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# PRELIMINARY STUDIES ON INDUCED ASEXUAL REPRODUCTION IN *HOLOTHURIA ATRA* AND *HOLOTHURIA PARDALIS* UNDER LABORATORY CONDITIONS

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**ABSTRACT:** Asexual reproduction was observed in *H.atra* and *H.pardalis* during 20th November 2019 to 20th February 2020. Asexual reproduction is a common phenomena in sea cucumbers (class Holothuroidea) and organisms are capable of regenerating completely or partially after fission and evisceration. The present research is a first attempt/trial on induce asexual reproduction in *H.atra* and *H. pardalis* under the laboratory conditions in Pakistan. The induced asexual reproduction is of high significance in aquaculture of holothuria and could lead towards commercial production. During the last two decades sea cucumber fishing showed a trend of overexploitation because they are easy to catch besides high market demand. Many countries including Australia, Papua New Guinea, Mauritius and Venezuela have imposed prohibition on sea cucmber fishing due to depletion of population in wild. The findings from present research will be substantive addidtion to the knowledge in the field of aquaculture and will be valuable to marine scientist and aquaculturist in their future research.

**KEYWORDS:** asexual reproduction; holothurian species; laboratory conditions

## INTRODUCTION

Holothurians are significant for commercial fishery and aquaculture. Approximately 66 holothurian species are commonly exploited throughout the world (Conand, 2008; Uthicke *et al.*, 2010). The loss of sea cucumber stocks is likely to have a significant negative impact on the ecosystem and the adjacent marine environment as a whole. Therefore, there is an urgent need for intensive studies of the biology, culture and fishery management of sea cucumbers species (Razek *et al.*, 2007).

Asexual reproduction is a common phenomena in sea cucumbers (class Holothuroidea) and organisms are capable of regenerating completely or partially after fission and evisceration. Sea cucumbers are referred as one of the most curious marine organisms having numerous distinctive morphogenetic properties. They are known for autotomy being capable to restore their lost organs after evisceration or asexual reproduction.

Most holothurian species which have adopted asexual reproduction follow the "twisting-and-stretching" mode of fission (Emson and Wilkie, 1980): the anterior and posterior sections slowly rotate in opposite directions, resulting in a constriction of the body. There are numerous studies by researchers who conducted experiments on "Artificial fission" in holothurians, which is a division of an organism into two parts by constricting

the body with a rubber band. By placing rubber band on the middle of the body is a simple technique to induce transverse fission.

The phenomena of asexual reproduction is of high significance in aquaculture and could lead towards commercial production hence need to be studied in depth on the regeneration and survival rates to develop a successful technique. This could be a milestone in the marine aquaculture market along with curtailing the negative impact of overexploitation and conserving the wild stock.

According to the Dolmatov, (2014) family-holothuroidae as being capable of asexual reproduction. Few studies on asexual reproduction of *H. atra* were reported by (Laxminarayana, 2006; Purwati *et al.,* 2009; Bonham and Held, 1963; Harriott, 1982; Conand and Ridder, 1990; Chao *et al.,* 1993; Conand, 1996; Boyer *et al.,* 1995; Jaquemet *et al.,* 1999; Lee *et al.,* 2008; Thorne *et al.,* 2013). *Holothuria atra* and *Holothuria pardalis* found abundant on the coast of Karachi. These species earlier reported from Pakistan coast by (Tahera, 1992; Ahmed and Ali 2014). Ahmed *et al.,* (2018) reported first time a-sexual reproduction by fission in *Holothuria (lessonothuria) verrucosa* (Selenka, 1867) from coastal waters of Karachi, Pakistan.

The aim of present study was to observe the asexual reproduction in sea cucumber by induce fission under laboratory conditions as no such data / studies available from Pakistan. Hence this is a first attempt/trial on induce asexual reproduction in *H. atra* and *H. pardalis* under the laboratory conditions. The results would provide in depth information on developing techniques for induced asexual reproduction under controlled conditions. These research techniques will be important and useful to develop cultivation technologies for commercial aquaculture and to curtail the fishing pressure on the natural stock/population.

## MATERIALS AND METHOD

Asexual reproduction was observed in *H. atra* and *H. pardalis* during 20$^{th}$ November 2019 to 20$^{th}$ February 2020. Twenty (20) specimens were collected from Buleji (24°50'20.41" N, 66°49'24.15" E) on 30$^{th}$ of September 2019 during low tide (-0.07m). Specimens were shifted to the laboratory by keeping them in polythene bag filled with sea water and supplied with aeration. Asexual reproduction of *H. atra* (N=10) and *H. pardalis* (N=10) was induced by fitting rubber bands in middle portion of the body (Abdel Razek, 2007).

The physicochemical parameters such as water temperature (℃), salinity and pH were measured by multi-parameters. For taxonomic studies and identification, morphological features were examined and microscopic studies were conducted. Ossicles were taken from three positions (dorsal and ventral body walls, and tentacles); wet mounts were prepared by placing a small piece of skin tissue on a slide and adding a few drops of 3.5% bleach, and the slides were then rinsed with drops of distilled water. The slides were examined under a microscope at 10 × 10 magnification. For observation of asexual reproduction of *H. atra* and *H. pardalis* was induced by fitting rubber bands of the middle portion of the body. Specimens were kept in glass aquaria and fiber glass tanks with a thin layer of fine sand on the bottom. Water salinity was maintained on 37 ppt.

Experiment was conducted for 90 days and water quality was monitored and maintained. Microphotography was also performed using a Digital camera (Fujifilm 16 MP).

## RESULTS AND DISCUSSION

Two specimens of *H. atra* and *H. pardalis* were collected from Buleji coast dated 30th September -2019. The specimens were collected during low tide from intertidal zone. The length and weight of the specimens were (*H. atra* = L: 21 cm; W: 78 gms and *H. pardalis* = L: 11.5 cm; W: 19gms). The experiment was conducted for 90 days and induced asexual reproduction was observed in *H. atra* and *H. Pardalis* by fitting rubber bands in the middle portion of the body. Experiment initiated on 20th November 2019 and ended on 20th February 2020. During the experiment water quality was closely monitored maintaining temperature range (17-27$^{O}$C), salinity (37ppt), pH (8) (Fig. 3).The Physico-chemical parameters from Buleji were presented in Table 1.On 1st to 2nd day the specimens were in stress body was more constricted at the constriction point. The posterior part was swollen and extended. Posterior and anterior parts rotated in opposite directions (Fig. 1: A-F and Fig. 2: A-E). Both specimens showed continuous twisting in the opposite directions and fission point was very clearly seen. On 5th day *H. pardalis* completely divided into two parts, (a=anterior); length 8 cm and (p=posterior); length 3.5 cm. (Fig. 2: F). Fission occurs in *H. Atra* on day 8. (a=anterior); length 14.5 cm and (p=posterior); length 7.5 cm. (Fig. 1: G-H).

It was observed that after fission specimen (p) were more active than specimen (a). Active (p) specimen were moving towards the wall of aquarium while, specimen (a) were stick on the bottom of the aquaria. On day 29th to 38th the wounds got healed in both the specimens. In *H. pardalis* the formation of tentacles outgrowth was observed on day 34th (Fig. 2: G) and anus development in specimen (a) was observed on day 46th (Fig. 2: H). In *H. atra* the formation of tentacles outgrowth was observed on day 47th (Fig. 1: I) and anus development in specimen (a) was observed on day 58th (Fig. 1: J).

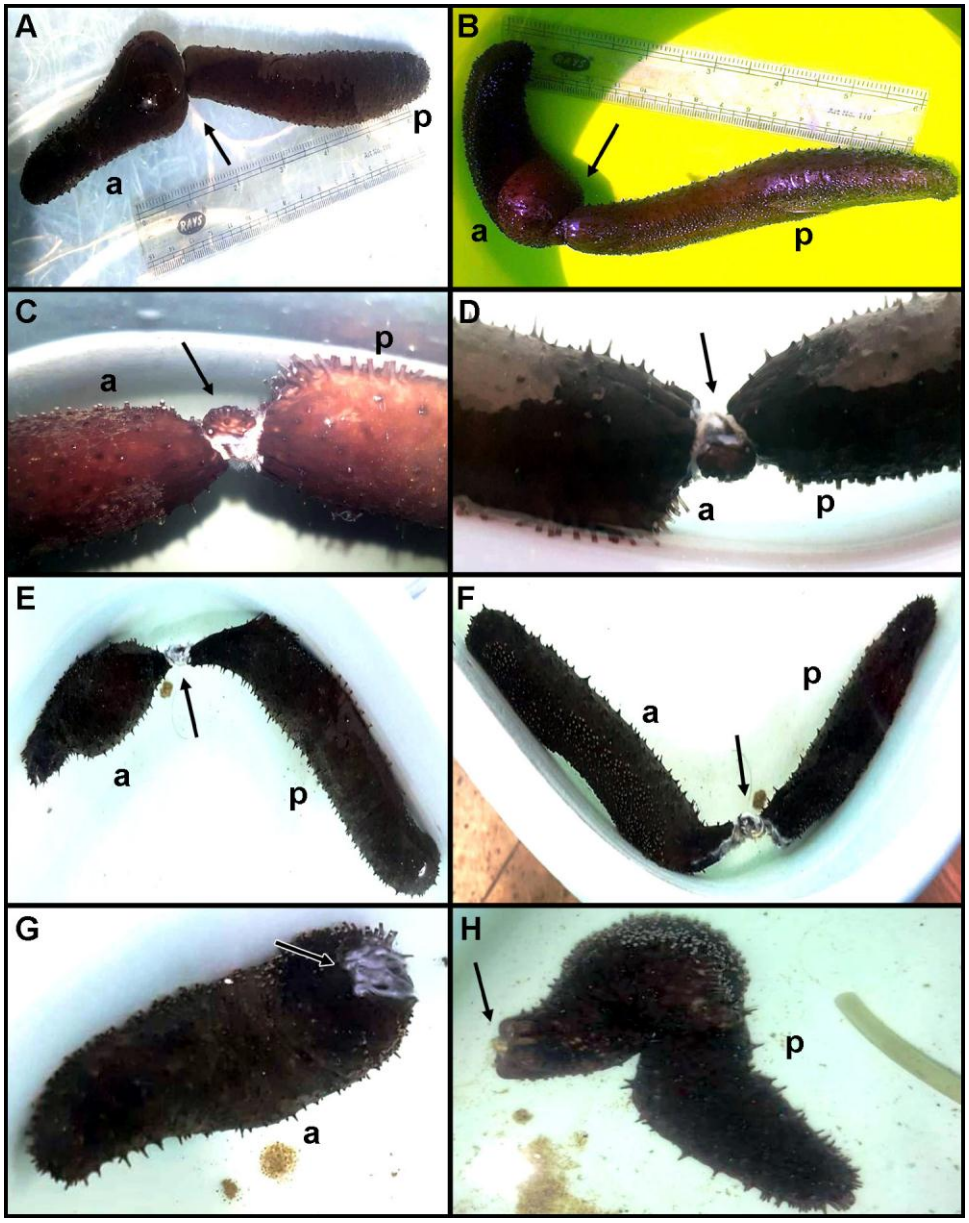
It was observed that (p) specimens of both species were more active than specimen (a). On day 64 and day 77 specimen (a) of both *H. atra* and *H. pardalis* could not survived and died whereas (p) specimen were alive and active and got developed the tentacles.

During the experiment decrease in weight of both specimen recorded; *H. atra* (3.7 gms), and *H. pardalis* (2.4 gms) because holothurians stop feeding prior to fission. According to Purwati (2004) asexual reproduction in holothurians is a very complex process that involves various mechanisms and organ systems. First, fission is accompanied by complex behaviors, such as stretching and twisting at the fission site.

The fission occurs in *H. pardalis* and *H. atra* on day 5 and 8, in *S. chloronotus*, duration of the process varies from a few minutes Uthicke, (2001) as in *H. surinamensis* and *Cladolabes schmeltzii* 1–5 days, (Dolmatov*et al.*, 2012; Crozier, 1917). The duration of fission most likely depends on the intensity of the transformation of the extracellular matrix of the body wall.

In most of the studied holothurian species, fission occurs approximately across the middle of the body (Dolmatov *et al.*, 2012; O'Loughlin *et al.*, 2009; Crozier, 1917; O'Loughlin, and O'Hara, 1992; Conand *et al.*, 1998; Emson and Mladenov, 1987).

After fission it was observed that the posterior portion of the specimens of *H. atra* and *H. pardalis* were attached to the bottom of the aquaria and anterior portion moved around the walls. Dolmatov et al., 2012 and Uthicke, (2001) reported similar observations, usually the posterior sections of the body are attached to a substrate by the tube feet whereas the anterior regions move forward or twist.

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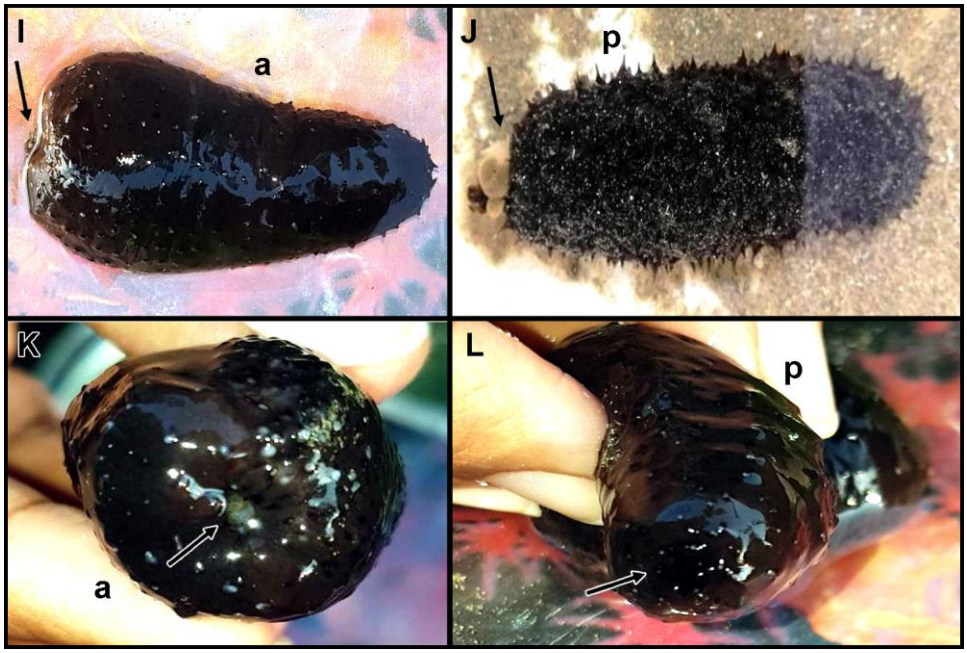


Fig. 1. A-F; twisting of *Holothuria atra* during fission (1-8 days): G-H; specimen (p) and specimen (a) after fission (9th day): I-J; Formation of tentacles outgrowth in specimen (p) and anus development in specimen (a) (39th -48th days): K-L; tentacles development in specimen (p) and anus development in specimen (a) (71th day).

Previously (Ahmed *et al.*, 2018) reported natural asexual reproduction in *H. verrucosa* under laboratory conditions, but present study was conducted on artificial asexual reproduction. Monticelli (1896) conducted the first such experiments. The artificial division of a holothurian into two sections is very far from natural stimulation of asexual reproduction and could be considered as only an imitation of fission. When a rubber band was used, behavioral reactions related to fission (constriction, twisting, and stretching) were absent (Purwati and Dwiono 2005).

Such studies are important and useful to learn more about regeneration abilities to develop cultivation technologies and increase holothurian populations (Dolmatov, 2014).

Present study shows that the posterior part (p) of the specimens survived after fission, Reichenbach and Holloway (1995) studied potential for asexual propagation of several commercially important species of tropical sea cucumber, both anterior and posterior fragments of *Actinopyga mauritiana* die after constricting of the body with a rubber band. In *Holothuria fuscogilva*, *A. miliaris*, and *Stichopus variegatus* only the posterior parts can regenerate into whole animals.

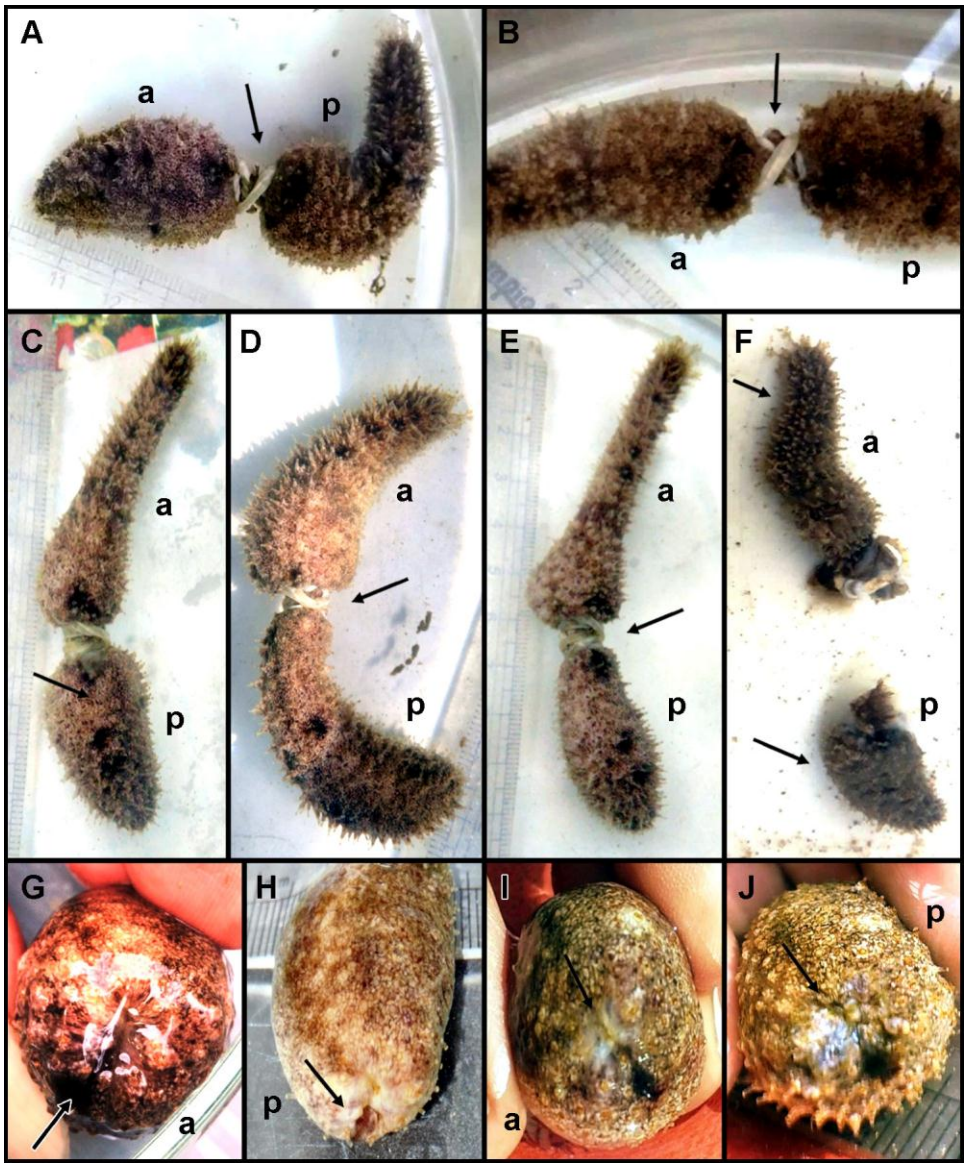


Fig. 2. A-E; twisting of *Holothuria pardalis* during fission (1-5 days): F; specimen (p) and specimen (a) after fission (5th day): G-H; Formation of tentacles outgrowth in specimen (p) (34th day) and anus development in specimen (a) on 46th I-J; tentacles development in specimen (p) and anus development in specimen (a) (58th day).

Reichenbach *et al.*, (1996) also studied on the other hand smaller (younger) individuals of *A. mauritiana*, *H. fuscogilva*, and *S. variegatus* have higher survivorship

and shorter regeneration time relative to adults. Only posterior parts of *Apostichopus japonicus*, *Holothuria scabra*, *Ohshimella ehrenbergi*, and *Colochirus quadrangularis* can regenerate lost anterior structures after such operation (Dolmatov, 2007, 2012, 2014).

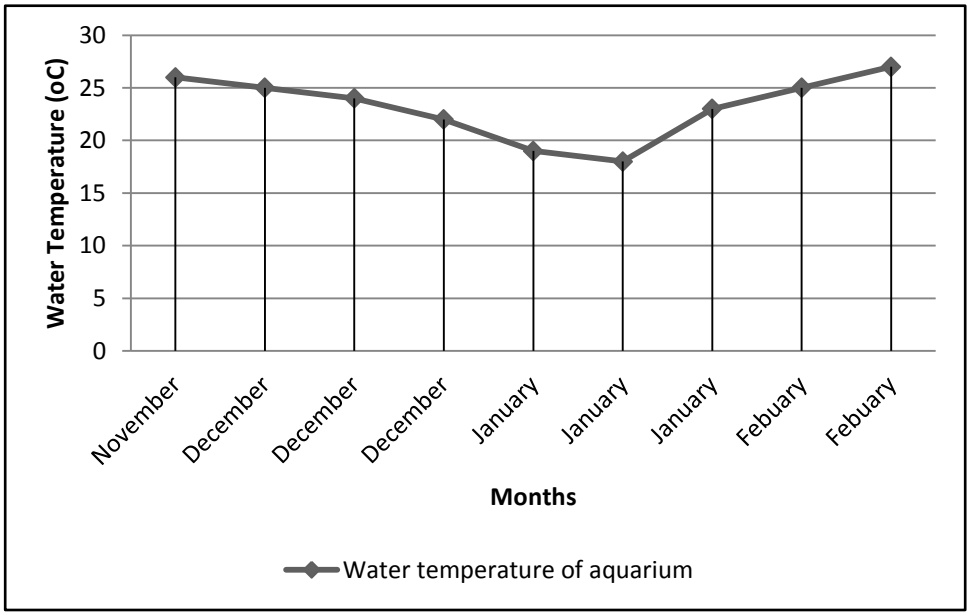


Fig. 3. Water temperature ($^{o}$C) of aquarium during experiment (Nov - 10 days), (Dec - 30 days), (Jan - 30 days), (Feb - 20 days).

**Table 1. Physico-chemical parameters from Buleji.**

| Date of collection | Locality | Tide (m) | Time | Water Temperature ($^{0}$C) | Salinity (‰) | pH |
|---|---|---|---|---|---|---|
| **30-09-2019** | Buleji | -0.07 | 17:41 | 32 | 37 | 7.9 |

Under laboratory conditions survival rate of *H. atra* and *H. pardalis* were recorded as (35%) and (45%) respectively. The survival rate of specimens (a) and (p) in *H. atra* were (15%) and (85%). in *H. pardalis* survival rate of specimens (a) and (p) were (5%) and (95%). Present study revealed that the survival rate of specimens (p) was greater than specimens (a).

The duration of the fission process and the growth of tentacles and formation anus may be affect by changes of temperature because Abdel Razek *et al.*, 2007 studied additionally, temperature showed a considerable effect on the fission process and the survival rate of the divided parts were increase when temperature were decreased. The wound-healing period also lasted longer at high temperatures than at low temperatures.

**Table 2. Asexual Reproduction in *Holothuria atra* during 20th November 2019 to 20th February 2020.**

| Days | Activity | Observations |
|---|---|---|
| 1 to 7th | ***H. atra*:** Asexual reproduction was induced by fitting rubber bands portion (a=anterior); Total length; 21 (cm): weight; (78 gms).<br><br>***H.pardalis:*** Asexual reproduction was induced by fitting rubber bands portion (a=anterior); length 11.5 (cm): weight; (19 gms). Photography was also performed using a digital camera. | ***H. atra* and *H. pardalis:*** Body was more constricted at the constriction point. The posterior part was swollen and extended. Posterior and anterior parts rotated in opposite directions.<br>Both specimens continuously twisting on the opposite side direction and fission point was very clear.<br>***H. pardalis:*** on day 5 specimen divided completely into two parts, (a=anterior); length 8 cm and (p=posterior); length 3.5 cm. |
| 8th to 18th | ***H. atra and H. pardalis:*** After fission specimens were shifted in two different well aerated aquariums.<br>Add fresh water for maintenance of salinity in aquarium.<br>Photography was performed using a digital camera. | ***H. atra:*** on day 8 specimen divided completely into two parts. (a=anterior); length 14.5 cm and (p=posterior); length 7.5 cm. |
| 19th to 28th | Add fresh water for maintenance of salinity in aquarium.<br>Photography was performed using a digital camera. | ***H. atra:*** After fission specimen (p) was more active then specimen (a). Healing of wounds.<br>***H. pardalis:*** same as above. |
| 29th to 38th | Add fresh water for maintenance of salinity in aquarium.<br>Photography was performed using a digital camera. | ***H. atra:*** Specimen (p) was active and moving on the tub wall however, specimen (a) was stick on the bottom of the tub. Healing of wounds.<br>***H. pardalis:*** Specimen (p) and specimen (a) both was active in aquarium. Wounds healed. Formation of tentacles outgrowth was observed on day 34th. |
| 39th to 48th | Add fresh water for maintenance of salinity in aquarium.<br>Photography was performed using a digital camera. | ***H. atra***: Formation of tentacles outgrowth was observed in Specimen (p) on day 47th. Wounds completely healed.<br>***H. pardalis:*** anus development in specimen (a) was observed on day 46th. |
| 49th to 58th | Add fresh water for maintenance of salinity in aquarium.<br>Photography was performed using a digital camera. | ***H. atra:*** anus development in specimen (a) was observed on day 58th. Specimen (p) was more active then specimen (a).<br>***H. pardalis:*** Specimen (p) and specimen (a) both was active in aquarium. |

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| | | |
|---|---|---|
| 59th to 68th | Add fresh water for maintenance of salinity in aquarium. Photography was performed using a digital camera. | ***H. atra:*** Specimen (a) died on day 64th. Specimen (p) was active and alive and continuously developing tentacles outgrowth. ***H. pardalis:*** both specimens (p) and (a) was active and alive. |
| 69th to 79 th | Add fresh water for maintenance of salinity in aquarium. Photography was performed using a digital camera. | ***H. atra:*** tentacles formation process continues in specimen (p). ***H. pardalis:*** Specimen (a) died on day 77th. |
| 80th to 90th | | ***H. atra:*** specimen (p) was healthy and active on day 90th. Tentacles formation process continues in specimen (p). ***H. pardalis:*** same as above. |

So water temperature is important factor during the whole process of fission.

## CONCLUSIONS

The phenomena of asexual reproduction is of high significance in aquaculture of holothuria and could lead towards commercial production hence need to be studied in depth on the regeneration and survival rates to develop a successful technique. This could be a milestone in the marine aquaculture market along with curtailing the negative impact of overexploitation and conserving the wild stock.

During the last two decades sea cucumber fishing showed a trend of overexploitation because they are easy to catch besides high market demand. Many countries including Australia, Papua New Guinea, Mauritius and Venezuela have imposed prohibition on sea cucmber fishing due to depletion of population in wild. *Isostichopus fuscus* was a vital fishery in the Galápagos during early 2000 but faced collapse due to overfishing. In the Mediterranean Egyptian holothurian fauna is facing extinction. Nine species of sea cucumbers are classified as vulnerable and seven as endangered by the International Union for Conservation of Nature (IUCN, 2020). Three species of sea cucumbers have been listed on Appendix II and one species *I. fuscus* listed on Appendix III by the Convention on International Trade in Endangered Species (CITES, 2020).

Present study is an humble effort and first step towards development of laboratory techniques on induced asexual reproduction in holothuria of Pakistani waters. But there is a dire need to initate extensive research for development of techniques on commercial exploitation. There are numerous questions to be answered including; factors stimulating fission and asexual reproduction, molecular and cellular mechanisms of fission, restoration of systems (digestive, reproductive etc.) in the divided individuals. The findings from present research will be substantive addidtion to the knowledge in the field of aquaculture and will be valuable to marine scientist and aquaculturist in their future research.

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