2.3 Quantitative method

2.3.1 Analysis of condition index (CI)

The water displacement method was used to determine the condition index. A total of 30 specimens (size range: 38–71 mm in length) from each sampling station were examined from June 2009 to September 2010. Each specimen was measured for the following: dry flesh weight, wet weight of shell in grams (g) and internal cavity volume (ml). Fresh cockle tissue including its shell was weighed using digital balances. The flesh was dried at 105 °C for 72 hours to a constant weight. Volume of the shell internal cavity volume was calculated by means of subtracting the volume of the shell (ml) from the total wet volume (ml). These data were used to calculate the condition index using the formula described by Lawrence and Scott (1982):

Condition index = dry flesh weight (gram) x 100 / shell internal cavity volume (cm^3)

118

119 *2.3.2 Analysis of gonadal index (GI)*

120 Gonadal index was calculated based on the formula proposed by Gosling (2003) and
121 Kim and Lee et al. (2008): *Gonadal index* = ∑ *n individual from each stage level x gonad*
122 *stage / n total specimen for each sampling batch.* The gonadal index (GI) was calculated for
123 each sampling month through gonadal microscopic fresh smear test and histological analysis
124 to estimate the proportion of the gonadal stages (indeterminate, developing, developed and
125 spawned). The GI value was ranked to: 1 (all individuals' gonads in the samples were in
126 spawned stage), 2 (all individuals' gonads in the samples were in indeterminate stage), 3 (all
127 individuals' gonads in the samples were in developing stage) and 4 (all individuals' gonads in
128 the samples were in developed stage).

129

130 *2.4 Statistical Analysis*

131 Raw data was compiled and entered into Microsoft Office Excel 2011 (Macintosh
132 version) for processing and analyzing of minimum and maximum value, average, and the
133 standard deviation as well as to generate graphs. One-Way ANOVA statistical analysis and
134 post hoc test were used to determine significance level ($P<0.05$ and $P<0.01$) in the values of
135 each data cluster. Pearson correlation test was also utilized to determine and understand the
136 relationship between differing variables (CI and GI). The principle component analysis
137 (PCA) was used to analyze the correlation between parameters which were affected by
138 reproductive activities in each sampling areas. These statistical analyses were conducted
139 using SPSS (*Statistical Package for Social Science*) release 20.0 for Macintosh.

140

141 **3. Results**

142 *3.1 Gonadal structure of Anadara granosa*

3.1.1. Gonadal microscopic fresh smear analysis

The description of gonad structure of *A. granosa* based on microscopic fresh smear analysis was categorized as shown below:

Stage 1 (indeterminate).

Male and female: Determination of sex cannot possibly be determined. Gonadal compound appeared to be empty and filled up only by network of connecting tissues. Unused residual of gametes can be found (Fig. 2)

Stage 2 (developing).

Male: The gonadal compound turned cream in color. Gametes have been very active and the testis was filled with spermatogonia and spermatid. Spermatozoa were also found in limited numbers and sometimes found in tailed form and actively swim (Fig 3a).

Female: The gonadal compound turned orange in color. Gametes in ovary have begun to appear, which are previtellogenic oogonia, oocytes and a limited number of oocytes vitellogenic. Oocytes were scattered and filled inside the follicle. Nucleus in oocytes vitellogenic have been started and are clearly visible. Oocytes have uneven sizes (Fig 4a).

Stage 3 (developed).

Male: The gonadal compound turned a more concentrated cream color as a result of highly condensed developed spermatozoa. The spermatozoa have already developed their tail and are swimming actively. Sometimes, spermatids can still be found in small numbers (Fig 3b).

Female: Gonadal compound turned intense, concentrated orange due to formation of highly condensed oocytes. Gametes were generally mature oocytes. Oocytes are in polyhedral form. The nucleus within the oocytes have matured and grown larger in

size. The yolks were found in most of the mature oocytes. Previtellogenic oocytes can still be found in small amounts (Fig. 4b).

Stage 4 (spawned).

Male: Gonadal compound reduced drastically. Spermatozoa have diminished. Unused residual spermatozoa can be found inside the lumen (Fig 3c).

Female: Gonadal compound turned bright orange due to the lowest concentration of oocytes. Mature oocytes were found in small amounts, but these are expected to be residue or absorbed as phagocytes. Most of the oocytes had no shape and the nucleus appeared to have shrunk and disappeared (Fig. 4c).

3.1.2. Gonadal histology analysis

Stage 1 (indeterminate).

Male and female: The stage is also called dormant stage; the sexes cannot be distinguished. Undeveloped gonads' content during this stage only consisted of connecting tissues and a handful of residual gametes leftover from the previous spawned stage (stage 4) (Fig. 5).

Stage 2 (developing).

Male: Gonad was gradually filled up with spermatogonia, spermatocyte, and a small quantity of spermatozoa. The average diameter of the follicles at this stage was 117.77 ± 19.58 µm. (Fig 6a).

Female: Oocytes occur in a range of sizes and were generally not the same shape (irregular). Gonad was gradually filled up with oogonia as well as vitellogonia oocyte and vitellogenic oocytes, the nucleus has uneven shapes. The average diameter of the follicles at this stage was 136.21 ± 22.12 µm, whereas the average diameter of oocytes was 24.81 ± 6.19 µm. (Fig 7a).

Stage 3 (developed).

Male: Gonad was mainly dominated by spermatozoa content. Interfollicular space at this stage was seen to be experiencing constriction due to the growing follicle size. Spematogonia were still found in limited number and typically found on the side wall of the follicle. The average diameter of the follicles was 186.16 ± 14.47 µm (Fig 6b).

Female: Gonad was characterized by the dominance of vitellogenic oocytes with a visibly large nucleus. Lumen space was dominated by the polyhedral oocyte vitellogenic shape which was untouched or free from the follicle wall. The cytoplasm of mature oocytes had been filled by a number of yolk granule. The average diameter of follicles was 215.13 ± 38.40 µm and oocytes were 30.01 ± 6.80 µm (Fig. 7b).

Stage 4 (spawned).

Male: Spermatozoa seemed to be reduced, as the follicle appeared almost empty. Spermatogonia were not found (Fig 6c).

Female: Residual oocytes were present. The follicles' wall seemed to be damaged and unfilled. Phagocytes were found around the residue oocytes (Fig. 7c).

3.1.3. Gonadal development cycle

The gonad percentage (for each stage) was compared between the three sampling locations: Banda Aceh (Indonesia), Lhokseumawe (Indonesia) and Pulau Pinang (Malaysia). Figures 8a, 9a, 10a, as well as 8b, 9b and 10b, depict the computation of gonad percentages per month for all the 4 phases discussed covering a span of 16 months, from June 2009 until September 2010, through gonadal microscopic fresh smear analysis and gonadal histology analysis, respectively. Figures 8c, 9c and 10c, as well as 8d, 9d and 10d, depict the monthly

condition index (CI), and monthly gonadal index (GI), respectively, covering of the same 16 months.

3.2. Environmental variable

Monthly seasonal variation of environmental parameters in three different sampling areas are reported in Table 1. During the study period, water temperature, salinity and phytoplankton density fluctuated significantly compared to other environmental parameters.

4. Discussion

4.1. Gonad development for Anadara granosa

The recorded CI values for the samples indicated significant varying values every month for samples of the same sampling location as well as those from different sampling locations. The difference in the trend of CI value indicated the status of the population of blood cockles throughout the year. A high CI value implies the gonad has already reached maturity. However, CI is not always linearly correlated to its breeding pattern. This can be shown from the comparison of the monthly CI vs GI values. The GI value is an assumed indication of the breeding status. A sudden drop in GI value signifies the occurrence of spawning activities. From this analysis, there was no linear correlation between CI and GI values for samples from Banda Aceh and Penang. However, a linear correlation between these values was noted for samples from Lhokseumawe. These were tested with the Pearson correlation test, which indicated CI values for samples from Banda Aceh ($r=0.469$ at $P>0.05$) and Penang ($r=0.123$ at $P>0.05$) have no significant correlation to their respective GI, but there is a mild correlation for samples from Lhokseumawe ($r=0.609$ at $P<0.05$). A negative correlation has also been reported in studies of other bivalve species. Hermann et al. (2009) reported a negative correlation between CI and gametogenesis cycle for *Amarilladesma mactroides* (Reeve, 1854). Mladineo et al. (2007) also reported zero correlation between CI and GI for the bivalve *Modiolus barbatus* (Linnaeus, 1758). The same applies to *Mercenaria*

241 *mercenaria* (Linnaeus, 1758) from the Gulf of Narragensett in the United States, as reported
242 by Marroquin-Mora and Rice (2008).

243 The GI values obtained throughout the year indicate high diversity in reproductive
244 patterns among the three sampling locations. This is expected due to the differences in the
245 habitat condition as well as the breeding season. Blood cockles from all three sampling
246 locations showed a rapid transition from gonad development to maturation phase. GI analysis
247 shows spawning activity happened every month throughout the year with varying intensity.
248 The GI value increases during gametogenesis and decreases after spawning. The fast-paced
249 transition could be a strategy for the blood cockles to increase the amount of gamete released
250 whilst favorable environmental conditions are present. This behavior is characteristic of
251 reproduction of invertebrates in tropical regions. Species have been shown to adopt
252 opportunistic strategies to develop the gonadal matter from energy available from food rather
253 than from energy stored inside somatic parts (Cárdenas and Aranda, 2000). Freites et al.,
254 (2010) found that *Anadara notabilis* exhibits a continuous reproductive cycle throughout the
255 year and that particulate organic matter, temperature and food availability were regulating
256 factors of the reproduction of *A. notabilis*.

257 This study of blood cockles' GI shows that it has a breeding cycle lasting an average
258 of 3~6 months across the three sampling locations (Banda Aceh, Lhokseumawe and Pulau
259 Pinang). During the 16 month sampling period, four reproductive cycles have been observed.
260 For the *A. granosa* population from Banda Aceh (Indonesia), cycle I occurred from June to
261 October 2009, cycle II from November 2009 to January 2010, cycle III from February to
262 April 2010, and cycle IV from April to September 2010. In Lhokseumawe (Indonesia), cycle
263 I started from June to August 2009, cycle II from September 2009 to January 2010, cycle III
264 from February to June 2010 and cycle IV from July to September 2010. For the *A. granosa*
265 population in Pulau Pinang (Malaysia), cycle I started from June to October 2009, cycle II

from November 2009 to February 2010, cycle III from February 2010 to April 2010 and cycle IV from April 2010 to September 2010. All three populations started the first cycle around June 2009 and ended the fourth cycle also around the same time, September 2010. The population from Lhokseumawe (Indonesia) showed a tendency to spawn faster compared to the other two populations. However, during the third cycle, populations from Banda Aceh (Indonesia) and Penang (Malaysia) exhibited a more rapid and shorter cycle lasting approximately 2~3 months, compared to Lhokseumawe (Indonesia) which took about 5 months.

4.2. Breeding pattern of Anadara granosa

Generally, the bivalve breeding process is characterized by a continual and seasonal pattern (Ceballos-Vazquez et al., 2000), and is iteroparous in nature, continually and repeatedly breeding throughout its entire life span (Dame, 1996). Bivalves give birth to their young by means of gametogenesis. This process is then followed by the release of one or several gametes. The process of rearranging empty gonad with new gametes for the next cycle always as a signals for the beginning of a new breeding cycle (Gosling, 2003). Variation in the breeding trend amongst cockle populations of different geographical locations makes it difficult to determine a pattern of gonad development. A well balanced distribution of males to females in blood cockles is supported by the sex ratio analysis done in this study. Gonad development and spawning period was determined to be parallel between the two opposing sexes, a scenario known as synchrony. According to Levitan (1993), synchrony in gonad development of bivalves is crucial to increase the possibility of effective mating. Extended spawning durations from one to two months is a common breeding strategy for bivalve species. Such a strategy is essential to maintain the cockle population over time within its habitat. Generally, sporadic gamete mating will happen

concurrently under suitable surrounding conditions. Blood cockles for all three sampling locations, and in general, exhibit a tendency to be characterized as bivalve brachidictics, which means they are capable of undergoing a continual breeding cycle throughout the year, with varying spawning intensity every month. Pathansali (1966), Narasimham (1988) and Broom (1983) reported that *A. granosa* in Peninsular Malaysia and India has a spawning season throughout the year with no apparent seasonal pattern. In comparison, the spawning season of Archidae (genus Anadara) is presented in Table 2.

The information on the reproductive cycle of *A. granosa* provided by this study is crucial for initiating its commercial aquaculture, as well as for the sustainable management of wild stocks. In the future, data on spawning periodicity might be used to identify trochophore or veliger larvae in the wild habitat and for seed collection activities. When bivalve culture production depends on natural seed supply, the timing of seed collection is critical since the potential brood stock are suitable for a short duration. Information presented here indicates that quantitative methods (condition index and gonadal index) are a precise indicator in *A. granosa* brood stock.

4.3. *Factors that affected reproduction cycle of Anadara granosa in the northern region of the Strait of Malacca*

Gametogenesis is affected by the change and interaction of exogenous (temperature, salinity, light, food), and endogenous factors (nervous system, hormones) that could determine the reproductive strategy of bivalve species (Ram et al., 1996; Utting & Millican, 1997; Louro et al., 2003; Barber and Blake, 2006; Magnesen & Cristophersen, 2008). Principle component analysis (PCA) was conducted to evaluate the comprehensive relationship between environmental factors and reproduction variables in the *A. granosa* populations in Banda Aceh, Lhokseumawe, and Pulau Pinang (Fig. 11).

316 The principle components which affected the reproductive cycle of the *A. granosa*
317 population in Banda Aceh were gonadal index, condition index, phytoplankton density,
318 orthophosphate, salinity, and water temperature. The principle component analysis for
319 Lhokseumawe showed that there were five variables affecting *A. granosa* reproduction,
320 namely interaction among gonadal index, condition index, phytoplankton density, ammonia,
321 and pH. The reproduction of the *A. granosa* population in Pulau Pinang also showed the
322 complex interaction of the seven principle variables, namely interaction between gonadal
323 index and environmental factors such as salinity, nitrite, ammonia, phytoplankton density,
324 turbidity and dissolved oxygen.

325 Principle component analysis indicated that the environment parameters modifying
326 the reproduction of *A. granosa* populations were diverse and complex. This analysis also
327 indicated that reproduction of *A. granosa* populations is significantly affected by interaction
328 of local environment parameters. For example, water temperature was found to be modestly
329 interacting with and affecting components of reproduction in *A. granosa* in Banda Aceh.
330 Dissolved oxygen, nitrite, ammonia, and turbidity variables were only found to be
331 specifically interacting and affecting the components of reproduction in the *A. granosa*
332 population in Pulau Pinang, however these variables were not the factors affecting the
333 reproduction in the *A. granosa* population in Banda Aceh and Lhokseumawe. Reproductive
334 physiology factors such as gonadal index and condition index were shown to be affected by
335 the interaction of several water environment parameters only that are dependent on adaptation
336 level (Gillmor, 1982; Beninger & Le Pennec, 1997).

337 One of the environmental factors that was known to be strongly correlated with *A.*
338 *granosa* reproduction was phytoplankton density. This variable was known to interact with
339 and affect gonadal index and condition index as determined by gonadal development stage in
340 the three *A. granosa* populations. Lodeiros and Himmelman (1999) had conducted statistical

341 analysis, namely multiple regression analysis, to see the relationship between environmental
342 factors and reproduction of the bivalve *Lima scabra*. The conclusion of that study found that
343 phytoplankton density was the only primary factor positively correlated to the reproduction of
344 *L. scabra*. Phytoplankton density is the principle factor influencing the reproduction of
345 bivalves (Wacker & von Elert, 2003; Villalejo-Fuerte et al., 2005; Kang et al., 2006; Liu et
346 al., 2006; Hernández-Olalde et al., 2007; Calderon- Aguilera et al., 2010). Phytoplankton are
347 also known to be the main source of diet to anadarinid animals (Kasigwa & Mahika, 1991).
348 Gonadal maturation and fertilization activities of *A. granosa* that correspond with the
349 high level of phytoplankton density is a strategy to increase planktotrophic larval autonomy
350 by increasing the larvae growth rate. The duration of planktonic phase is able to be reduced
351 through optimal utilization of the food source (phytoplankton). Himmelman (1975) showed
352 that a high content of phytoplankton in the aquatic environment will stimulate the
353 reproductive period of invertebrate organisms, particularly species that have pelagic larvae.
354 Jeffre et. al. (1992) found that phytoplankton were known to release a type of chemical
355 substance that could stimulate the nervous system of bivalves to make them release gametes.
356
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363
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Captions for figures

Fig. 1. Sampling location

(Insert after line 80, page 4)

Fig. 2. Gonadal structure of *Anadara granosa* based on microscopic fresh smear analysis at indeterminate stage.

(Insert after line 149, page 7)

Fig. 3. Gonadal structure of male *Anadara granosa* based on microscopic fresh smear analysis:

(a) Stage 2 (Developing)

(b) Stage 3 (Developed)

(c) Stage 4 (Spawned).

Spz: Spermatozoa; St: spermatid; VSz: vitellogenic spermatozoa.

(Insert after line 176, page 8)

Fig. 4. Gonadal structure of female *Anadara granosa* based on microscopic fresh smear analysis:

(a) Stage 2 (Developing)

(b) Stage 3 (Developed)

(c) Stage 4 (Spawned).

EVO: early stage of vitellogenic oocyte; LVO: late stage of vitellogenic oocyte; Nl: Nucleus; RO: residual oocyte; YG: yolk granule.

(Insert after line 176, page 8)

Fig. 5. Gonadal structure of *Anadara granosa* based on histology analysis at indeterminate stage.

FW: follicle wall; Lu: Lumen; EL: empty lumen; Ct: connective tissue.

(Insert after line 182, page 8)

Fig. 6. Gonadal structure of male *Anadara granosa* based on histology analysis:
(a) Stage 2 (Developing)
(b) Stage 3 (Developed)
(c) Stage 4 (Spawned).
FW: follicle wall; Lu: lumen; Spz: spermatozoa; MS: mature spermatozoa; SD: sperm ductus; St: spermatid; DS: degenerative space; DSz: degenerative spermatozoa; FF: follicle fragment; EF: empty follicle; Ct: connective tissue.

(Insert after line 207, page 9)

Fig. 7. Gonadal structure of female *Anadara granosa* based on histology analysis:
(a) Stage 2 (Developing)
(b) Stage 3 (Developed)
(c) Stage 4 (Spawned).
FW: follicle wall; Lu: Lumen; EVO: early stage of vitellogenic oocyte; LVO: late stage of vitellogenic oocyte; MO: mature oocyte Nl: nucleus; FF: follicle fragment; EF: empty follicle; RO: residual oocyte; IS: interfolicular space; YG: yolk granule.

(Insert after line 207, page 9)

Fig. 8. *Anadara granosa* gonadal development pattern from Banda Aceh, Indonesia (June 2009-September 2010).

(Insert after line 217, page 10)

Fig. 9. *Anadara granosa* gonadal development pattern from Lhokseumawe, Indonesia (June 2009-September 2010).

(Insert after line 217, page 10)

Fig. 10. *Anadara granosa* gonadal development pattern from Pulau Pinang, Indonesia (June 2009-September 2010).

(Insert after line 217, page 10)

585 **Fig. 11.** Principle component analysis (PCA) plot for the reproductive factor component on

586 *Anadara granosa* population.

587 (a.) Banda Aceh, Indonesia
588 (b.) Lhokseumawe, Indonesia
589 (c.) Pulau Pinang, Malaysia
590 (Insert after line 215, page 11)

Captions for table

Table 1. Mean monthly seasonal environmental parameter at the sampling areas from June 2009 to September 2010.

(Insert after line 221, page 10)

Table 2. Comparison of spawning period with the highest intensity of releasing gamete in genus Anadara.

(Insert after line 297, page 13)

From: **Editorial Office OSJ** em@editorialmanager.com
Subject: OSJO: Submission Confirmation for OSJO-D-16-00027R2
Date: 5. August 2016 at 03:44
To: Munawar Khalil khalil@unimal.ac.id

Ref.: Ms. No. OSJO-D-16-00027R2
Reproductive biology of blood cockle Anadara granosa (Bivalvia: Arcidae) in the northern region of the Strait of Malacca

Dear Mr Khalil,

Ocean Science Journal has received your revised submission.

You may check the status of your manuscript by logging onto Editorial Manager at http://osjo.edmgr.com/.

Kind regards,

Editorial Office
Ocean Science Journal

From: **Jong Seong Khim** em@editorialmanager.com
Subject: OSJO: Your manuscript entitled Reproductive biology of blood cockle Anadara granosa (Bivalvia: Arcidae) in the northern region of the Strait of Malacca
Date: 19. October 2016 at 13:51
To: Munawar Khalil khalil@unimal.ac.id

JK

Ref.: Ms. No. OSJO-D-16-00027R2
Reproductive biology of blood cockle Anadara granosa (Bivalvia: Arcidae) in the northern region of the Strait of Malacca
Ocean Science Journal

Dear Mr Khalil,

Reviewers have now commented on your paper. You will see that they are advising that you revise your manuscript. If you are prepared to undertake the work required, I would be pleased to reconsider my decision.

The reviewers' comments can be found at the end of this email or can be accessed by following the provided link.

This is your login information:
Your username is: khalil84
Your password is: available at this link http://osjo.edmgr.com/Default.aspx?pg=accountFinder.aspx&firstname=Munawar&lastname=Khalil&email_address=khalil@unimal.ac.id

When revising your work, please submit a list of changes or a rebuttal against each point which is being raised when you submit the revised manuscript.

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Your revision is due by 18 Dec 2016.

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Yours sincerely

Jong Seong Khim, Ph.D.
Editor-in-Chief
Ocean Science Journal

Reviewers' comments:

Reviewer #2: Title: Reproductive biology of blood cockle Anadara granosa (Bivalvia: Arcidae) in the northern region of the Strait of Malacca.

Comapred to the first draf, the revised and resubmitted manuscript contained more biological and environmental information about habitat and general biology of the cockle in the study area. The revision also reflected most of the reviwers' comments, and accordingly I recommend this manuscript to the journal to accept and publish, after few minor revisions as suggested below.

1. Quality of Figures 1, 2 the gonadal smears are unacceptable, due to its poor resolution and low magnification. It is recommnened to drop these pictures in the final draft.
2. Effects of environmental variabls, such as water temperature, salinity, and the food level as chl. a, on the annual reproductive cycle of A. granosa were analyzed usijng principle component analysis. However, the results were presented not in the section Results, but in the discussion. Accordingly, it is suggested to present the PCA analysis results in the Result section, while interpretations of the PCA analysis is remained in the discussion.

Reviewer #3: The revised manuscript has been improved very much to meet the suggestions of the reviews' comments. However, the MS still have a couple of problem. I fully understand the importance of gonad smear analysis under a light microscopic observation to judge gonadal development of bivalves, however, the Figures 1 and 2 do not provide any information of the morphological characteristics of the gonads during the early and spent phase of development because of too small size of gonadal cells. And the papers you listed were not proper examples of gonad smear analysis. They do not have any pictures of gonads. Thus, these two figures should be deleted. In addition, the abstract doesn't give a compressed result of your findings; what is the main physical and biological factors that rule out the reproductive cycle of A. granosa, comparing the three locations.

—

COVER LETTER FOR SUBMISSION OF REVISION MANUSCRIPT

Ocean Sciences Journal (OSJ)

COVER LETTER FOR SUBMISSION OF MANUSCRIPT
Date: October, 22nd 2016

We appreciate the opportunity to revise our manuscript. With this cover letter, we will submit the revised manuscript (No. OSJO-D-16-00027) entitled, "Reproductive biology of blood cockle *Anadara granosa* (Bivalvia: Arcidae) in the northern region of the Strait of Malacca" for publication in OSJ. We carefully considered on the comments offered by the reviewers. We would like to thank referees for the careful and constructive reviews. Detailed corrections have listed below point by point and the major revised parts are highlighted in **red** color in revised manuscript. We want to extend our appreciation for taking the time and effort necessary to provide such insightful guidance.
Based the comments from the referees, we have made changes of the manuscript, which are detailed below.
We would like to express our appreciation for your extremely thoughtful comments and constructive criticisms on our manuscript. As you will see below we have been able to revise and improve the paper as a result of your valuable feedback. Detailed corrections have listed point by point and the major revised parts are highlighted in **red** color in revised manuscript.

Reply to the evaluation by the Second Referee:

1. Quality of Figures 1, 2 the gonadal smears are unacceptable, due to its poor resolution and low magnification. It is recommnened to drop these pictures in the final draft.
   Answer: All figures in gonadal smears were droped.

2. Effects of environmental variabls, such as water temperature, salinity, and the food level as chl. a, on the annual reproductive cycle of A. granosa were analyzed usijng principle component analysis. However, the results were presented not in the section Results, but in the discussion. Accordingly, it is suggested to present the PCA analysis results in the Result section, while interpretations of the PCA analysis is remained in the discussion.
   Answer: PCA analysis was moved in result section.

Reply to the evaluation by the Third Referee:

1. The revised manuscript has been improved very much to meet the suggestions of the reviews' comments. However, the MS still have a couple of problem. I fully understand the importance of gonad smear analysis under a light microscopic observation to judge gonadal development of bivalves, however, the Figures 1 and 2 do not provide any information of the morphological characteristics of the gonads during the early and spent phase of development because of too small size of gonadal cells. And the papers you listed were not

proper examples of gonad smear analysis. They do not have any pictures of gonads. Thus, these two figures should be deleted. In addition, the abstract doesn't give a compressed result of your findings; what is the main physical and biological factors that rule out the reproductive cycle of A. granosa, comparing the three locations.

Answer: All figures in gonadal smears were droped and the author added (in abstract section): The Principle component analysis (PCA) indicated that A. granosa reproduction was affected by interaction between internal physiological factors and indigenous environmental factors. In all sampling areas, phytoplankton density played as a main key role for the reproductive cycle in A. granosa

Abstract

A study on the reproductive cycle of the blood cockle *Anadara granosa* (Bivalvia: Arcidae) was conducted at three different areas in the northern region of the Strait of Malacca. A total of 1,920 samples of adult *A. granosa* (38–71 mm length) were collected from June 2009 until September 2010. Qualitative techniques (gonadal microscopic fresh smear test and histology analysis) as well as quantitative techniques (analysis of condition index and gonadal index) were used to predict monthly gonadal development stages of *A. granosa*. The gonadal index of *A. granosa* from Banda Aceh (Indonesia) (r=0.469, P>0.05) and Pulau Pinang (Malaysia) (r=0.123, P>0.05) did not show any correlation to their condition index, whereas gonadal index of *A. granosa* from Lhokseumawe (Indonesia) (r=0.609, P<0.05) showed moderate positive correlation to the condition index. During the 16 month sampling period, four reproductive cycles were observed: each from three to six months. The process of releasing gametes is dribble spawning, and is the same in all populations. The Principle component analysis (PCA) indicated that *A. granosa* reproduction was affected by interaction between internal physiological factors and indigenous environmental factors. In all sampling areas, phytoplankton density played as a main key role for the reproductive cycle in *A. granosa*. Information on the reproductive biology of this species is essential for species management and to improve the sustainability practices of the fisheries industry. These findings provide basic information on the biology of the blood cockle *A. granosa* for stock management in the region.

Keywords: blood cockle, reproductive cycle, gametogenesis, gonadal index, condition index

1. Introduction

Anadara granosa is one of 7500 bivalve species in the family Arcidae, often called "blood arks" or "blood cockles" (Gosling, 2003; Arapov et. al., 2010). Their common name refers to the hemoglobin and hemocyanin pigments in their blood and tissue cells, giving their blood a dark red color (Ruppert and Barnes, 1994) which has allowed this species to live in oxygen-critical habitat (Broom, 1985; Terwilliger and Terwilliger, 1985; Cilenti et al., 2010). The species is indigenous to intertidal mudflats of many Southeast Asian countries, particularly Indonesia, Malaysia and Thailand. *Anadara granosa* are mainly distributed in mangrove forests, muddy vegetation and mixed areas. The intertidal species *A. granosa* is known as a keystone species in mangrove habitats in several areas in the northern region of the Strait of Malacca. This species has also been one of the most important fisheries commodities in Southeast Asia for many years (Borrero, 1986; Broom, 1985; Suwanjarat et al., 2009).

The northern Strait of Malacca is an important nursery area for many intertidal organisms and a feeding area for migrating species. Being the most important species in terms of fisheries production, this cockle has become the target of an extensive culturing operation in West Malaysia (Broom, 1983). At the same time, harvesting of wild stock of cockles in Sumatra and Java (Indonesia) is at an all-time high to meet the demand for shellfish. In Malaysia, the annual production of blood cockles in 2009 exceeded metric 65,000 tonnes, which is valued at US $36.60 million (Jabatan Perikanan Malaysia (Malaysian Fisheries Department), 2010). The main blood cockle production areas in Malaysia are concentrated in Kedah (Merbok), Pulau Pinang (Juru), Perak (Kuala Gula, Kula Sangga-Matang, Kuala Trong, Sungai Jarum), Selangor (Kuala Selangor) and Johor (Muar). In Indonesia, this species can be found in abundance on the coast of West Sumatra, Central and South Java, East and West Kalimantan and other muddy bottoms in Sulawesi, Maluku and

Papua (Khalil et al., 2009). The most recent data available on annual cockle production in Indonesia is from 2009 when it reached 47,437 metric tonnes, or equal to US$ 23.72 million (Kementerian Kelautan dan Perikanan Indonesia (Ministry of Marine Affairs and Fisheries Republic Indonesia), 2010).

The Northern Straits of Malacca is an important area for the harvesting and culture of the blood cockle *A. granosa* due to suitability of the habitats for spawning and growth (Mirzaei, 2015). However, annual production statistics indicate a decrease in stocks in the last decade. This situation may be due to inadequate management of wild cockle populations. Fisheries management is needed to improve policies for the sustainability of the fisheries industry. Thorough information on reproductive cycles is necessary for predicting annual recruitment, as well as interpreting growth, mortality, and survival data in the marine culture of species (Shaw, 1965; Manzi et al., 1985; Sbrenna and Campioni, 1994). This data is lacking for the blood cockle *Anadara granosa* but is essential to optimize aquaculture of this species. This bivalve species can be managed more effectively after evaluating the regeneration capabilities of natural stocks and interpreting growth patterns. Detailed and comprehensive information on gonadal development is also important for economic management of this species (Gribben et al., 2004; Peharda et al., 2006). This study aimed to investigate the seasonal gonadal cycle of the cockle *A. granosa* by using quantitative techniques (gonadal index and condition index) through gonadal fresh smear test and gonad histology (a qualitative technique) from specimens collected from the northern region of the Strait of Malacca.

2. Materials and Methods

2.1 Collecting of samples

A total of 120 samples of adult *A. granosa* were collected monthly from June 2009 till September 2010 from the natural habitat in Banda Aceh (5°32'34.67"N-95°17'2.54"E), Lhokseumawe (05°09'35.3"N-097°08'29.4"E) in Aceh, Indonesia and Pulau Pinang (5°16'9.66"N-100°23'27.37"E) in Malaysia (Fig. 1). The total number of specimens sampled was 1,920 and the adult cockle sizes ranged from 38–71 mm in length. The sampling area was characterized by muddy substrate which was surrounded by mangroves, no wave action and high salinity. The specimens were collected at a depth of 5-30 cm and salinity ranged from 10-33 ppt. Sampling activity in the field was conducted once a month over the specified time frame during low tides. The live specimens were collected manually with the aid of harrow, which was run through the muddy area to pull bivalves to the surface. After collecting, the specimens were stored in isotherm containers and immediately transported to the laboratory. The samples were cleaned to fully remove all fouling organisms and other adherences.

2.2 Qualitative technique

2.2.1 Gonadal microscopic fresh smear test

A total of 40 specimens per sampling site were randomly allocated for the gonadal microscopic fresh smear test each month. All the specimens were dissected with a dissecting needle and pipette. The fresh smear procedure was adopted to observe the gonad content under a compound light microscope (magnification = 100 x) to analyze the stages of the gonadal development. The sex and gametogenesis stages were identified using image analysis, which included 4 stages: (+1) indeterminate, (+2) developing, (+3) developed and (+4) spawned (Rajagopal et al., 2006).

2.2.2 Histology analysis

A total of ten gonad specimens from each of the three sampling sites were allocated for histological analysis each month. Slides were prepared through the process of embedding paraffin wax into the tissue. Haematoxyline and Eosin coloration were used for tissue coloring (Howard et al., 1983). The initial process requires dehydration of the specimen tissue. Dehydration was done through a series of steps of immersing the sample into varying concentrations of alcohol. The sample was embedded into a mold of wax next and kept in a refrigerator overnight before preparing it for HE coloration. The solutions used for histology included bouins, alcohol (50%, 70%, 80%, 90%, 95% and absolute alcohol), xylene, liquid wax, histosolve, HE solution and 1.5% ammonia. A microtome was used to cut 5-7 µm thick tissue sections which were mounted on a glass microscope slide. The light compound microscope was used to analyze the gonad structure to recognize the sex and gametogenesis stages (divided into: (+1) indeterminate, (+2) developing, (+3) developed and (+4) spawned).

2.3 Quantitative method

2.3.1 Analysis of condition index (CI)

The water displacement method was used to determine the condition index. A total of 30 specimens (size range: 38–71 mm in length) from each sampling station were examined from June 2009 to September 2010. Each specimen was measured for the following: dry flesh weight, wet weight of shell in grams (g) and internal cavity volume (ml). Fresh cockle tissue including its shell was weighed using digital balances. The flesh was dried at 105 °C for 72 hours to a constant weight. Volume of the shell internal cavity volume was calculated by means of subtracting the volume of the shell (ml) from the total wet volume (ml). These data were used to calculate the condition index using the formula described by Lawrence and Scott (1982):

Condition index = dry flesh weight (gram) x 100 / shell internal cavity volume (cm^3)

121

122 *2.3.2 Analysis of gonadal index (GI)*

123 Gonadal index was calculated based on the formula proposed by Gosling (2003) and
124 Kim and Lee et al. (2008): *Gonadal index* = ∑ *n individual from each stage level x gonad*
125 *stage / n total specimen for each sampling batch.* The gonadal index (GI) was calculated for
126 each sampling month through gonadal microscopic fresh smear test and histological analysis
127 to estimate the proportion of the gonadal stages (indeterminate, developing, developed and
128 spawned). The GI value was ranked to: 1 (all individuals' gonads in the samples were in
129 spawned stage), 2 (all individuals' gonads in the samples were in indeterminate stage), 3 (all
130 individuals' gonads in the samples were in developing stage) and 4 (all individuals' gonads in
131 the samples were in developed stage).

132

133 *2.4 Statistical Analysis*

134 Raw data was compiled and entered into Microsoft Office Excel 2011 (Macintosh
135 version) for processing and analyzing of minimum and maximum value, average, and the
136 standard deviation as well as to generate graphs. One-Way ANOVA statistical analysis and
137 post hoc test were used to determine significance level ($P<0.05$ and $P<0.01$) in the values of
138 each data cluster. Pearson correlation test was also utilized to determine and understand the
139 relationship between differing variables (CI and GI). The principle component analysis
140 (PCA) was used to analyze the correlation between parameters which were affected by
141 reproductive activities in each sampling areas. These statistical analyses were conducted
142 using SPSS (*Statistical Package for Social Science*) release 20.0 for Macintosh.

143

144 **3. Results**

145 *3.1 Gonadal structure of Anadara granosa*

3.1.1. Gonadal microscopic fresh smear analysis

The description of gonad structure of *A. granosa* based on microscopic fresh smear analysis was categorized as shown below:

Stage 1 (indeterminate).

Male and female: Determination of sex cannot possibly be determined. Gonadal compound appeared to be empty and filled up only by network of connecting tissues. Unused residual of gametes can be found.

Stage 2 (developing).

Male: The gonadal compound turned cream in color. Gametes have been very active and the testis was filled with spermatogonia and spermatid. Spermatozoa were also found in limited numbers and sometimes found in tailed form and actively swim.

Female: The gonadal compound turned orange in color. Gametes in ovary have begun to appear, which are previtellogenic oogonia, oocytes and a limited number of oocytes vitellogenic. Oocytes were scattered and filled inside the follicle. Nucleus in oocytes vitellogenic have been started and are clearly visible. Oocytes have uneven sizes.

Stage 3 (developed).

Male: The gonadal compound turned a more concentrated cream color as a result of highly condensed developed spermatozoa. The spermatozoa have already developed their tail and are swimming actively. Sometimes, spermatids can still be found in small numbers.

Female: Gonadal compound turned intense, concentrated orange due to formation of highly condensed oocytes. Gametes were generally mature oocytes. Oocytes are in polyhedral form. The nucleus within the oocytes have matured and grown larger in size. The yolks were found in most of the mature oocytes. Previtellogenic oocytes can still be found in small amounts.

Stage 4 (spawned).

Male: Gonadal compound reduced drastically. Spermatozoa have diminished. Unused residual spermatozoa can be found inside the lumen.

Female: Gonadal compound turned bright orange due to the lowest concentration of oocytes. Mature oocytes were found in small amounts, but these are expected to be residue or absorbed as phagocytes. Most of the oocytes had no shape and the nucleus appeared to have shrunk and disappeared.

3.1.2. Gonadal histology analysis

Stage 1 (indeterminate).

Male and female: The stage is also called dormant stage; the sexes cannot be distinguished. Undeveloped gonads' content during this stage only consisted of connecting tissues and a handful of residual gametes leftover from the previous spawned stage (stage 4) (Fig. 2).

Stage 2 (developing).

Male: Gonad was gradually filled up with spermatogonia, spermatocyte, and a small quantity of spermatozoa. The average diameter of the follicles at this stage was 117.77 ± 19.58 µm. (Fig 3a).

Female: Oocytes occur in a range of sizes and were generally not the same shape (irregular). Gonad was gradually filled up with oogonia as well as vitellogonia oocyte and vitellogenic oocytes, the nucleus has uneven shapes. The average diameter of the follicles at this stage was 136.21 ± 22.12 µm, whereas the average diameter of oocytes was 24.81 ± 6.19 µm. (Fig 4a).

Stage 3 (developed).

Male: Gonad was mainly dominated by spermatozoa content. Interfollicular space at this stage was seen to be experiencing constriction due to the growing follicle size. Spematogonia were still found in limited number and typically found on the side wall of the follicle. The average diameter of the follicles was 186.16 ± 14.47 µm (Fig 3b).

Female: Gonad was characterized by the dominance of vitellogenic oocytes with a visibly large nucleus. Lumen space was dominated by the polyhedral oocyte vitellogenic shape which was untouched or free from the follicle wall. The cytoplasm of mature oocytes had been filled by a number of yolk granule. The average diameter of follicles was 215.13 ± 38.40 µm and oocytes were 30.01 ± 6.80 µm (Fig. 4b).

Stage 4 (spawned).

Male: Spermatozoa seemed to be reduced, as the follicle appeared almost empty. Spermatogonia were not found (Fig 3c).

Female: Residual oocytes were present. The follicles' wall seemed to be damaged and unfilled. Phagocytes were found around the residue oocytes (Fig. 4c).

3.1.3. Gonadal development cycle

The gonad percentage (for each stage) was compared between the three sampling locations: Banda Aceh (Indonesia), Lhokseumawe (Indonesia) and Pulau Pinang (Malaysia). Figures 5a, 6a, 7a, as well as 5b, 6b and 7b, depict the computation of gonad percentages per month for all the 4 phases discussed covering a span of 16 months, from June 2009 until September 2010, through gonadal microscopic fresh smear analysis and gonadal histology analysis, respectively. Figures 5c, 6c and 7c, as well as 5d, 6d and 7d, depict the monthly condition index (CI), and monthly gonadal index (GI), respectively, covering of the same 16 months.

3.2. *Environmental variable*

Monthly seasonal variation of environmental parameters in three different sampling areas are reported in Table 1. During the study period, water temperature, salinity and phytoplankton density fluctuated significantly compared to other environmental parameters. Principle component analysis (PCA) was conducted to evaluate the comprehensive relationship between environmental factors and reproduction variables in the *A. granosa* populations in Banda Aceh, Lhokseumawe, and Pulau Pinang (Fig. 8).

4. Discussion

4.1. *Gonad development for Anadara granosa*

The recorded CI values for the samples indicated significant varying values every month for samples of the same sampling location as well as those from different sampling locations. The difference in the trend of CI value indicated the status of the population of blood cockles throughout the year. A high CI value implies the gonad has already reached maturity. However, CI is not always linearly correlated to its breeding pattern. This can be shown from the comparison of the monthly CI vs GI values. The GI value is an assumed indication of the breeding status. A sudden drop in GI value signifies the occurrence of spawning activities. From this analysis, there was no linear correlation between CI and GI values for samples from Banda Aceh and Penang. However, a linear correlation between these values was noted for samples from Lhokseumawe. These were tested with the Pearson correlation test, which indicated CI values for samples from Banda Aceh ($r=0.469$ at $P>0.05$) and Penang ($r=0.123$ at $P>0.05$) have no significant correlation to their respective GI, but there is a mild correlation for samples from Lhokseumawe ($r=0.609$ at $P<0.05$). A negative correlation has also been reported in studies of other bivalve species. Hermann et al. (2009) reported a negative correlation between CI and gametogenesis cycle for *Amarilladesma*

245 *mactroides* (Reeve, 1854). Mladineo et al. (2007) also reported zero correlation between CI
246 and GI for the bivalve *Modiolus barbatus* (Linnaeus, 1758). The same applies to *Mercenaria*
247 *mercenaria* (Linnaeus, 1758) from the Gulf of Narragensett in the United States, as reported
248 by Marroquin-Mora and Rice (2008).

249 The GI values obtained throughout the year indicate high diversity in reproductive
250 patterns among the three sampling locations. This is expected due to the differences in the
251 habitat condition as well as the breeding season. Blood cockles from all three sampling
252 locations showed a rapid transition from gonad development to maturation phase. GI analysis
253 shows spawning activity happened every month throughout the year with varying intensity.
254 The GI value increases during gametogenesis and decreases after spawning. The fast-paced
255 transition could be a strategy for the blood cockles to increase the amount of gamete released
256 whilst favorable environmental conditions are present. This behavior is characteristic of
257 reproduction of invertebrates in tropical regions. Species have been shown to adopt
258 opportunistic strategies to develop the gonadal matter from energy available from food rather
259 than from energy stored inside somatic parts (Cárdenas and Aranda, 2000). Freites et al.,
260 (2010) found that *Anadara notabilis* exhibits a continuous reproductive cycle throughout the
261 year and that particulate organic matter, temperature and food availability were regulating
262 factors of the reproduction of *A. notabilis*.

263 This study of blood cockles' GI shows that it has a breeding cycle lasting an average
264 of 3~6 months across the three sampling locations (Banda Aceh, Lhokseumawe and Pulau
265 Pinang). During the 16 month sampling period, four reproductive cycles have been observed.
266 For the *A. granosa* population from Banda Aceh (Indonesia), cycle I occurred from June to
267 October 2009, cycle II from November 2009 to January 2010, cycle III from February to
268 April 2010, and cycle IV from April to September 2010. In Lhokseumawe (Indonesia), cycle
269 I started from June to August 2009, cycle II from September 2009 to January 2010, cycle III

from February to June 2010 and cycle IV from July to September 2010. For the *A. granosa* population in Pulau Pinang (Malaysia), cycle I started from June to October 2009, cycle II from November 2009 to February 2010, cycle III from February 2010 to April 2010 and cycle IV from April 2010 to September 2010. All three populations started the first cycle around June 2009 and ended the fourth cycle also around the same time, September 2010. The population from Lhokseumawe (Indonesia) showed a tendency to spawn faster compared to the other two populations. However, during the third cycle, populations from Banda Aceh (Indonesia) and Penang (Malaysia) exhibited a more rapid and shorter cycle lasting approximately 2~3 months, compared to Lhokseumawe (Indonesia) which took about 5 months.

4.2. Breeding pattern of Anadara granosa

Generally, the bivalve breeding process is characterized by a continual and seasonal pattern (Ceballos-Vazquez et al., 2000), and is iteroparous in nature, continually and repeatedly breeding throughout its entire life span (Dame, 1996). Bivalves give birth to their young by means of gametogenesis. This process is then followed by the release of one or several gametes. The process of rearranging empty gonad with new gametes for the next cycle always as a signals for the beginning of a new breeding cycle (Gosling, 2003). Variation in the breeding trend amongst cockle populations of different geographical locations makes it difficult to determine a pattern of gonad development. A well balanced distribution of males to females in blood cockles is supported by the sex ratio analysis done in this study. Gonad development and spawning period was determined to be parallel between the two opposing sexes, a scenario known as synchrony. According to Levitan (1993), synchrony in gonad development of bivalves is crucial to increase the possibility of effective mating. Extended spawning durations from one to two months is a common

295 breeding strategy for bivalve species. Such a strategy is essential to maintain the cockle
296 population over time within its habitat. Generally, sporadic gamete mating will happen
297 concurrently under suitable surrounding conditions. Blood cockles for all three sampling
298 locations, and in general, exhibit a tendency to be characterized as bivalve brachidictics,
299 which means they are capable of undergoing a continual breeding cycle throughout the year,
300 with varying spawning intensity every month. Pathansali (1966), Narasimham (1988) and
301 Broom (1983) reported that *A. granosa* in Peninsular Malaysia and India has a spawning
302 season throughout the year with no apparent seasonal pattern. In comparison, the spawning
303 season of Archidae (genus Anadara) is presented in Table 2.

304 The information on the reproductive cycle of *A. granosa* provided by this study is
305 crucial for initiating its commercial aquaculture, as well as for the sustainable management of
306 wild stocks. In the future, data on spawning periodicity might be used to identify trochophore
307 or veliger larvae in the wild habitat and for seed collection activities. When bivalve culture
308 production depends on natural seed supply, the timing of seed collection is critical since the
309 potential brood stock are suitable for a short duration. Information presented here indicates
310 that quantitative methods (condition index and gonadal index) are a precise indicator in *A.*
311 *granosa* brood stock.

312

313 4.3. *Factors that affected reproduction cycle of Anadara granosa in the northern region of*
314 *the Strait of Malacca*

315 Gametogenesis is affected by the change and interaction of exogenous (temperature,
316 salinity, light, food), and endogenous factors (nervous system, hormones) that could
317 determine the reproductive strategy of bivalve species (Ram et al., 1996; Utting & Millican,
318 1997; Louro et al., 2003; Barber and Blake, 2006; Magnesen & Cristophersen, 2008). The
319 Principle component analysis (PCA) was shown that the principle components which affected

320 the reproductive cycle of the *A. granosa* population in Banda Aceh were gonadal index,
321 condition index, phytoplankton density, orthophosphate, salinity, and water temperature. The
322 principle component analysis for Lhokseumawe showed that there were five variables
323 affecting *A. granosa* reproduction, namely interaction among gonadal index, condition index,
324 phytoplankton density, ammonia, and pH. The reproduction of the *A. granosa* population in
325 Pulau Pinang also showed the complex interaction of the seven principle variables, namely
326 interaction between gonadal index and environmental factors such as salinity, nitrite,
327 ammonia, phytoplankton density, turbidity and dissolved oxygen.

328 Principle component analysis indicated that the environment parameters modifying
329 the reproduction of *A. granosa* populations were diverse and complex. This analysis also
330 indicated that reproduction of *A. granosa* populations is significantly affected by interaction
331 of local environment parameters. For example, water temperature was found to be modestly
332 interacting with and affecting components of reproduction in *A. granosa* in Banda Aceh.
333 Dissolved oxygen, nitrite, ammonia, and turbidity variables were only found to be
334 specifically interacting and affecting the components of reproduction in the *A. granosa*
335 population in Pulau Pinang, however these variables were not the factors affecting the
336 reproduction in the *A. granosa* population in Banda Aceh and Lhokseumawe. Reproductive
337 physiology factors such as gonadal index and condition index were shown to be affected by
338 the interaction of several water environment parameters only that are dependent on adaptation
339 level (Gillmor, 1982; Beninger & Le Pennec, 1997).

340 One of the environmental factors that was known to be strongly correlated with *A.*
341 *granosa* reproduction was phytoplankton density. This variable was known to interact with
342 and affect gonadal index and condition index as determined by gonadal development stage in
343 the three *A. granosa* populations. Lodeiros and Himmelman (1999) had conducted statistical
344 analysis, namely multiple regression analysis, to see the relationship between environmental

345 factors and reproduction of the bivalve *Lima scabra*. The conclusion of that study found that
346 phytoplankton density was the only primary factor positively correlated to the reproduction of
347 *L. scabra*. Phytoplankton density is the principle factor influencing the reproduction of
348 bivalves (Wacker & von Elert, 2003; Villalejo-Fuerte et al., 2005; Kang et al., 2006; Liu et
349 al., 2006; Hernández-Olalde et al., 2007; Calderon- Aguilera et al., 2010). Phytoplankton are
350 also known to be the main source of diet to anadarinid animals (Kasigwa & Mahika, 1991).
351 Gonadal maturation and fertilization activities of *A. granosa* that correspond with the
352 high level of phytoplankton density is a strategy to increase planktotrophic larval autonomy
353 by increasing the larvae growth rate. The duration of planktonic phase is able to be reduced
354 through optimal utilization of the food source (phytoplankton). Himmelman (1975) showed
355 that a high content of phytoplankton in the aquatic environment will stimulate the
356 reproductive period of invertebrate organisms, particularly species that have pelagic larvae.
357 Jeffre et. al. (1992) found that phytoplankton were known to release a type of chemical
358 substance that could stimulate the nervous system of bivalves to make them release gametes.
359

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366

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Captions for figures

Fig. 1. Sampling location

(Insert after line 83, page 4)

Fig. 2. Gonadal structure of *Anadara granosa* based on histology analysis at indeterminate stage.

FW: follicle wall; Lu: Lumen; EL: empty lumen; Ct: connective tissue.

(Insert after line 184, page 8)

Fig. 3. Gonadal structure of male *Anadara granosa* based on histology analysis:

(a) Stage 2 (Developing)

(b) Stage 3 (Developed)

(c) Stage 4 (Spawned).

FW: follicle wall; Lu: lumen; Spz: spermatozoa; MS: mature spermatozoa; SD: sperm ductus; St: spermatid; DS: degenerative space; DSz: degenerative spermatozoa; FF: follicle fragment; EF: empty follicle; Ct: connective tissue.

(Insert after line 209, page 9)

Fig. 4. Gonadal structure of female *Anadara granosa* based on histology analysis:

(a) Stage 2 (Developing)

(b) Stage 3 (Developed)

(c) Stage 4 (Spawned).

FW: follicle wall; Lu: Lumen; EVO: early stage of vitellogenic oocyte; LVO: late stage of vitellogenic oocyte; MO: mature oocyte Nl: nucleus; FF: follicle fragment; EF: empty follicle; RO: residual oocyte; IS: interfolicular space; YG: yolk granule.

(Insert after line 209, page 9)

Fig. 5. *Anadara granosa* gonadal development pattern from Banda Aceh, Indonesia (June 2009-September 2010).

(Insert after line 219, page 9)

Fig. 6. *Anadara granosa* gonadal development pattern from Lhokseumawe, Indonesia (June 2009-September 2010).

(Insert after line 219, page 9)

Fig. 7. *Anadara granosa* gonadal development pattern from Pulau Pinang, Indonesia (June 2009-September 2010).

(Insert after line 219, page 9)

Fig. 8. Principle component analysis (PCA) plot for the reproductive factor component on *Anadara granosa* population.

(a.) Banda Aceh, Indonesia
(b.) Lhokseumawe, Indonesia
(c.) Pulau Pinang, Malaysia

(Insert after line 226, page 10)

Captions for table

Table 1. Mean monthly seasonal environmental parameter at the sampling areas from June 2009 to September 2010.

(Insert after line 226, page 10)

Table 2. Comparison of spawning period with the highest intensity of releasing gamete in genus Anadara.

(Insert after line 303, page 13)

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Thank you for submitting your work to this journal.

With kind regards

Jong Seong Khim, Ph.D.
Editor-in-Chief
Ocean Science Journal

—

From: 서만덕 mdseo@kiost.ac.kr
Subject: [Ocean Science Journal] English Proof (Please, send final version of MS to me)
Date: 2. January 2017 at 07:52
To: khalil@unimal.ac.id, zulfigarusm@yahoo.com, aileen@usm.my

만덕

Dear. Dr. Munawar Khalil

First of all, We appreciate for your contribution to Ocean Science Journal.
Your manuscript has been accepted, and now, we send revised manuscript by English editor's proofreding.

Please, check your manuscript for errors or corrections to make, and send the final version of manuscript to me.

1) Check your references again following OSJ's reference style.

* OSJ's reference style : http://www.springer.com/cda/content/document/cda_downloaddocument/Instructions+for+Authors.pdf?SGWID=0-0-45-722098-p173840203

2) Please send me high resolution figures and tables in your manuscript.

3) Let us know "running title".

After proofreading, we will send a printing format of MS to you for page proofs.

Thank you again for your submission to Ocean Science Journal.

Sincerely,

Man Deok Seo

Man Deok Seo, Ph.D.
Specialist / Archivist
Editorial Secretary of Ocean Science Journal

Ocean Science Library
Korea Institute of Ocean Science & Technology
Ansan P.O.Box 29, Seoul 425-600, KOREA

Tel:+82-31-400-6540 / 010-3313-0887

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From: 서만덕 mdseo@kiost.ac.kr
Subject: Re: RE: Re: RE: [Ocean Science Journal] Request: 1st Galley Proof (required reading)
Date: 13. February 2017 at 04:59
To: Munawar Khalil khalil@unimal.ac.id

Dear Dr. Munawar Khalil

We've modified some errors.

Please check your manuscript and make a reply about corrections to our e-mail.

Sincerely yours,

Man Deok SEO

--- Original Message ---
From : "Munawar Khalil"<khalil@unimal.ac.id>
To : "'서만덕'"<mdseo@kiost.ac.kr>
Date : 2017/02/10 금요일 오후 2:24:10
Subject : RE: Re: RE: [Ocean Science Journal] Request: 1st Galley Proof (required reading)

Dear Dr. Man Deok SEO

Thank you for your attention and help. Please check the attachment file for final errors and corrections check.

If you need more further information, please do not hesitate to contact me.

Best Regard,

Munawar Khalil

Indonesia

From: 서만덕 [mailto:mdseo@kiost.ac.kr]
Sent: Tuesday, 7 February, 2017 8:44 AM
To: Munawar Khalil <khalil@unimal.ac.id>
Subject: Re: Re: RE: [Ocean Science Journal] Request: 1st Galley Proof (required reading)

Dear Dr. Munawar Khalil

Thank you for your response.

If you have additional errors and corrections, please make a reply to our e-mail.

Sincerely yours,

Man Deok SEO

--- Original Message ---
From : "Munawar Khalil"<khalil@unimal.ac.id>
To : mdseo@kiost.ac.kr
Date : 2017/02/06 월요일 오전 11:21:08
Subject : Re: RE: [Ocean Science Journal] Request: 1st Galley Proof (required reading)

Dear Dr. Man Deok SEO

Thank you for yours review and response. You are right. Please change Oct. 2010 to Oct. 2009 in Table 1.

Thank you

Best Regard,

Khalil

Indonesia

Sent from my Mi phone

On 서만덕 <mdseo@kiost.ac.kr>, Feb 6, 2017 8:27 AM wrote:

> Dear Dr. Munawar Khalil
>
> We have a question.
>
> We thinhk that the date range in Table 1(p. 9) is incorrect.
>
> Please check "Oct. 2010" in Table 1.
>
> Sincerely yours,
>
> Man Deok SEO
>
> --- Original Message ---
> **From :** "Munawar Khalil"<khalil@unimal.ac.id>
> **To :** "'서만덕'"<mdseo@kiost.ac.kr>
> **Date :** 2017/02/04 토요일 오전 11:23:30
> **Subject :** RE: [Ocean Science Journal] Request: 1st Galley Proof (required reading)
>
> Dear Dr. Man Deok SEO
>
> Thank you so much for the printed version. Everything look great. But I have some little comment in Table 2, page 12. Please check the attachment file.
>
> 1. My zip code is: 24351
> 2. Thank you with the Reproduction Rights of Articles

2. The running title is : Reproductive Biology of Anadara granosa

if you need more further information, please do not hesitate to contact me. Thank you

Best Regard,

Munawar Khalil

Indonesia

From: 서만덕 [mailto:mdseo@kiost.ac.kr]
Sent: Friday, 3 February, 2017 8:44 AM
To: khalil@unimal.ac.id
Subject: [Ocean Science Journal] Request: 1st Galley Proof (required reading)

Dear Dr. Munawar Khalil

Now, we send a printed-version of your manuscript for 1st galley proof.

Please check your Manuscript and make a reply about errors and corrections to our e-mail.

Let us know additional information as below.

1) zip code (postal code) of Universitas Malikussaleh.

Department of Aquaculture, Universitas Malikussaleh, Aceh **zip code(?)**, Indonesia

- OSJ address style : department name, university name(or institute name), city name zip code, nation

2) running title

Sincerely yours,

Man Deok SEO

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Editorial Secretary of Ocean Science Journal

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E-mail: mdseo@kiost.ac

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From: **Springer** SpringerAlerts@springeronline.com
Subject: Your article in Ocean Science Journal is now online
Date: 25. February 2017 at 00:31
To: khalil@unimal.ac.id

S

Springer

Online First: your article is published 2017-02-25

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Dear Author,

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Title
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Journal
Ocean Science Journal, (), 1-15

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10.1007/s12601-017-0010-y

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