Effect of temperature increase on gametes release of *Holothuria scabra*

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Abstract. Temperature is an important factor that affects the spawning of sea cucumber, however, there is little information on the effects of temperature on gamete release. We evaluated the effect of temperature increase (+2, +4, +6, +8 and +10°C) on gamete release (proportion of individuals that release gametes, duration, and mechanism of gametes releases) of *Holothuria scabra*. No individual released gametes at control and at a temperature increase of + 2°C. The maximum number of individuals that released gametes occurred at a temperature increase of +4°C (33.33±4.54%), a significantly higher number than those exposed to temperature increases of +10°C and +12°C. Proportion of males that spawned was higher than females. The release of sperm occurs through three mechanisms, namely: (1) sperm release through the gonopore (temperature increase of +4°C, +6°C, and +10°C); (2) sperm release through the anus (temperature increase of +10°C and +12°C) and (3) sperm release along with the evisceration (temperature increase of +10°C and +12°C). Duration of sperm release at +8°C (35.00±11.16 minutes) was significantly higher than other groups. Eggs release takes place in seconds (2 to 4 seconds). Females started spawning approximately 60–82.50 minutes after the males.
Key Words: eggs, gametes, *Holothuria scabra*, sperm, temperature.

Introduction. *Holothuria scabra,* commonly known as sandfish, is an Aspidochirotid sea cucumber, a well-studied species (Agudo 2006; Hamel et al 2001; Purcell et al 2012). Sea cucumbers are exported to Asian seafood markets, primarily as a dried product called beche-de-mer. Sandfish is considered an economically important organism in East Asia and a global trade commodity due to its nutritional and medical value (Hamel et al 2001; Bell et al 2005; Purcell 2014; Xia & Wang 2015). The huge proportional increases in sale prices of *H. scabra* compared to export price underpin the intense demand for these species (Purcell 2014). The high commercial value and the increasing demand has intensified exploitation in the wild (Conand 2004; Purcell et al 2010; Sicuro & Levine 2011; Toral-Granda 2008). Global demand for sandfish, has led to signs of overexploitation reported in several countries (Purcell 2014). Therefore, the importance of sea cucumber aquaculture has increased considerably over the last few decades (Lovatelli et al 2004). Aquaculture could be a sustainable alternative to meet the current market demand (Anderson et al 2011; Bartley & Bell 2008) by reducing pressure on wild stocks and by providing restocking actions. Aquaculture production secured 25% of the supply in 2011 (Eriksson & Clarke 2015), however, this production can be mainly attributed to the increased aquaculture production of *Apostichopus japonicus* (94% in 2011), largely in China (Eriksson & Clarke 2015).

Currently, *H. scabra* is considered a promising mariculture candidate in the tropical region (Hamel et al 2001; Eriksson & Clarke 2015). Aquaculture appears to be a viable solution to overcome the wild exploitation for human consumption as food, as well as supplying the biomedical, pharmaceuticals and nutraceuticals sectors (Benkendorff 2009; Anderson et al 2011; Riani et al 2016). Moreover, hatchery-produced juveniles could be used to reconstitute wild breeding populations. Aquaculture of *H. scabra* has been applied in several countries such as Vietnam, India, Australia, the Philippines, New Caledonia, other Pacific regions, and Africa (James et al 1994; Pitt & Duy 2004; Agudo 2006; Bowman 2012; Juinio-Meñez et al 2012; Robinson et al 2013).

Sea water temperature is one of the most important variables for the aquaculture of sea cucumbers. This is due to the fact that sea cucumbers are ectothermal organisms (Li et al 2002; Dong & Dong 2006; Dong et al 2006) and their body temperature closely follows the ambient seawater temperature which in turn modulates most of their biochemical and physiological processes. Several studies reported that changes in seawater temperature can alter important parameters for the aquaculture of sea cucumbers, such as their feeding behavior, metabolism, growth and even their survival rate (Mercier et al 1999; Dong & Dong 2006; An et al 2007; Dong et al 2008; Ji et al 2008). Temperature affects the growth and physiological performance of sea cucumbers.

Sea cucumbers are intertidal species. As an intertidal species, the sea cucumber undergoes temperature fluctuations. Temperature fluctuations also occur when thermal shock is given during artificial spawning. Environmental temperature fluctuations affect the physiological system of sea cucumbers. Temperature fluctuations also influence sea cucumber growth (Dong et al 2006); very high or low temperatures inhibit growth and increase the incidence of sea cucumbers (Dong et al 2006; Dong et al 2008). Several studies show that temperature fluctuations can enhance growth of some aquatic organisms (Cox & Coutant 1981; Miao & Tu 1996; Sierra et al 1999; Zdanovich 1999; Dong et al 2006).

Related studies report that a drastic increase in temperature affects the biological activity of sea cucumbers such as *H. scabra* (Cheng & Chen 2000; Cheng et al 2004; Coates et al 2012), burrowing behavior (Mercier et al 1999; Wolkenhauer 2008), feeding activity (Mercier et al 1999), juvenile growth (Lavitra et al 2010), locomotor function (Purcell et al 2006; Wolkenhauer 2008), feeding period (Wolkenhauer 2008) and energy consumption (Kühnhold et al 2016). A Research by Sun et al (2018) showed that temperature shock affects feeding behavior, movement, and digestive physiology of *Apostichopus japonicus.*

There is a lack information on the effects of temperature regarding the spawning process, especially about the gamete release. Few studies reported that spawning in echinoderms such as *H. scabra* is triggered by changes in water temperature (Krishnaswamy & Krishnan 1967; Engstrom 1980; Himmelman 1980; Cameron & Fankboner 1986; Pearse et al 1986; Ramofafia et al 2000; Ramofafia et al 2003). Previous studies have indicated that males are more sensitive to temperature changes than females. A change in temperature can affect the physiological lipids, plasma membrane and sperm cell enzyme activity, and alter the sperm plasma composition of *Holuthuria scabra* (Dadras et al 2017). It also has an effect on sperm physiology (Mansour & Lahnsteiner 2012; Dadras et al 2017; Fenkes et al 2017). Males always release gametes before females.

Considering the above, it is important to understand the effect of temperature increase on gamete release of *Holothuria scabra*. Therefore, the main objective of the present study was to investigate the effects of temperature stress (i.e. temperature shock) on time, duration, and mechanism of gamete release of *H. scabra*.

Material and Method

Specimen collection. *Holothuria scabra* specimens were collected in April 2019 from Saleh Bay, Sumbawa district, West Nusa Tenggara, Indonesia (8°42'890''S, 117°47'882''E) (Figure 1). *H. scabra* are collected at night to avoid stress caused by high temperatures and are taken at low tide. Individual sandfish were placed in plastic bags with 0.5 l of seawater (salinity of 35‰), sealed and transported in containers. Each container held between 10 and 15 animals. Containers were transported by boat for 1-2 h. The average collection and transport interval to the laboratory was 8 hours.

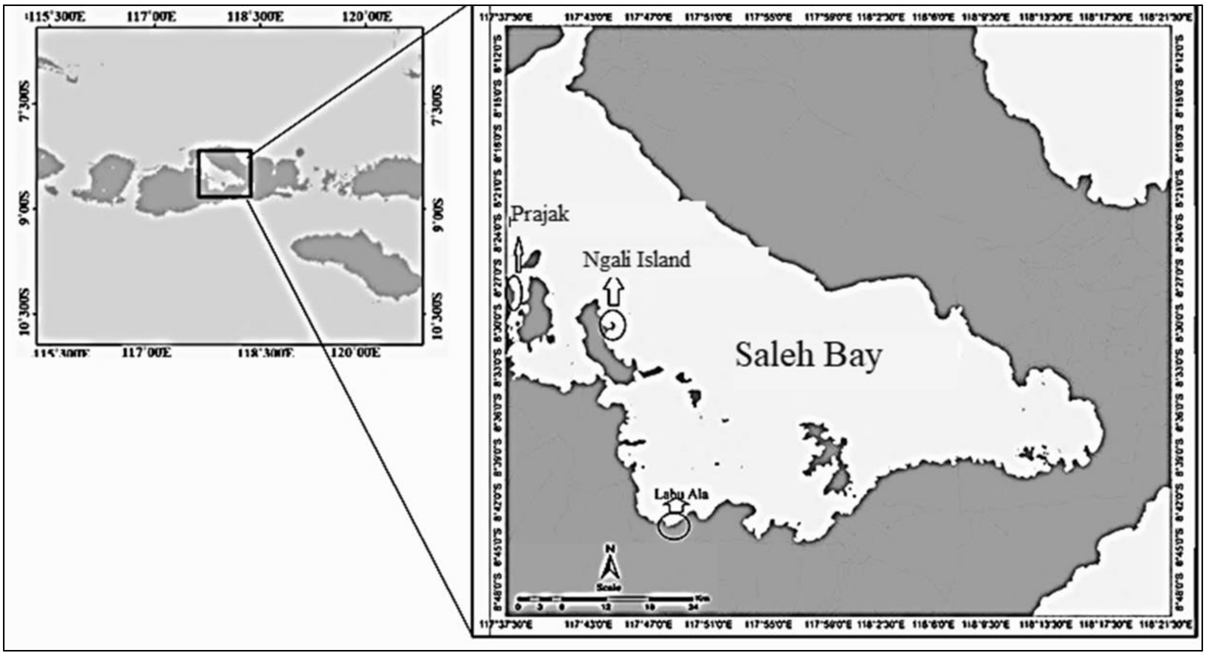

Figure 1. Location of sandfish (*Holothuria scabra*) collection.

***Experiment design**.* Healthy *Holothuria scabra* of similar size, with a contracted body length of 19.86±2.78 cm and a wet body mass of 329.81±32.73 g, were selected for the experiments. The specimens underwent a 12-hours acclimation at an ambient temperature of 27°C. After acclimation, sea cucumbers were arbitrarily collected and equally divided into experimental groups. For this study we chose six temperature increase treatments from ambient temperature (+2°C, +4°C, +6°C, +8°C, +10°C and +12°C) and a control treatment (at a temperature of 27°C). Each treatment contained three replications. Sea cucumber were randomly distributed into 21 containers (55×30×35 cm, water volume of 45 L) at different temperature increases with 18 individuals per container. After one hour, sea cucumbers are put into a container that has a temperature of 27°C. The mechanism, time, and duration of the release of gametes (sperm and eggs) were observed during the study. Observation lasts for 4 hours with check-ups every 30 minutes. Duration of gamete release was determined by observing the whole period between first release of gametes by an individual to the end of the gametes release process. The color of the sperm released by males was beige. The color of the eggs released by females was white. Seawater used in the experiment was filtered using a sand filter. During the experiment, seawater pH and salinity was controlled at 8.6±0.5 and 35‰ respectively.

***Statistical analysis**.* To evaluate the homogeneity and normality of the variances of the data, the Levene and Shapiro-Wilk tests were applied. Analysis was carried out using the SPSS version 24.0 statistics software. Where assumptions of normality and homogeneity of variances were fulfilled, one way-ANOVA test followed by post hoc multiple comparisons with Bonferroni test was used to compare significant differences on data. Where assumption of data was not fulfilled, the non-parametric Kruskal-Wallis test was used to compare significant differences on data. When significant differences were found, Mann-Whitney tests were used for multiple comparisons. The proportion of male and female (independent) did not fulfill the assumptions of normality and homoscedasticity, for which a non-parametric test was applied. The results are presented as means ± standard error (SE). The significance level for all statistical analysis was set at $p \leq 0.05$.

Results

Proportion of specimens releasing gametes (sperm and eggs). Results of this study showed that sperm and eggs release occur at temperature increases of +4°C, +6°C, +8°C, +10°C and +12°C. No individual released sperm and eggs at the control treatment and at a temperature increase of +2°C. The maximum number of individuals (male and female) that released gametes was observed at a temperature increase of +4°C (33.33±4.54%), with no significant difference when compared with +6°C and +8°C temperature increases (Mann-Whitney, p>0.05). However, the proportions of *H. scabra* that release gametes at +4°C and +6°C temperature increases were significantly higher than those of the +10°C and +12°C temperature increases groups (Mann-Whitney, p<0.05). The proportion of individuals that release gametes at +10°C and +12°C temperature increases (11.11±4.54%) was the lowest, significantly lower than that observed at +4°C and +6°C temperature increases (p<0.05), but no significant difference was observed with the +8°C temperature increase.

The highest proportion of males releasing sperm occurred in *H. scabra* exposed to a temperature increase of +4°C with 25.93%±3.2, followed by the temperature increase of +6°C (24.07±3.20) and +8°C (24.07±6.42). The lowest proportion of males that release sperms corresponded to the individuals exposed to temperature increases of +10 and +12°C (9.26±3.20, 9.26±6.41). Kruskal-Wallis test followed by Mann-Whitney test showed that there were no significant differences between +4°C, +6°C and +8°C and between +10°C and 12°C temperature increases (p>0.05). However, the proportion of males that release sperm in these three groups were significantly different with +10°C and +12°C groups (p < 0.05) (Table 1).

Table 1

Proportion of males and females that release gametes at temperature increase variation

Temperature increase	*Proportion of H. scabra releasing gametes (%)*	*Composition of males and females (%)*		*Ratio of Males and females*
		Male	*Female*	
Control	0.00	0.00	0.00	-
+2°C	0.00	0.00	0.00	-
+4°C	33.33±4.54	25.93±3.21	7.41±3.21	3.50:1.00
+6°C	31.48±6.93	24.07±3.21	7.41±6.42	3.25:1.00
+8°C	27.78±4.54	24.07±6.42	3.70±3.21	6.50:1.00
+10°C	11.11±4.54	9.26±3.21	1.85±3.21	5.00:1.00
+12°C	11±4.54	9.26±6.42	1.85±3.21	1.50:1.00

No significant difference in the proportion of females that release eggs was observed between the treatments of +4°C, +6°C, +8°C, +10°C and +12°C (Mann-Whitney test, p=0.304). Results in this study show that proportion of males that spawned was higher than the number of females that spawned. The ratio between females and males that release gametes ranges from 1:3.5 to 1:5.

Organs and mechanism for release of gametes (sperm and eggs). The results of this research show that the release of sperm occurs through three mechanisms, namely: (1) sperm release through the gonopore, which is in the anterior part of the body; (2) sperm release through the anus, which is in the posterior part of the body and (3) sperm release along through evisceration (Figure 2). In contrast to the release of sperm that occurs through these three mechanisms, the release of eggs in all treatments shows the same mechanism, namely through the gonopore in the anterior part of the body.

Sea cucumbers that release sperm during shock treatment at temperature increases of +4°C, +6°C and +8°C release sperm through the gonopore which is situated anteriorly. At +10°C and +12°C, the most sperm release occurs through the anus, situated posteriorly (Table 2).

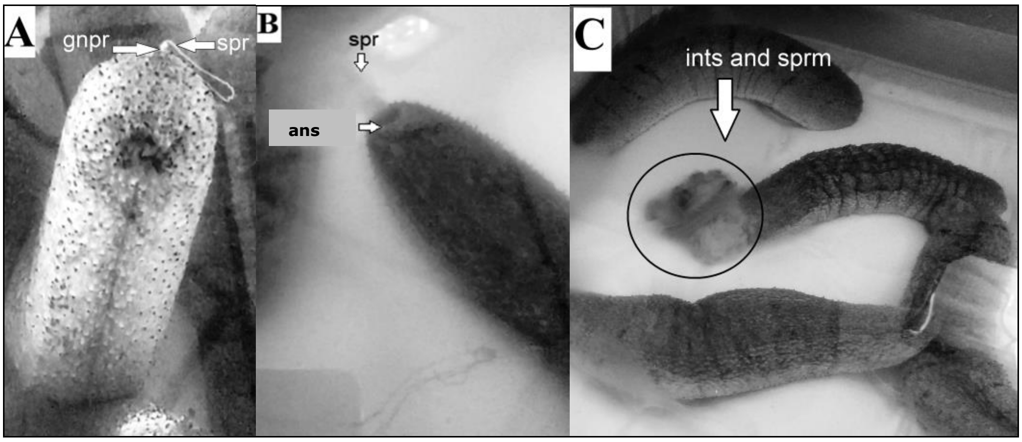

Figure 2. Mechanism of sperm release. A. sperm (spr) release through the gonopore (gnpr); B. sperm (spr) release through the anus (ans); C. sperm (sprm) release together with intestine (ints) (evisceration).

Table 2

Female (F) and male (M) gamete release mechanisms functioning at different levels of increased temperature

Temperature increase (°C)	*n*		*Proportion of individuals releasing gametes (%)*					
			Gonopore		*Anus*		*Evisceration*	
	F	*M*	*F*	*M*	*F*	*M*	*F*	*M*
control*	-	-	-	-	-	-	-	-
+2*	-	-	-	-	-	-	-	-
+4	14	4	100	100.00	0.00	0.00	0.00	0.00
+6	13	4	100	100.00	0.00	0.00	0.00	0.00
+8	13	2	100	100.00	0.00	0.00	0.00	0.00
+10	5	1	100	40.00	0.00	20.00	0.00	40.00
+12	5	1	100	0.00	0.00	40.00	0.00	60.00

Time and duration of gamete release. Kruskal-Wallis test showed that there was a significant difference ($p<0.05$) in the duration of sperm release among treatment groups. Duration of sperm release at a temperature increase of +8°C (35.00±11.16 minutes) was significantly higher than that at +4°C, +6°C, +10°C and +12°C (Mann-Whitney test, $p<0.05$). However, duration of sperm release at +10°C and +12°C (5.30±3.62 minutes, 3.00±1.54 minutes) was significantly lower than that at +4°C and +6°C ($p<0.05$) (Table 3). The duration of sperm release is also influenced by the mechanism of sperm release. The release of sperm through the gonopore has a longer duration than the release through the anus and through evisceration (Table 3). Sperm release through evisceration is the mechanism that has the shortest duration of sperm release compared to other mechanisms.

The duration of the release of eggs was shorter than the duration of the release of sperm. Egg release only takes place in seconds (2 to 4 seconds) and there is no significant difference among treatments. Females started spawning approximately 60–82.50 minutes after the males released the sperm. This powerful ejection dispersed the eggs much more widely relative to the streams of sperm released by the males.

Sperm release occurs most quickly at high temperature shocks of +10°C and +12°C. In both treatments, the sperm is first released in the first 30 minutes from the start of the treatment. At +4°C and +6°C temperature increase treatment, the release of sperm is slower than for the other treatments and it begins only between the 90^{th} and the 120^{th} minute from the start of the treatment (Figure 3).

Table 3

Duration of gamete release at temperature increase variation

Thermal shock (°C)	Duration of sperm release (minutes)				Duration of eggs release (seconds)
	Duration	Duration at variation mechanism			
		Gonopore	Anus	Evisceration	
Control	-	-	-	-	-
+2°C	-	-	-	-	-
+4°C	24.43±4.53 $^{n=14}$	24.43±4.53	-	-	3.50±0.57$^{n=4}$
+6°C	26.30±11.67 $^{n=13}$	26.30±11.67	-	-	3.25±0.50$^{n=4}$
+8°C	35.00±11.16 $^{n=13}$	35.00±11.16	-	-	2.50±0.71$^{n=2}$
+10°C	5.30±5.62 $^{n=5}$	8.75±0.35	4.25±0.35	0.5*	3$^{n=1}$
+12°C	3.00±1.54 $^{n=5}$	-	4.25±1.06	2.17±1.25	2$^{n=1}$

*=only one individual

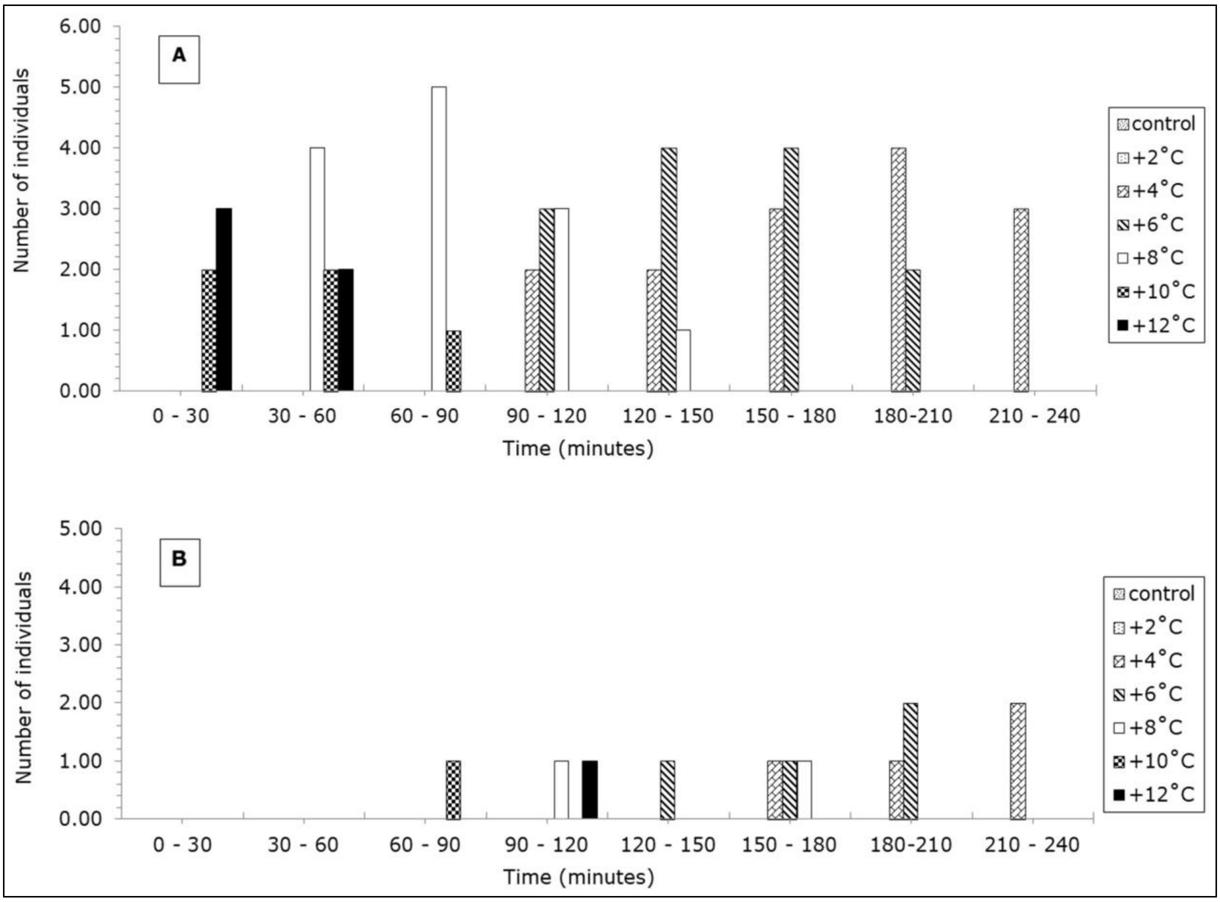

Figure 3 Number of individuals that released gametes at different temperature increases. (A): Number of males that released sperm; (B): Number of females that released eggs.

Discussion

Effect of temperature on the proportion of Holothuria scabra that released gametes. Water temperature is a crucial factor influencing the physiology of organisms (Kortet & Vainikka 2008; Muñoz et al 2015; Sinclair et al 2016). It has been shown that changes in water temperature can evoke acute or chronic stress in a variety of organisms. Temperature changes influence the growth rate, susceptibility, and the general health status of invertebrates (Hughes et al 2003; Cheng et al 2004; Purcell &

Simutoga 2008; Bowman 2012). Relatively small changes in the sperm thermal environment have many fundamental influences on the physiology and function of sperm (Mansour & Lahnsteiner 2012; Dadras et al 2017; Fenkes et al 2017). Temperature can affect the physiological state of lipids, the properties of plasma membranes and the activity of sperm cell enzymes, and alter the composition of sperm plasma (Dadras et al 2017).

The result of this present study showed that of *H. scabra* releases gametes at temperature shocks of +4°C, +6°C, +8°C, +10°C and +12°C. There were no individuals releasing gametes found in the control group and at a temperature shock of +2°C. There were a greater number of males spawning than the number of females. Several theories and research results state that the number of male individuals compared to females in releasing gametes is a strategy of males competing for the fertilization of a female's eggs (Hardege & Bentley 1997; Lamare & Stewart 1998; Marshall 2002; Marshall et al 2004). Sperm competition is probably intense for most male spawners.

The number of individuals releasing gametes in this study was higher than those from other related studies. Kumara & Dissanayake (2017) reported that the number of *H. scabra* individuals releasing gametes at a temperature shock of 3-5°C ranged from 0.00 to 26% with male and female compositions of 1.00:1.00 to 2.60:1.00. The least percentage of individuals of sea cucumbers releasing gametes have been found for other species of sea cucumbers such as *Athyonidium chilensis* (only 30%) (Guisado et al 2012) and *Holothuria leucospilota* (0.00% to 16.7%) (Huang et al 2018).

The difference in the number of individuals releasing sperm proves that temperature is particularly important regarding sperm release. The absence of individuals releasing gametes in the control group and at the temperature increase of +2°C shows that the temperature does not cause stress on the sea cucumber *H. scabra*. Temperature stress can stimulate spawning (James et al 1988; Mercier et al 1999; Morgan 2000; Battaglene et al 2002; Giraspy & Ivy 2005).

This study shows that the gonopores gradually open with an increase in temperature, which makes it easier for sperm to be released. Temperature also plays a role in increasing metabolism and enzymes work including enzymes that influence the release of sperm and eggs. Dadras et al (2017) reported that temperature influences the activity of sperm cell enzymes and an increase in temperature until the optimum value can improve the performance of the enzyme. An increase in temperature can also induce the maturation of sea cucumber gonads (Muthiga et al 2009; Guzmán et al 2003). The results of this study also showed that an increase in temperature of +12°C tends to decrease the number of sea cucumbers that release sperm. This is presumably because an increase in temperature of +12°C exceeds the optimum temperature for the release of sperm. Some research results show that the success of spawning of *H. scabra* only occurs at a temperature stimulation of 3-5°C (Agudo 2006; Ivy & Giraspy 2006; Kumara & Dissanayake 2017). The results of this study show that *Holothuria scabra* can be spawned by increasing the temperature with up to +8˚C.

Effect of temperature shock on time, mechanism, and duration of gametes release. In organisms that carry out external fertilization, males often release gametes before females (Guisado et al 2012). In this study, the distance between the time of release of sperm and the time of release of eggs ranged from 60 to 82.50 minutes. This proves that the release of eggs is slower than the release of sperm. Sperm release before the release of eggs for *H. scabra* is also evidenced by other research results (Ivy & Giraspy 2006; Purcell et al 2006). Sperm release before the egg has been commonly found in other sea cucumbers such as *Holothuria tubulosa* (Rakaj et al 2018), *Holothuria leucospilota* (Huang et al 2018), *Cucumaria frondosa* (Hamel & Mercier 1996), *Cucumaria lubrica*, *Cucumaria miniata* (McEuen 1988), *Holothuria poli* (Rakaj et al 2019). Sperm release by males has the potential to stimulate females to release eggs (Battaglene et al 2002). The length of egg release compared to sperm release is also thought to be due to females being less sensitive to temperature changes than males.

At a temperature increase of +4°C, +6°C and +8°C, all individuals released sperm through the gonopore located in the anterior part of body. However, at +10°C and

+12°C, it appears that the release of sperm is done through all three mechanisms, namely 1) release through the gonopore contained in the anterior part of the body, 2) release through the anus contained in the posterior part of the body and 3) release along with the process of evisceration. Sperm release through gonopores is a common mechanism in sandfish and other Holothuroidea. McEuen (1988) and Huang et al (2018) report that for Holothuroidea, sperm is excreted through the gonopore in the anterior part of the body. Sperm release through the anus is rarely encountered in sea cucumbers. In this study, sea cucumbers that release sperm through the anus are only present at temperature increases of +10°C and +12°C. The number of sea cucumbers releasing sperm through the anus at a temperature increase of +10°C is three individuals or 60% of the total males releasing sperm. At a temperature increase of +12°C, the number of males releasing sperm through the anus is 40% of the total males releasing sperm. The release of sperm through the anus was previously discovered by Hamel and Mercier (1996) for *Cucumaria frondosa*. Hamel and Mercier (1996) state that this happens in rare cases (1/60 for males, 1/200 for female). The results of this study proved that sperm release through the anus occurred at high temperature shock stimulation of +10°C and +12°C. Apart from the gonopores, sperm release also occurred with the process of evisceration.

The different mechanisms of sperm release have influenced the time and duration of sperm and egg release. At a temperature increase of +10°C and +12°C, sperm release is faster than that in other groups. At a temperature increase of +10°C and +12 °C, sperm release occurs from the first minute to the 60th minute. It is because some sea cucumbers release sperm through the anus and through evisceration. At a temperature increase of + 4 and +6°C, sperm release starts after 90 minutes and at a temperature increase of +8°C, the release of sperm begins after 60 minutes. The release of sperm in these three temperature shocks was slower than for the temperature shocks of +10°C and +12°C. This has been caused by the release of sperm through the gonopore.

Overall, the duration of sperm release ranges from approximately 3 to 35 minutes. The longest duration of sperm release was at a temperature increase of +8°C with (35±11.16 minutes). The shortest duration of sperm release was at +12°C (3.00±1.54 minutes). The long duration of the sperm release at the temperature increase of +8°C was due to the sperm release by all individuals carried out through the gonopore. The released sperm through the gonopore is in the form of small, long streams. The small flow of sperm out through the gonopore causes the release of all sperm of the sea cucumber to be slow (long duration). The duration of sperm release through the anus was shorter than the duration of sperm release through the gonopore. This may occur because the anal canal is wider than the gonopore, so that it is easier for sperm to pass through the anal canal than through the gonopore. The wide anal canal also causes large volumes of sperm to be excreted in a short time. Sperm that comes out through the anus looks like a burst of white smoke.

In contrast to the release of sperm which has a long duration and varied mechanisms, the release of eggs by the female is short and is only released through the gonopore. The strong spray of releasing eggs aims to disperse the eggs throughout the water. The spread of eggs in the waters can facilitate fertilization. This is caused by sperm cells that also spread in the water. The duration of the release of eggs is only in the range of 2 to 4 seconds.

The duration of the sperm and eggs release in *Holothuria scabra* in this study did not differ greatly with the duration of the sperm and egg release of other sea cucumbers. The results of the study by Rakaj et al (2018) reported that *Holothuria tubulosa* spawned with a shock temperature increase of 3-5°C and has a duration of sperm release of about 2 to 2.5 hours while the release of eggs has a very short duration, that is 4-5 seconds. Ivy and Giraspy (2006) reported that females excrete eggs in the form of strong sprays. The eggs that have been released sink to the bottom of the water later and look like grains that are yellow to orange.

Some research also shows that the release of sperm in organisms with external spawning shows that males tend to release gametes in a long duration compared to females. It is intended so that sperm can flow further in the water column (Thomas

1994; Marshall 2002; Marshall et al 2004). A longer duration of sperm release than of the eggs indicates that the number of sperm in one spawning is more than the number of eggs. The large number of sperm is one of the male competition strategies in fertilizing eggs (Bateman 1948; Parker 1990, 1993, 1998; Parker & Ball 2005). The long duration of sperm release is predicted as a strategy of aquatic organisms in supporting the success of spawning (Olito & Marshall 2019). Sperm released in the water have many obstacles to reach the egg, so as an effort to increase the success of spawning, large quantities of sperm is released (Levitan & Petersen 1995; Yund 2000).

Conclusion. *Holothuria scabra* displayed sensitivity to a temperature increase ≥4°C from ambient temperature (27°C). Temperature increase up to +8°C can be tolerated by *Holothuria scabra* and it triggers gamets (sperm and eggs) release. New discoveries in this study are that *Holothuria scabra* at an increase in temperature ≥10°C can release sperm through the anus. Thermal shock >8^{o}C causes a high evisceration rate of *Holothuria scabra* and is not recomended for artificial spawning.

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