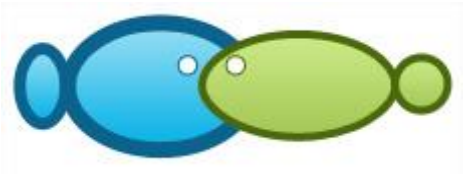

Partial inclusion of macroalgae into formulated feed to induce gonad maturation in tropical abalone, *Haliotis squamata*, broodstock

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Abstract. The reproductive performance of tropical abalone is crucial for sustainable aquaculture practices, particularly in improving broodstock management strategies. This study evaluated the reproductive performance of tropical abalone (*Haliotis squamata*) broodstock, induced by feeding them formulated feed containing macroalgae as one of its protein sources. The experimental broodstock specimens were fed a formulated diet consisting of a combination of three macroalgae species: *Gracillaria* sp., *Ulva* sp., and *Sargassum* sp., with increasing protein levels (30, 35, 40, and 45%). The broodstocks were reared in a flow-through water system and fed the respective diet twice a day (5 AM and 8 PM). The gonad maturity level, gonad somatic index, fecundity, and hatching rate were measured at the end of the experiment. This study indicates that the gonad somatic index, fecundity, and hatching rate of the eggs were significantly influenced by the protein level of the diet, with higher values being obtained from the broodstock fed with high protein diets (40-45% crude protein). However, the gonad maturity level showed no difference across all treatments. This study suggests that broodstock reproductive performance can be improved by feeding them a high protein diet with macroalgae as one of the protein sources.

Key Words: macroalgae, reproductive performance, tropical abalone, protein.

Introduction. The availability of sustainable abalone larvae supply is one of the keys to the success of abalone aquaculture in Indonesia (Grandiosa 2020). However, the decrease in wild seed supply due to habitat destruction and overexploitation has become a major problem in developing abalone aquaculture in many countries (Jarayabhand & Paphavasit 1996). Moreover, obtaining ready-to-spawn broodstock completely from natural stocks is difficult (Rusdi et al 2016). This is because wild broodstock have different gonad maturity levels, likely influenced by their consumption of different macroalgae. Rusdi et al (2010) observed higher gonad maturity level of tropical abalone *Haliotis squamata* broodstock fed with the combination of macroalgae diet, compared to those fed single macrolagae diet. Therefore, despite tropical abalone's ability to spontaneously spawn year-round (Capinpin et al 1998; Singhagraiwan & Doi 1992), there is an unpredictable supply of high-quality larvae. Hence, abalone hatcheries must continuously maintain ready-to-spawn broodstock.

Gonad maturity can be induced through hormone manipulation, environmental manipulation, and feed formulation (Haryati 2021). High-quality feed can induce gonad maturity, as the nutritional content of feed plays a significant role in the reproductive performance of aquatic animals, particularly in gonad maturity and fecundity (Watanabe 1988). In order for the abalone brood to possess desirable traits, such as the ability to spawn continuously and produce quality eggs and larvae, the broodstock must be provided with high-quality feed with a balanced composition to trigger the maturity of the abalone broodstock gonad.

The development of abalone species as a promising aquaculture commodity in Indonesia has been recognized as a potential avenue to bolster national revenue, akin to other aquaculture species. Nonetheless, this growth met challenges, particularly in the absence of specialized feed tailored for abalone (Grandiosa 2020). Consequently, there has been a surge of research focused on abalone, particularly in the domains of abalone nutrition and feed. An array of investigations on abalone feed has centered on the utilization of seaweed as a primary raw material in feed formulations (Fajriah et al 2017; Giri et al 2015; Giri et al 2016; Kuncoro et al 2013; Marzuqi et al 2012; Nurfajrie et al 2014; Susanto et al 2010; Bilbao et al 2012; Duong et al 2020; Duong et al 2021; Roussel et al 2019). Seaweed, or macroalgae, represents a novel component in formulated feeds for aquatic organisms due to its abundant production capacity, which circumvents the need for costly arable land, and its accessibility from coastal areas or seaweed farmers (Wan et al 2019). Notably, seaweed is replete with essential nutrients, including protein, carbohydrates, fats, crude fiber, and ash. Furthermore, it is enriched with enzymes, nucleic acids, amino acids (up to 10-20 times more than terrestrial plants), various vitamins, and essential minerals (Priono 2016).

Numerous investigations have explored the utilization of seaweed as a natural dietary component to stimulate gonad maturity in tropical abalone broodstock (Effendy & Patadjai, 2016; Wa et al 2016; Mulki et al 2019; Purwaningsih et al 2013; Rusdi et al 2016; Bautista-Teruel et al 2001; Fitri 2014) However, no research has hitherto investigated the incorporation of seaweed as a partial inclusion in artificial feed as a protein source to induce the development and maturation of gonads in tropical abalone species, particularly *H. squamata*. Previous studies have focused on *Gracillaria* sp. and *Ulva* sp. as natural feed options to enhance the reproductive performance of tropical abalone broodstock (Fitri 2014; Rusdi et al 2016). In this study, these specific macroalgae species will serve as the protein source in the formulated feed.

Given the limited information regarding the use of seaweed as a partial protein source in abalone feed to stimulate gonad maturity of the broodstock, it is important to address this knowledge gap. The study seeks to contribute to the field by examining the incorporation of seaweed as a viable protein source in formulated feed to trigger the development and maturation of gonads in tropical abalone, specifically *H. squamata*. By determining the ideal proportion of seaweed incorporated into the diet, this study aims to provide significant insights into the effective management of abalone broodstock. These aspects include reproductive performance indicators such as gonad maturity level, gonad somatic index, fecundity, and hatching rate, which are pivotal in assessing the overall success and sustainability of abalone aquaculture. Consequently, the findings derived from this research will offer invaluable insights into the formulation of specialized feeds for abalone broodstock, enhancing the prospects for improved reproductive outcomes and fostering the advancement of sustainable aquaculture practices.

Material and Method

Experimental animals. Tropical abalone broodstocks (±57.90 mm in length, ±37.22 in width, and ±31.40 g body weight), were used in this study (Figure 1).

Figure 1. *Haliotis squamata* broodstock: A. Female, B. Male.

The abalone broodstocks utilized in this experiment were sourced from a controlled spawning event within hatchery environment of the Center for Marine Aquaculture Research and Fisheries Extension, specifically raised and bred in the hatchery, and represent the F2 generation. The broodstocks were selected properly and checked for their condition. Abalone with damaged shell or wound in their muscle were not used.

Experimental system. The experiment was conducted in a flow-through water system consisting of 24 plastic baskets, each with a specific size. In each treatment combination, 20 abalone broodstocks were placed into three replicate plastic baskets. To avoid feed loss during the experiment, the bottom of the plastic baskets was layered with a plastic sheet. Additionally, each basket was equipped with PVC pipes to serve as shelter (3 inches) The water flow rate in the system was maintained at 15 L min^{-1} as reported by Giri (2016), and the salinity was maintained at 35-36 ppt.

Prior to stocking, the abalone broodstock were individually weighed and checked for their gonad maturity level. Subsequently, the animals underwent an acclimation process lasting approximately 3 weeks, during which they were fed the experimental diets. Any mortalities that occurred during this period were replaced with animals from the same population (F2 generation). Throughout the feed trial, no mortality was recorded. The abalone were fed at a rate of 5% of their biomass in all treatment combinations. Feeding was carried out twice a day, at 5 PM and 8 PM. To maintain water quality and cleanliness, any uneaten feed and overnight feces were cleaned using a siphon every morning.

Experimental design. In this experimental study, a total of 240 *H. squamata* broodstock were utilized, distributed across four distinct treatments. Each treatment was replicated three times, resulting in a total of 12 experimental units. Within each replicate, 20 abalone broodstock were assigned. During the experiment's progression, a staged approach was implemented to handle the abalone broodstock. From each group of 20 abalone broodstock within a replicate, a subset of 5 individuals was deliberately isolated for deferred spawning at a later stage, ensuring equitable representation across all treatments and replicates. Consequently, the remaining 15 abalone broodstock within each replicate were retained for the specific purpose of assessing gonad maturity levels and calculating the Gonad Somatic Index (GSI). This experimental design allowed for a comprehensive evaluation of the treatment effects on gonad maturity and fecundity, while maintaining sufficient broodstock for subsequent spawning, and ensured robustness in data analysis and interpretation.

Experimental feeds. The diets used in this experiment were formulated using a combination of three seaweed species: *Ulva* sp., *Sargassum* sp., and *Gracillaria* sp. The seaweed was obtained from Klungkung regency, Bali. Prior to processing, the seaweed was sun-dried and then ground to make a fine powder. The seaweed powder was subsequently incorporated into the feed as a protein source.

The feed was formulated to have four protein levels: 30, 35, 40, and 45% crude protein. The feed ingredients were weighed according to the formula (Table 1), manually mixed, and then made into pellets using a pellet machine. The feed was then oven-dried at 70 to 100°C until no moisture was detected. Subsequently, the feed was stored in the fridge prior to feeding. The proximate analysis of the feed was conducted using the Kjeldahl method as described in the Association of Official Analytical Chemists (AOAC 1990) guidelines for chemical testing. Lipid content was determined by chloroform-methanol extraction as described by Bligh & Dyer (1959). Ash content was determined gravimetrically by incineration, and NFE (Nitrogen Free Extract) was calculated by difference (Table 1).

Table 1
Ingredients and nutritional composition of experimental diets with increasing protein level

Ingredients (%)	*Protein level (%)*			
	30 (A)	*35 (B)*	*40 (C)*	*45 (D)*
Fish meal	22	22	22	22
Casein	7	13	19	25
Seaweed powder*	15	20	25	30
Corn flour	19	14	9	3
Flour	19	13	7	2
Fish oil	4	4	4	4
Vitamin mix	4	4	4	4
Mineral mix	4	4	4	4
CMC	5.5	5.5	5.5	5.5
Chemical composition (g (100 g)$^{-1}$ DM), analyzed and calculated				
Moisture (%)	7.87	7.93	8.58	7.84
Crude protein (%)	27.68	32.36	39.84	44.88
Crude lipid (%)	8.10	7.37	7.72	7.25
NFE (%)	42.93	32.79	25.03	17.37
Crude Fiber (%)	1.34	1.38	1.51	1.80
Ash (%)	19.96	26.10	25.90	28.70

NFE (Nitrogen free extract): 100% - (Protein% + Lipid% + Ash %); *combination of *Gracillaria* sp., *Ulva* sp. and *Sargassum* sp.

Measured parameters. Gonad Maturity Level (GML), Gonad Somatic Index (GSI), fecundity, and hatching rate data were collected after 47 days of the experiment. The GML of the abalone broodstocks was observed visually using the classification guide by Setyono (2003). At the end of the study, the broodstocks were dissected, and the gonad was weighed to determine the Gonad Somatic Index. The GSI was calculated using the formula (Webber & Giese 1969):

$$GSI = \frac{Gonad\ Weight}{Body\ Weight}\ x\ 100$$

The artificial spawning process of abalone involves several sequential stages. Firstly, a selection of mature gonads from both male and female individuals is performed. Since the maturity level of gonads varies among individuals, only those with mature and ready-to-spawn gonads are chosen for the process. Spawning induction is carried out by subjecting the selected abalone individuals to a dry-up period lasting 1-2 hours, during which they are placed in waterless baskets. Subsequently, the chosen male and female abalones are introduced into separate 20-liter box containers with a ratio of 1 male to 2 females. The containers are filled with filtered seawater treated with a sand filter and are then kept in a dark room. The next step involves stimulating spawning by adding pure oxygen through aeration for a period of 3 hours in the spawning boxes, followed by the continuous addition of regular aeration until spawning occurs. After successful spawning, fertilization of the eggs takes place, followed by siphoning and thorough washing of the eggs to ensure cleanliness.

Fecundity was determined by calculating the number of eggs produced by the broodstock using the volumetric method. A 1 mL sample was taken, and the number of eggs was calculated under the microscope. The procedure was repeated three times, and the average number of eggs was determined. The average value of eggs in the 1 mL sample was then multiplied by the total sample volume using the following formula (Murtidjo 2005):

$$F = \frac{n}{v}\ x\ V$$

Where:
F - fecundity;
n - the number of sampled eggs;
v - the volume of sampled eggs;
V - the total sample volume.

Hatching rate was calculated by multiplying the number of eggs hatched by 100 and dividing it by the number of fertilized eggs (Hui et al 2012):

$$HR = \frac{number\ of\ hatched\ eggs}{number\ of\ fertilized\ eggs}\ x\ 100$$

Statistical analysis. Residual plots were used to examine the homogeneity and normality of the data. The analysis of Gonad Maturity Level, Gonad Somatic Index, Fecundity and Hatching rate was conducted using ANOVA. Subsequently, Tukey's post-hoc tests were used to distinguish significant differences between treatment means. All statistical analysis were done using IBM SPSS version 24 for Macintosh (IBM SPSS Inc., Chicago, IL). Data are presented as mean ± SE.

Results. The gonad maturity level and gonad somatic index of tropical abalone *H. squamata* broodstock fed four diets with increasing protein levels are presented in Table 2. The gonad maturity level (GML) of the abalone broodstock in all treatments was not significantly different and did not appear to be affected by the level of crude protein content in the diet. However, the gonad somatic index (GSI) showed a significant difference, with the diet containing 45% protein exhibiting a higher GSI (17%) compared to diets containing 30 and 35% protein.

Table 2

Gonad maturity level and gonad somatic index of abalone broodstock fed experimental diets

Treatment code	*Protein level*	*Gonad maturity level (Percentage of GML 2)*	*GSI (%)*
A	30%	2.0000±3.46410	14.1033±0.76002[a]
B	35%	2.6667±4.61880	14.9233±0.44467[a]
C	40%	17.0000±19.05256	15.8100±0.33956[ab]
D	45%	21.6667±9.71253	17.0000±1.10964[b]

Data presented as Mean±SE. A significance level of $p<0.05$ was used in all statistical analysis. Values that share the same superscript are not significantly different.

Table 3

Fecundity and hatching rate of abalone broodstock fed experimental diets

Treatment code	*Protein level*	*Fecundity (eggs)*	*Hatching rate (%)*
A	30%	3,059.998±33,223.12[a]	60.86±1.01[a]
B	35%	3,106.665±14,788.52[a]	62.70±1.03[a]
C	40%	3,713.332±77,595.98[b]	67.22±0.85[b]
D	45%	4,040.000±57,717.03[c]	70.44±0.76[b]

Data presented as Mean±SE. A significance level of $p<0.05$ was used in all statistical analysis. Values that share the same superscript are not significantly different.

Furthermore, the fecundity and hatching rate of the broodstock fed with four protein levels were significantly affected by the protein content of the diet (Table 3). The abalone broodstock fed with a diet containing 45% protein demonstrated the highest fecundity (4,000 eggs) compared to those fed with diets containing lower protein content. Similarly, the hatching rate of the eggs from broodstock fed with 40 and 45% protein

level diets (67 and 70%, respectively) was higher than those from broodstock fed with only 30 and 35% protein level diets.

Discussion. The current study examined the gonad maturity level, gonad somatic index, fecundity and hatching rate of tropical abalone broodstock *H. squamata* fed four different macroalgae-based diets with increasing protein level. The results demonstrated that high protein diet (40-45%) affected the fecundity, gonad somatic index and hatching rate, even though the gonad maturity level seemed not to be affected by the diet protein level. Optimal gonad development in abalone broodstock can be achieved through proper diet conditioning. Therefore, determining the nutritional requirements for broodstock conditioning is crucial. Notably, the protein level in the diet plays a significant role in gonad morphology and fecundity (Bilbao et al 2012). The study used seaweed as a partial alternative protein source to reduce the reliance on fishmeal in abalone feed. Macroalgae contain essential nutrients such as protein, dietary fibers, and phytochemicals, enhancing the feed's nutritional quality (Morais et al 2020). *Gracillaria* sp. contains 5.6-24% of protein (Marrion et al 2005), *Ulva* sp. 8.5-17.44% of protein (Marsham et al 2007; Yaich et al 201; Yıldırım et al 2009), and *Sargassum* sp. 8.54-18.21% (Debbarama et al 2016; Dewinta et al 2020). However, due to certain properties, macroalgae can only be used as a partial substitute in formulated feed and cannot completely replace other ingredients. A replacement above 10% of the total concentration in animal feed may result in low palatability (Morais et al 2020). The gonad maturity level in this study was not significantly affected by the protein level in the diet. However, the treatment with the highest protein content (45%) showed the highest percentage of broodstock reaching gonad maturity level 2 (maturing). Despite only 22% of the broodstock achieving GML 2 (mature) during the 47-days experiment with a diet containing 45% crude protein, this relatively slow progress of gonadal development aligns with the findings of Permana et al (2018) who reported that tropical abalone *H. squamata* reared in the hatchery and fed with macroalga *Ulva* sp. reached gonad maturity level 3 (ripe) in 90 days of experimentation. In comparison, wild *Haliotis varia* requires 7 months to spawn from the beginning of gametogenesis (Najmudeen 2007). Furthermore, *H. squamata* broodstock fed with a combination of *Gracillaria* sp., *Ulva* sp., and *Sargassum* sp. during a 70-days experiment produced the highest percentage (45%) of gonad maturity level 3 (Rusdi et al 2010). Additionally, Fitri (2014) reported that there was a significant effect on the gonad maturity level of the broodstock *H. squamata* fed the combination of 50:50 *Gracillaria* sp. and *Ulva* sp. This effect is likely due to the nutritional content of these macroalgae, particularly their fatty acids, which play an essential role in promoting gonadal development (Viera et al 2011).

The gonadosomatic index (GSI) serves as a valuable tool to determine the reproductive cycle based on the premise that gonad maturity and spawning align with the maximum gonad weight (Lebour 1938). In this study, the highest GSI value (17%) was observed in the broodstock population fed with a diet containing 45% protein. This signifies that the gonadal development of the broodstock fed the high protein diet was superior to those fed lower protein diets. The diet, as an exogenous factor, plays a crucial role in inducing gonad maturity in mollusks (Runham 1988). During gonad maturity, there are changes in the biochemistry composition of the gonad and digestive gland (Litaay 2005). Additionally, abalone gametogenesis involves an increase in the number of lipids and proteins in the gonad of the family Haliotidae (Najmudeen 2007a). Moreover, essential nutrients such as protein, lipid, and fatty acids (ARA, EPA, and DHA) have been reported to influence the reproductive performance of *Haliotis asinina* (Bautista-Teruel et al 2001). Red algae (Rhodophyta) are considered one of the main sources of fatty acids (Nelson et al 2002). *Gracillaria* sp., a red algae species, has been recognized as an excellent feed source to promote gonad development in tropical abalone *H. asinina* (Singhagraiwan 1993). Additionally, *Ulva reticulata* contains several fatty acids, including ARA, EPA, and DHA (Ratana-Arporn & Chirapart 2006), known to significantly enhance gonad maturity in *H. asinina* (Bautista-Teruel et al 2001; Litaay 2005).

Fecundity, defined as the number of eggs produced by broodstock after a spawning event (Bilbao et al 2012), and hatching rate, calculated by dividing the number

of hatched eggs by the total number of fertilized eggs and multiplied by one hundred percent (Hui et al 2014), were assessed in the present study. The results revealed a significant difference between treatments, with the highest fecundity and hatching rate observed in the broodstock fed with a diet containing 45% protein. This finding suggests that a higher protein diet consumed by the abalone broodstock leads to a higher percentage of fecundity and hatching rate. The observed increase in fecundity and hatching rate could be attributed to the fact that the higher protein diet provides sufficient nutrients to stimulate the reproductive performance of the broodstock. This outcome aligns with the study conducted by Bautista-Teruel et al (2001), which observed an increase in fecundity of *H. asinina* in response to a higher protein level provided in the diet. The study suggested that the availability of high macronutrient components in the abalone feed, such as proteins and lipids, could enhance the fecundity and hatching rate of *H. asinina*. Moreover, proteins and essential fatty acids, which are usually allocated to the gonads, play essential roles in reproductive performance (Harrison 1991). The use of macroalgae as one of the protein sources could be an alternative to reduce the inclusion of fish meal into diet.

Conclusions. The study demonstrates that the protein level in the diet significantly affects fecundity, hatching rate, and gonad somatic index of tropical abalone *H. squamata* broodstock. While gonad maturity level seems to be less influenced by diet, the study underscores the importance of proper nutrition in achieving optimal reproductive performance and highlights the potential benefits of incorporating macroalgae as a protein source in abalone feed formulations.

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