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Factors Influencing the Prospect of Asexual Reproduction in *Holothuria Arenicola* (Semper, 1868)

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Research Article

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Abstract

Asexual reproduction by transverse fission was studied in populations of *Holothuria arenicola* through investigation of different factors affecting the fission and survival rates. Size, fission position, and temperature impacts were experimentally assessed. It was observed that higher fission rate was recorded in small specimens as compared to large ones. The fission position at 30% yielded higher fission rate (90%) as compared to that obtained in 45% fission (70%). Temperature showed a considerable effect on either fission or survival rate with a direct proportional relationship between temperature and both rates. At 20°C, fission started in the 2nd day to be at rate of 20% which was gradually increased until reaching 100% by the 8th day; sharp decline of survival rate was occurred reaching 35% by the 9th day. At 25°C, fission reached 70% by the 8th day; the survival rate became constant at 50% from the 17th day onward. At 30°C, the highest fission rate was attained in the 4th day but the survival rate was zero in the 5th day. It was noted that the wound-healing period took longer time at 30°C than at 20°C and 25°C. Additionally, changes of anterior parts (A) and posterior parts (P) internal organs were followed weekly over two months after fission induction to detect both of the dissolved structure and regenerated one.

Keyword: *Holothuria Arenicola*; Asexual reproduction; Fission rate; Fission position; Temperature; Regeneration

Introduction

Populations of sea cucumber are being overfished worldwide. Some studies indicate that sea cucumber populations in overexploited fishing grounds may require as many as 50 years in the absence of fishing pressure to rebuild [1-3]. The rapid decline in sea cucumber populations worldwide to support the beche-de-mer market [4] has led to the start of fishing activities in the Egyptian Red Sea coast in late 1996 and in Egyptian

Mediterranean waters in 2000. Several years later, a severe depletion in sea cucumber stocks took place in many areas. This depletion occurred in populations of all sea cucumber species of different economic values. The loss of critical stocks of sea cucumber is likely to have a significant impact on the ecosystem condition and the adjacent marine environment, as a whole [5]. Therefore, there is an urgent need for developing strategies for managing and enhancing the sea cucumber natural stocks.

Possible approaches to enhance and increase yield of sea cucumber stocks may include induced asexual reproduction through fission [2]. Inducing fission in order to double individual numbers would be a promising alternative for holothurian re-population, as suggested by Lokani, et al. [6]. In addition to doubling of a sea cucumber stock, its advantages are: inducement can be done on any number of individuals; survival rates can be very high, as the produced individuals are already adapted to the habitat and begin growing from a relatively large size; threats (predation, etc.), which normally occur during the larval and juvenile stages, are reduced; and costs and technology are low [7].

The most important and abundant holothurian sea cucumber species in Egyptian Mediterranean coast is *Holothuria Arenicola* [8]. Researches on *H. arenicola* are scarce worldwide, Mosher [9] studied its distribution in the Bahamas with observations on habitat, behavior, and feeding activity, Tahera and Tirmizi [10] recorded in Pakistan, Iran [11]. Also, the chemical composition of *H. arenicola* was studied [12]. In Egypt, few published studies are available on *H. arenicola* distribution [8]; asexual reproduction [13,14], extraction of bioactive material [15], and captive spawning [16].

The present work aims to study the factors affecting the asexual reproduction yield of *H. arenicola* to provide the optimum conditions needed for attaining the best fission and survival rates.

Materials and Methods

The collected specimens were maintained in fiberglass tanks (300L) with a thin layer of fine sand on the bottom. The tanks were aerated with an air-blower. Water salinity was 39 ppt. The ambient temperature was 26 ± 1°C. Water in tanks was changed once daily. Specimens allowed being adapted for one week before conducting experiments.

Prior to fission inducement, samples were anaesthetized in a magnesium chloride (2.5%) solution, and then total length (TL) and total weight (TW) were measured for each sample. Asexual reproduction was induced through tightening up the animal's body with rubber bands. The rubber was placed tightly around each animal. Rubber bands were frequently checked and replaced since specimens sometimes managed to rid themselves of rubber bands. Constricted samples were reared in small glass tank filled with fresh, filtered and slowly aerated seawater. Water was fully changed twice each day. No food was added.

The number of divided, undivided and dead samples was reported daily. The percentages of fission and survival rates were calculated for each size group.

$$\text{Fission Rate} = N / T \times 100$$

Where,
N = number of divided specimens
T = total number of specimens

Survival rate was calculated as reported by Reichenbach and Hollway [17] as follow:

$$\text{Survival Rate} = [(A+P)/ 2T] \times 100$$

Where,
A+P = number of the anterior parts (A) and posterior parts (P)
T = total number of specimens that had undergone fission

Study of Size Effect

The experiment was investigated to assess the effect of animal size on fission and survival rates. Specimens were separated into two size groups, each group was held in separate tank. The first group (small size) was ranged from 8.6cm to 10.2 cm TL and from 17.56 to 45.27g TW. The second group (large size) was in range of 11.9-15.1cm TL and range of 39.96 to 74.42g TW. Experiment was conducted in winter and summer. Samples were fitted with rubber bands at 45% of body length (from anterior end). Temperature was kept at 26 ± 1°C. The experiment lasted for 30 days.

Study of Fission Position Effect

The effect of fission position on fission efficiency and survival rate was determined at two levels.

Samples at range of 9.4 to 12.3cm TL and 51.3 to 53.44g TW were fitted at 45% of the total body length (from anterior end) with rubber bands. Samples ranged from 8.2 to 10.8cm TL and 31.26 to 43.62g TW, were induced to asexual reproduction at 30% of total length (from anterior end). Both of fission and survival rates were determined over 30 days.

Study of Temperature Effect

The environmental conditions affecting the fission and survival rate were studied in terms of temperature effect in summer. Twenty specimens were placed in 20, 25 and 30°C for 2 days prior to fission inducement. Twenty samples at 20°C were at range of 10.2 to 14.7cm TL and range of 36.65 to 69.11g TW. The sample group at 25°C ranged from 11.9 to 15.1 cm TL and from 39.96 to 74.42g TW. The range of samples at 30°C was 9.4 to 15.3 cm TL and 26.78 to 66.96g TW.

30°C was achieved by using the aquarium thermostat heaters, while 20°C and 25°C were achieved by conducting the experiment in air conditioned room. Samples were fitted with rubber bands at 45% of body length (from anterior end). The experiment lasted for 30 days.

Study the Difference in Anatomy before and after Fission Induction

To investigate the fission effect on the internal organs, dissection was performed in normal specimens and divided parts (anterior and posterior parts) resulted from fission induction. Both of anterior and posterior parts were noted over two months to determine the time of anus and mouth formation in anterior and posterior parts, respectively. TL and TW were measured weekly for anterior and posterior parts. Weekly dissection was done for specimens of anterior and posterior parts to follow the organ absorption and regeneration.

Results

Study of Size Effect

The size was noted to have an effect on fission and survival rates. Fission and survival rate were significantly different between small and large size samples ($P < 0.05$). Higher fission rate was recorded in small specimens as compared to large ones. The survival rate in small specimens (8.6-10.2 cm TL and 17.56 - 45.27g TW) was ranged from 42 to 75% (Figure 1). Lower range of survival rate was noted in large specimens (11.9-15.1cm TL and 39.96 - 74.42g TW) to be from 25 to 54%.

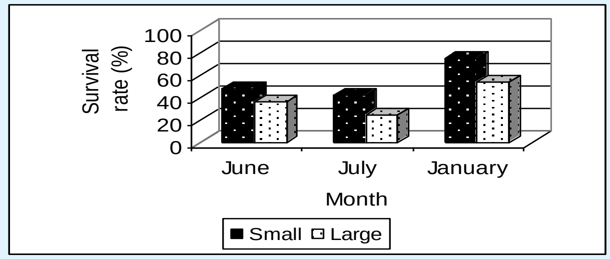

Figure 2: Effect of size on survival rate (%) of Holothuria arenicola divided parts resulted from fission induction.

Regardless of specimen size, the highest fission and survival rate were obtained in winter (January) while the lowest were in summer (June and July). The wound healing period was ranged from day to two days in both of small and large sized specimens. Fission lasted for 6 days (after fission induction) in summer while it took only 4 days in winter (Figure 2).

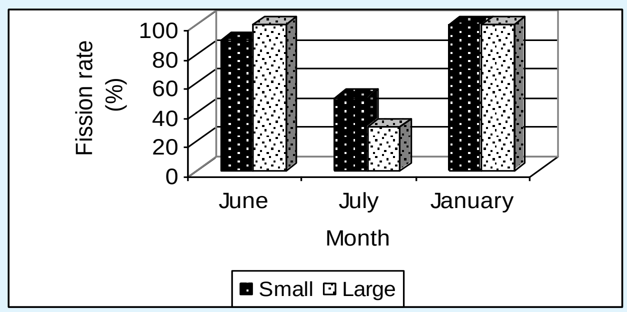

Figure 1: Effect of size on fission rate (%) of Holothuria arenicola divided parts resulted from fission induction.

Study of Fission Position Effect

Fission position was noted to have a considerable effect on both of fission and survival rates. There was a significant difference in fission and survival rates between 30% and 45% fission position ($P < 0.05$). The fission position at 30% yielded higher fission rate (90%) as compared to 70% fission rate obtained in 45% fission. The fission duration was five days in both specimens fissioned at 30% and 45% fission position (Table 1). The survival rate of divided parts resulted from fission at 30% and 45% fission position was 81% and 50%, respectively. The survival rate of anterior and posterior parts was 61% for each by the end of experiment.

Month	Studied Parameter		Av.TL (cm)	Av.TW(g)	Fission Duration (days)	Fission (%)	Survival (%)
January			8.5±1.5	25.48±1.63	6	100	64
April	Fission position	45%	10.3±1.82	48.66±2.85	5	70	61
		30%	9.3±2.4	39.22±1.69	5	90	61
June			11.5±0.85	50.23±1.23	6	90	48
July	Size	Small	9.7±1.03	32.06±2.16	6	50	42
		Large	13.3±1.9	48.3±1.65	6	30	25
July	Temperature	20°C	13±2.3	51.84±1.89	8	100	30
		25°C	13.3±1.28	48.3±1.96	8	70	50
		30°C	12±1.49	40.7±1.65	8	90	0

Table 1: Fission duration, fission rate and survival rate of Holothuria arenicola under different conditions.

Study of Temperature Effect

Temperature showed a considerable effect on either fission or survival rate. There was a significant difference in fission and survival rate between 20, 25 and 30°C ($P < 0.05$). It was observed that as temperature decrease, the fission duration decrease. The survival rate became constant by the 15th and 19th day in 20 and 25°C, respectively.

At 20°C, no fission was observed in the 1st day after fission induction. Fission started in the 2nd day to be at rate of 20% which was gradually increased until reaching 100% by the 8th day (Figure 3). Two or three days were required for complete wound healing. 100% survival rate was attained until the 5th day after fission induction. Afterward, sharp decline of survival rate was occurred reaching 35% by the 9th day. Onward, survival rate was remained stable with 30% until the end of experiment (Figure 4). Most of recorded mortality was for specimens eviscerated from the constricted point.

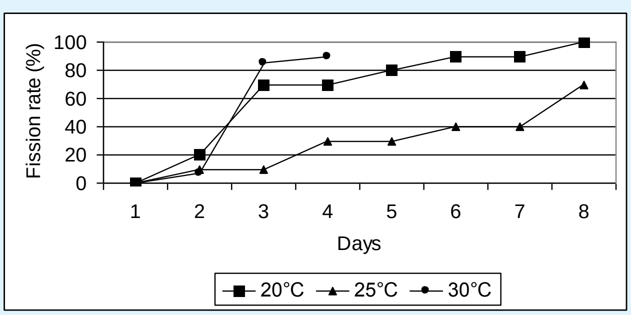

Figure 3: Effect of different temperatures on fission rate of Holothuria arenicola divided parts resulted from fission induction.

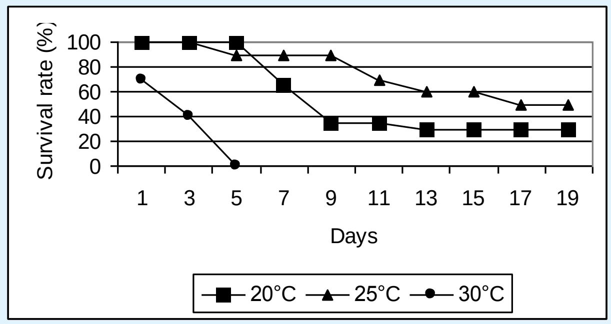

Figure 4: Effect of different temperatures on survival rate of Holothuria arenicola divided parts resulted from fission induction.

At 25°C, the trend of survival rate was similar to fission rate. Fission was started in the 2nd day to be at rate of 10% which was gradually increased until reaching 70% by the 8th day (Figurer 3). The survival became constant at 50% from the 17th day onward. The wound healing period ranged from 1-2 days.

At 30°C, the survival rate was sharply declined from 70% in the 1st day (after fission induction) to zero in the 5th day (Figure 4). Within the four days after fission induction, the highest fission rate was recorded in 30°C. Most of specimens were eviscerated at the constriction point. Wound healing was observed in few divided specimens which dead by the 5th day. In 30°C, the wound-healing period took longer time than at 20°C and 25°C.

Study the Difference in Anatomy before and after Fission Induction

Before Fission Induction: *H. arenicola* with dorsal and ventral views was illustrated in Figure 5. The body is slender, elongate, and tapered toward both ends. It is brown with contrasting flecks of darker brown and black color. The tegument of *H. arenicola* is thick, smooth and has a tendency to disintegrate rapidly. The weight of the tegument (muscle included) is ranged between 24.94 and 38.96gm, and it is 3 mm thick. Its mouth is located at the anterior end of its body, and is surrounded by 20 small, yellowish, transparent tentacles. Tentacles (averaging 0.7cm in length) are used to collect food. Small, cylindrical tube feet are scattered across the body, but are denser on the lateral margins and the ventral surface. Toward the anterior and posterior ends, the tube feet are found on irregular warts. Two longitudinal rows of 20 dusky blotches often run along the dorsal surface.

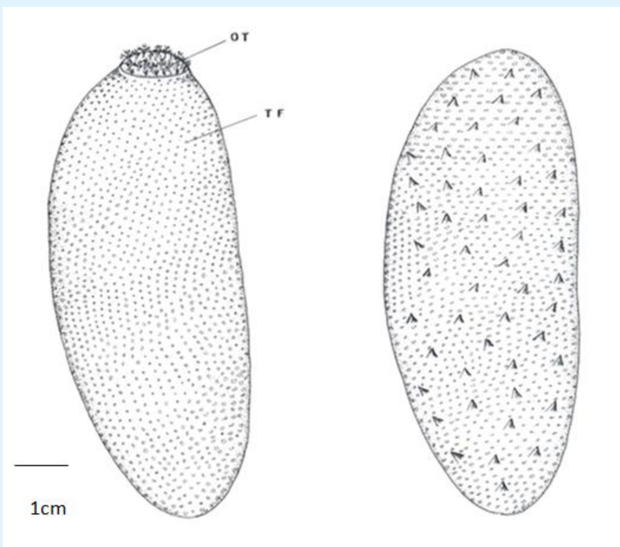

Figure 5: *Holothuria arenicola*. A- ventral view. B- Dorsal view showing (OT) oral tentacles, (TF) tube feet. Scale bar represent 1cm.

The muscles of normal specimens characterize by five pairs of longitudinal muscular bands that attach from the peripharyngeal calcareous ring up to cloaca. Each muscular band has width ranged from 2-3 mm and is attached to the tegument in its median part (Figure 6). The average length of the digestive tract is 9.5 cm. The gonads are in the form of one tuft of branched tubules. The gonad weight was in range of 3-7.3gm**.** The respiratory system is formed from two branched tubes called respiratory trees, on both sides. They opened into cloaca and extend into the coelomic cavity.

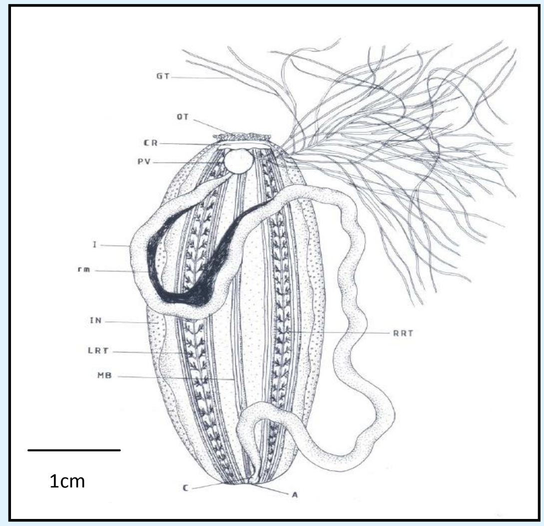

Figure 6: Dissection of Holthuria. arenicola specimen before fission induction showing (A) anus, (C) cloaca, (CR) calcareous ring, (GT) gonad tubules, (I) intestine, (IN) integument, (LRT) left respiratory tree, (MB) muscle band, (OT) oral tentacles, (PV) polian vesicle, (rm) rete mirabile, (RRT) right respiratory tree. Scale bar represents 1cm.

After Fission Induction:

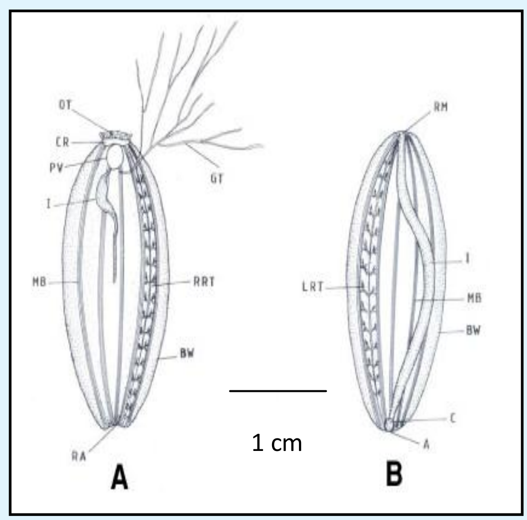

Figure 7: Dissection of Holothuria arenicola after fission induction. A-anterior part, B- posterior part. (A) anus, (BW) body wall, (C) cloaca, (CR) calcareous ring, (GT) gonad tubules, (I) intestine, (LRT) left respiratory tree, (MB) muscle band, (OT) oral tentacles, (PV) polian vesicle, (RA) regenerated anus, (RM) regenerated mouth, (RRT) right respiratory tree. Scale bar represents 1cm.

I-The anterior part (A)
The anterior part (A) has very few organs. The only ones remaining are the mouth area and its appendages; the gonads (if any); part of the intestine; polian vesicle, and right respiratory tree (Figure 7A).
B-The posterior part (P): The posterior parts (p) are characterized by presence of the short digestive tract, and left respiratory tree (Figure 7B).

After Two Months after Fission Induction: Changes of internal organs were followed weekly after fission induction through dissection of anterior and posterior parts over two months to detect both of the dissolved structure and regenerated one. The data were summarized in Table 2 over eight weeks.

Time after fission (Week)	Anterior					Posterior				
	DT	LRT	RRT	A	S.R (%)	DT	LRT	RRT	M	S.R (%)
2	*+	-	*+	-	58	*+	*+	-	-	100
4	*+	-	*+	-	58	-	*+	-	-	100
6	-	-	*+	+	48	-	*+	-	+	60
8	-	-	*+	+	48	-	*+	-	+	60

Table 2: Survival rate and changes of internal anatomy of *Holothuria arenicola* after fission induction over eight weeks.
(*+) indicates the remaining structures after fission; (-) indicates that structure are dissolved or not yet regenerated, and (+) indicates that the structure is regenerated; (DT) digestive tract; (LRT) left respiratory tree; (RRT) right respiratory tree; (A) anus and (M) mouth.

A-The anterior part (A): The anterior part (A) dissolves most of the remaining organs (intestine, gonad) after three weeks after fission, and then regenerates them, probably through materials left over after dissolution. The right respiratory tree and polian vesicle are kept from dissolution. The anal area started to regenerate on 28th day after fission.

B-The posterior part (P): The posterior part regenerates the missing organs (the oral area; gonads; polian vesicle; *rete mirabile*; digestive tract, and right respiratory tree). It keeps only the left respiratory tree from dissolution. The beginning of mouth area regeneration commenced at the same time of anal area regeneration, on 28th day.

Discussion

Holothurians are known for their ability to reproduce asexually by fission. Asexual reproduction in sea cucumbers by fission has been studied by several researchers [18-27]. In the present study, *H. arenicola* was successfully induced to asexual reproduction by using rubber bands as simple effective technique to induce transverse fission. *H. arenicola* small specimens at range of 8.6-10.2 cm TL and 17.56 - 45.27g TW showed higher survival and fission rate than in large specimens at range of 11.9-15.1cm TL and 39.96 - 74.42g TW. In other words, the fission ability increases as the animal size decrease. This finding is in agreement with Uthicke [28] who suggested that the body size of sea cucumber could have an effect on the likelihood of asexual reproduction taking place. Similarly, smaller specimens of *Holothuria fuscogilva, H. nobilis, Actinopyga mauritiana*, and *Stichopus variegatus* (both anterior and posterior parts) had higher survivorship and shorter regeneration times relative to adult animals of the same species [23,25] reported that the survival rate of *Actinopyga mauritiana* of the small size group (average length 12 cm and average weight 130 g) was 75 %, while the survival rate of the large size group (average length 19 cm and average weight 330 g) was 58%.

Regardless of specimen size, both of fission rate and survival rate were observed to be decreased from June to July and attained the highest percentage in January. This finding can be interpreted by the inversely proportional relationship of fission rate and survival rate with ambient temperature. As temperature increased from June to July, there was a decrease in fission rate (36-48%) and survival rate (90-100%) in June to (25-42%) and (30-50%) in July, respectively. While as temperature decrease in January, high values were recorded for both of fission (100%) and survival rates (54-75%). Further confirmation for this suggestion achieved by comparing the present results with others obtained previously in study of fission induction of *H. arenicola* in Mediterranean Sea in November [13]. They reported 63.3% and 63% survival rate and fission rate, respectively. Moreover, the fission duration was noted to be decreased from 6 days in summer (June and July) to 4 days in winter (January). Similarly, Laximinarayana [26] reported that asexual reproduction in nature is a seasonal event mainly occurring in winter in natural populations. Although the timing differed among different species, fission is a winter phenomenon for all [29]. Therefore, it can be concluded that the efficiency of fission process in *H. arenicola* and rate of survival of divided parts are temperature related.

Moreover, food availability may influence the occurrence of asexual reproduction. During the cold winter months, bacterial, detritus and diatoms, which some holothuroids feed on, are generally less abundant. Since sea cucumbers cease to feed during the period of regeneration, reduced food availability may be a signal that fission should take place [24].

Concerning fission position, Reichenbach and Holloway [17] induced fission in eight holothurians species. Their results showed a higher survival rate when the rubber bands were placed on the animal's natural fission plane, as in *Stichopus chloronotus,* the tegument, as well as the width of the muscles and their attachments marked the area being regenerated [30]. In the present study, it was found that the survival rate of anterior and posterior parts after fission at 30% of total length was significantly higher than that at 45%. Therefore, it is concluded that the position of the split in *H. arenicola* is in 30% of the total length. Similarly, fission in *H. leucospilota* occurs at 20–33% of its body length when measured from the anterior end [31-33]. However, fission occurred at about 52 % of the total length in *Stichopus chloronotus*, since anterior specimens were slightly larger than posterior ones [30]. The survival rate of posterior parts in *H. arenicola* was significantly higher than that of anterior parts regardless of the studied parameter. This indicates that posterior part has high regenerative ability than anterior one. Likewise, Kilada, et al. [13] reported the survival rate of *H. arenicola* as 90% and 40% for posterior and anterior parts, respectively over three weeks. This was similarly reported in *H. fuscogilva, H. nobilis, Actinopyga mauritiana*, and *Stichopus variegates*, since posterior parts had similar or higher survivorship relative to the anterior parts [23]. Further confirmation for the present obtained result was achieved through studying the survival rate of *H. arenicola* over 75 days, none of anterior parts still alive and only 16% of posterior parts survived. These findings mean that the posterior parts have the ability to regenerate to whole animals, while anterior parts have low or no potential for regeneration. As wise, Reichenbach and Holloway [17] reported that both of *Stichopus chloronotus* and *Actinopyga mauritiana* regenerate only the posterior parts into whole animal. Also, *Stichopus variegatus* was able to regenerate only the posterior part into a whole animal in around 100 days, with 0% survival of the anterior parts and 80 % survival of the posterior parts [23].

Because of the great critical effect of temperature among other environmental parameters on asexual reproduction, temperature effect was investigated experimentally on the entire fission process. Total mortality in 30°C could be considered as sign of the intolerable thermal stress. Also, higher fission rate in 20, 30°C than in 25°C may be related to the thermal stress. This interpretation is supported by Uthicke [28] who suggested that stress resulted from unstable habitat, including large fluctuations in water temperature, salinity and food sources may lead to an increase in mortality rates.

There are some conflictions between observations obtained from fission induction in cold month (20°C) and others recorded from fission induction in low temperature (20°C). First, the fission duration was four days in January when the ambient temperature around 20°C, while the fission duration under laboratory controlled temperature (20°C) was 8 days in the experiment done in June. Second, it was noted that higher fission rate correspond to higher survival rate, while at 20°C, high fission rate and low survival rate were observed. These two conflictions in addition to high survival rate in January could be attributed to physiological state of animal in which its energy was completely directed toward sexual reproduction during the summer season, not for fission and regeneration. As the spawning season end, gonads resorbed, temperature decreased, and energy redirected to binary fission and regeneration in winter. This hypothesis is supported by the fact that sexual reproduction has metabolic costs. It takes energy to generate and ripen gametes, as well as to release them. In most invertebrates, at least half of the total energy taken in is diverted into gamete production [34]. If the environmental conditions were not favorable for embryonic development, it is best not to waste this energy when nothing may come of it. Hence, it was suggested that high fission at 20°C was a result of thermal stress more than being a reaction to similar environmental condition of asexual reproduction period. Moreover, results at 25°C were the highest in survival rate with the shortest wound healing period as compared to 20°C and 30°C because of exerting only fission induction stress under ambient temperature rather doubling stress (fission induction and thermal stress) at 20°C and 30°C.

It is concluded that the optimum conditions for *H. arenicola* high asexual reproduction efficiency are: fission induction in small size specimens in range of 8.6-10.2 cm TL and 17.56 - 45.27g TW; fitting the rubber band at 30% from the anterior end; conducting the fission induction in winter season or in summer (at 25°C laboratory temperature), and start feeding the fissioned parts by the day 94th. Further investigations are necessary to substantiate the current findings together to assess the fission induction as alternative for *H. arenicola* re-population.

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