

Effect of Cadmium on the Embryonic Development and Hatching Rhythm of Big Fin Squid *Sepioteuthis lessoniana*, Lesson (1830) from Palk Strait, East Coast of India

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Abstract

In the present study, the impact of cadmium on embryonic development, morphology of eggs and newly hatched larvae of the *Sepioteuthis lessoniana* were described using the laboratory-reared specimens originating from Thondi, South East coast of India. The eggs were exposed to a concentration of 25 µg Cd/L for up to hatching. The average incubation period of the eggs varied between 18-23 days and the estimated mean temperatures were 18.6 ± 0.7°C for control, and 19.2 ± 1.1°C for Cd exposed groups. The results revealed that a total hatching rate of 99.2 ± 1.6% was achieved in control group and only 65.2 ± 8.8% in treated group. The deformities of the paralarvae were illustrated with light and scanning electron microscopy. In conclusion, the present concentration exhibited toxic effects to the eggs and paralarvae of *S. lessoniana*. Therefore, Cd level as high as we used in this study could give good information on dangerous Cd levels on squid.

Keywords: Squid; Cadmium; Paralarvae; Larval bioassay; Embryos; Heavy metals

Introduction

Heavy metals enter aquatic ecosystems from various sources such as municipal wastewater, liquid industrial waste discharges, and river runoff. They can cause adverse effects on aquatic organisms if adequate concentration of chemicals comes into contact with a biological membrane of an organism or system [1]. At higher levels of biological organization (tissue, organ and whole organism), heavy metals induce changes in metabolism, biochemistry, physiology, and histology, and inhibit synthesis of proteins and nucleic acids [2-4]. Most of the studies have highlighted the very high ability of cephalopods to concentrate various toxic elements such as Ag or Cd [5-8].

Cadmium (Cd) is among the most toxic metals in the aquatic environment, possesses no known biological role and exhibits high toxicity if allowed to accumulate in metabolically-active tissues [9]. Cadmium is a biologically non-essential heavy metal that occurs naturally and is released from various sources [10]. The cadmium contamination in surface and groundwater is believed to be of non-point source as a result of agricultural and urban run-off. Concentrations of Cd measured in environmental water samples vary considerably from 1 ppb to over 400 ppb in contaminated sites [11]. Most of Cd forms complexes with ions such as Cl [12], and only a small fraction of cadmium in the form of free hydrated metal ion form, Cd²⁺, is biologically available to the organisms [13]. Its average concentration in unpolluted waters is about 0.05 µg Cd/L Cd. Concentrations of Cd in coastal waters tend to increase because of anthropogenic input and local geological conditions [14].

Squid accumulate trace amount of heavy metals including Cd, in its liver, digestive gland, mantle, ovary, and eggs from water and food [15]. Cd is transferred from water and food to the squid and from squid to their predators through the food chain. Therefore, squid play an important role in the chain [6,8,15-19]. By another hand, no information exist on the incorporation of elements by eggs in cephalopod species that lack eggshell, as in the incirrate octopods (*Octopus vulgaris*) which egg chorion is in direct contact with seawater. The egg capsules of *Sepioteuthis lessoniana* are laid on the underside of rocky overhangs,

on branched sessile organisms or on fishing lines and hang down in the water [20-24]. Hence, the egg capsules stand wherever laid and expose to every kind of heavy metals from not only the water but also the sediment environments. Moreover, as reported by Bustamante et al. [8], heavy metal levels in cephalopods, which are extensively fished and consumed by human, are also of direct significance to the public health. However, there is only one study related to the effects of the some heavy metals on *Loligo vulgaris* embryos [24], but seemingly no available study exists in literature on the effects of any heavy metal on development and hatching of eggs of *Sepioteuthis lessoniana*. Knowledge of the early life history of *S. lessoniana* would provide basic information for fishery biology. It would also be invaluable in the analysis of the phylogeny of the Cephalopoda by clarifying the embryogenesis of squid species that has seldom been previously pursued by researchers. Therefore, the aim of the current study is to determine the effect of Cd on development and hatching of *S. lessoniana* eggs collected from the Palk Strait region, South East coast of India.

Materials and Methods

Egg collection

Recently spawned egg masses of *S. lessoniana*, probably by different breeders, had been collected from fishing lines deployed in the Palk Strait region, Southeast coast of India. This region is comparatively less polluted or unpolluted as the site was not surrounded by any industries/urban pollution sources. The egg capsules were transported

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to the laboratory on the same day. Eggs were separated into smaller clusters of four or five egg capsules and placed in the glass aquarium containing filtered sea water and covered with black cloth to avoid the growth of algae. The egg masses were sorted according to embryonic development stages based on Naef's [25] (Roman numerals) and Arnold's [26] (Arabic numerals) criteria, and then their morphometric characteristics were measured (n=30). During the experimental period, embryonic development stages of the eggs were observed under a stereo microscope (20–40X magnification). The embryonic development stage was 13 (II) at the commencement of the trials, which is the gastrula stage.

Stock solution

Cadmium chloride (CdCl₂) (95% purity) was purchased from Himedia (Himedia, India). Two hundred and fifty milligrams of CdCl₂ was dissolved in 100 ml of double distilled water.

Experimental design

All trials were performed on two bunches of egg capsules including average 110 ± 9.8 eggs in triplicate (n=6 egg capsules per concentration) without any acclimation process. The egg capsules were grouped into two, one group served as control and other group exposed to a concentration of 25 µg Cd/L. The experiments were conducted with continuous aeration, optimum temperature and salinity. Sea water containing experimental Cd concentrations was changed every day by replacing 100% of the beaker volume. Illumination (a 40 W fluorescents tube) was controlled by a timer to provide a photoperiod of 12:12 h of light: dark periods. All embryos in the egg capsules were examined every day in the control and exposed groups until hatching. The main criterion of death of egg was whitening of the yolk of embryo. The length of the incubation period (D), total hatching (TH = [number of hatching eggs (premature and swimming paralarva at nearly the water surface)/ number of incubated eggs] * 100), and hatching success (HS = [number of healthy and swimming paralarvae at nearly water surface/number of incubated eggs] * 100) of the eggs were estimated for both control and each experimental group (Sen 2005a, b). The length of egg capsules were measured with measurement board to the nearest mm, egg dimensions (length and width) were measured by millimetric ocular to the nearest 0.01 mm, and numbers of eggs per capsule were counted by macrometric observations. During hatching, newly hatchlings (paralarvae) were harvested from the beakers and counted daily.

Histology

For histological studies, paralarvae were taken from both control and experimental groups and fixed in Bouin's fixative. The samples were dehydrated through graded alcohol series and embedded in paraffin wax. Sections of 6–8 mm thickness were taken, dehydrated and stained with hematoxylin and eosin. The stained sections were mounted using DPX and photomicrographs were taken using Leica 2500 microscope (Germany).

Scanning electron microscopy (SEM)

Paralarvae of both control and Cd treated were fixed in 2.5% glutaraldehyde in seawater for 15 h, washed in seawater followed by dehydration in an increasing concentration of ethanol (20, 30 and 50%) and stored in ethanol 70% at 4°C. At the beginning of the SEM preparation, the samples were again dehydrated in an increasing concentration of ethanol (80, 90, and 95%) until they were saturated in absolute ethanol. Each ethanol bath lasted 10 min. After complete

dehydration in the ethanol series, the samples were dried to the critical point, which was carried out in a Bal-Tec CPD 030 Drier using CO₂ as the transition liquid. After the drying stage, samples were mounted on stubs with double-sided conductive sticky tape to orientate them in the preferred position. The mounted samples were sputter coated with gold–palladium in a Polaron Sputter Coater SC500. Finally, the samples were observed using a scanning electron microscope (Hitachi S3500N).

Statistics

The obtained data were given as mean \pm SD values in the text, and were compared using one-way analysis of variance (ANOVA) to determine significant differences among means (p<0.05). Statistical analyses were carried out by SPSS 11.0 software.

Results

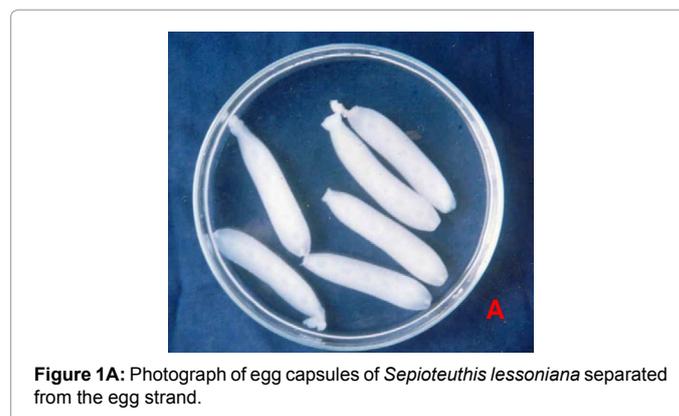
During the experimental period, the ambient temperature ranged between 16.3 and 20.5°C, and the estimated mean temperatures were $18.6 \pm 0.7^\circ\text{C}$ for the control, and $19.2 \pm 1.1^\circ\text{C}$ for Cd exposed groups. There were no significant differences in water temperatures between control and Cd exposed groups (p>0.05).

Egg morphology

Morphometric characteristics of the egg capsules and eggs were given in Table 1 (Figure 1A). The total incubation period of the big fin squid *S. lessoniana* varied between 18–23 days. As the development of the embryos progressed within, the egg capsule became more transparent and slightly enlarged with the absorption of water. Embryos were present in most of the capsules seen through as a white mass and occasionally moving within the capsules of both control and Cd treated groups. From thirteenth to seventeenth day, the yolk totally reduced and the organs of the embryo started to develop. Decreasing of yolk is another important character to confirm the development of embryo. Before hatching they floated in the capsule for some time and wriggled out through a small slit in the capsule (Figure 1B). By the time the embryo became a full-grown young within the capsule, the yolk would have been fully absorbed. But in Cd exposed group the diminished yolk

Value	Capsule Length (mm)	Eggs per Capsule (nos)	Egg length (mm)	Egg width (mm)
Min	50	4	20	10
Max	80	7	25	18
Mean	69	5	23	14
SD	8	0.002	2	3

Table 1: Morphometric characteristics of the egg capsules and eggs.



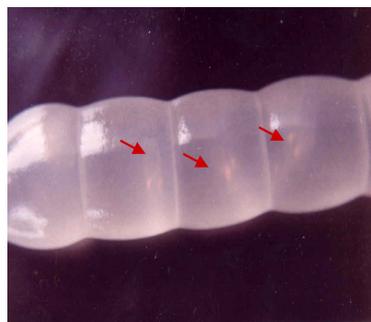


Figure 1B: 12 Days old embryo inside the chorionic capsule.



Figure 1C: Cadmium exposed eggs showing yolk sac within the capsules.

sac still persisted and yolk sac could be seen within the empty capsule (Figure 1C). The embryo with its mantle, head and arms resting on the globular yolk sac was more visible through the capsule. Hatching took place between the hours 432 and 456 in control group and between the hours 456 and 480 in Cd exposed groups. Total hatching rate was $99.2 \pm 1.6\%$ in control group, while the estimated hatching success rates were $65.2 \pm 8.8\%$ in the treated group. Significant differences were found in total hatching and hatching success rates among the groups ($p < 0.05$).

Morphology of the paralarvae

Control: In control, the paralarvae showed a normal morphological aspect and swam actively in the glass tank. Before hatching they floated in the capsule for some time and wriggled out through a small slit in the capsule. By the time the embryo became a full-grown young within the capsule, the yolk would have been fully absorbed. The hatched young ones of *S. lessoniana* were the miniature replicas of the adult cuttlefish externally. At this stage they measured 6.5-7 mm in dorsal mantle length. The chromatophores were very clear and distributed all over the body with prominent brown dots (Table 2). By the end of the experiment, chromatophores on the body were clear and heart beating was evident in some of the embryos. Nevertheless, mean survival was relatively high in the control group, no malformations were observed and paralarvae were capable to swim actively (Figures 2A and 2B).

Experimental group: In Cd treated group, most of the embryos remained in early developmental stages, the only observable evidence of organ formation being the rudiments of the pigmented eyes, stunted growth of arms and mantle. Therefore, the paralarvae showed large amounts of vitelline reserves in their external yolk sac (Figure 3A). Seven days (168 h) after fertilization, premature hatching was observed (Figure 3B). In some cases, few embryos reached late stages of

development but showed malformations such as eyes further apart from each other and the internal yolk sacs broader than in morphologically normal embryos and no one succeeded to hatch (Figure 3C). Also, they were poorly active when stimulated by light during the incubation. Chromatophore formation has not seen in the body. Embryos were unable to break their chorionic membranes, remaining at this stage for few days, until they completely consumed their vitelline reserves and died. Almost all premature hatchlings died within the capsule. Statistical analyses suggest that survival was significantly different between treatment and control groups.

Histological changes associated with cadmium toxicity

Section through the paralarvae of control showed large rounded prominence on each side of the head marks, the position of the eye-stalk. The eye stalks are now very conspicuous projections from the sides of the dorsal end of the embryo. Eye primordia were clearly visible in the paralarvae and comprised of a shallow hemispherical

Days	No of Hatching	Rate of Mortality
16	2 ± 0	-
17	8 ± 1	5 ± 1
18	57 ± 3	10 ± 3
19	140 ± 5	59 ± 7
20	33 ± 2	166 ± 10

Table 2: Hatching and mortality rate of the paralarvae of big fin squid *Sepioteuthis lessoniana*.

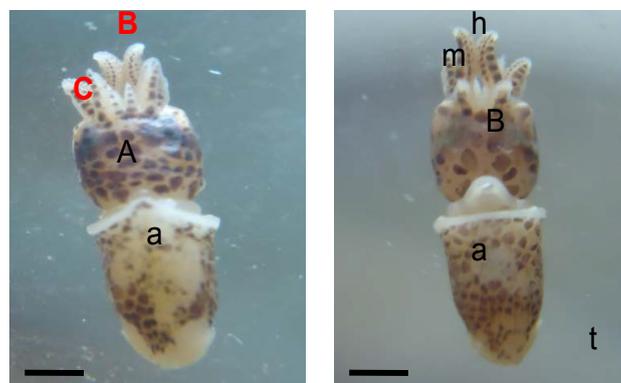


Figure 2: (2A) Dorsal and (2B) ventral view of the paralarvae of *Sepioteuthis lessoniana* showing the shape and disposition of the chromatophores in the arms (a), tentacles (t), head (h), mantle (m) and body (b). Scale bar: 11mm.

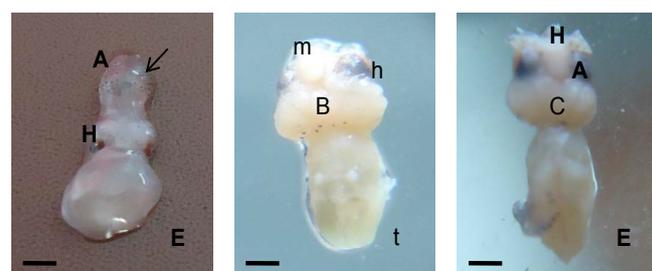


Figure 3A-C: Premature hatchlings in cadmium treated group (3A). Early developed embryo with vitelline reserves (VR) on the dorsal side, rudimented eye (E) Scale bar: 8mm. (3B). Stunted arm development (A), rudimented eye (E), distorted head (H) Scale bar: 9 mm (3C) 17th day paralarvae with malformations in the eye (E), arms (A) and head (H) Scale bar: 11mm.

cup of undifferentiated neural retina which, along with presumptive lentigenic and iris cell tissues, enclosed a posterior eye chamber (Figure 4A). The ridges of the head divided into three arms, upon each side of the body, about half-way between the mantle and the opposite pole of the egg (Figure 4B). However, in cadmium treated paralarvae, the rudiments of the eye-stalk could be seen on one end of the dorsal view (Figure 4C). Eye primordial was not clear as in control. The pair of arms makes their appearance as buds upon the next pair and appeared as elevations of the integument upon the surface of the body (Figure 4D).

Ultrastructural changes associated with cadmium toxicity

Under scanning electron microscopy, control showed well developed eye-invaginations. The eye-stalks are much more prominent, and the head, mantle and body are divided into three well-marked regions. The funnel folds arise as bulge of tissue which form a V-shaped structure medial to the eyes, posterior to the arms, and anterior to the down-growing mantle (Figure 5A). The three pairs of arms, are much elongated, and begin to bend away from the surface and the suckers have appeared upon the longest pair (Figure 5B). The mantle is smooth and connects well with the body (Figure 5C). In contrast, the head arms and mantle of Cd treated paralarvae are very much smaller as compared with the control. The formation of funnel fold is poorly developed on the mantle (Figure 5D). The eye invaginations are not visible. Arm ridges are stunted and irregularly arranged (Figure 5E). The surface topography of the mantle showed rough, narrow downwards and weakly joined to the body (Figure 5F).

Discussion

Squid egg strand is characterized by a complex structure in which several small oocytes surrounded by a chorion are united by oviducal jelly, and enveloped by a common sheet of nidamental secretions [27]. Cuttlefish hatchlings are essentially isometric copies of adults, late embryos and hatchlings of squid are much smaller and are termed "paralarvae" because of their incomplete development when compared with adults [28]. As in cuttlefish eggs, some structural changes occur during the embryonic development. In cuttlefish eggs,

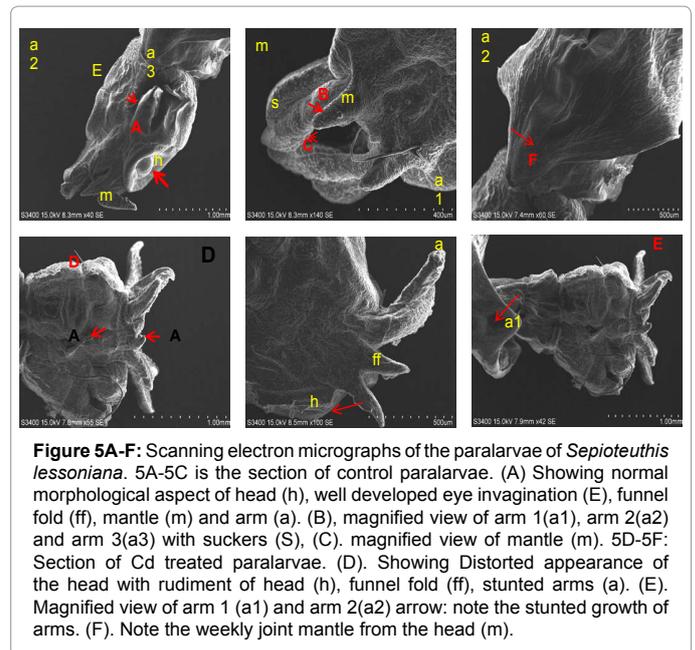


Figure 5A-F: Scanning electron micrographs of the paralarvae of *Sepioteuthis lessoniana*. 5A-5C is the section of control paralarvae. (A) Showing normal morphological aspect of head (h), well developed eye invagination (E), funnel fold (ff), mantle (m) and arm (a). (B), magnified view of arm 1(a1), arm 2(a2) and arm 3(a3) with suckers (S), (C), magnified view of mantle (m). 5D-5F: Section of Cd treated paralarvae. (D), Showing Distorted appearance of the head with rudiment of head (h), funnel fold (ff), stunted arms (a). (E), Magnified view of arm 1 (a1) and arm 2(a2) arrow: note the stunted growth of arms. (F). Note the weekly joint mantle from the head (m).

it has been demonstrated that the increased permeability of the egg to some metals is correlated with this swelling process [29,30]. As indicated by Bustamante et al. [7], irrespective of their morphological and evolutionary variety and heterogeneity in their geographical distribution, cephalopods have in common the capability to concentrate Cd at extremely high levels. Therefore, Cd concentrations could be risky for the squid populations in natural conditions. Spawning behaviour observed in this study was similar to those shown in previous studies [31]. Temperature is the main abiotic factor influencing growth during the embryonic and hatchling stages of cephalopods [32,33]. This is a relatively normal result due to the use of ambient temperature for incubation in the current study. However, at constant incubation temperatures, body size becomes larger in hatchlings that hatch later in some cephalopod species [34] but it does not vary in other species [35]. As in other coleoid cephalopods, *S. atlantica* showed an inverse relationship between temperature and developmental duration [36]. Our comparison between the tested temperatures did not show apparent differences in. Sen and Sunlu [1] explained that cadmium adversely affected development, survival and hatching of *L. vulgaris* eggs. In the present study the nominal concentration of 25 µg Cd/L was used to augment the deformities associated with cadmium in the embryonic and post embryonic behavior of squid.

Treatments without Cd showed no chorion expansion and resulted in 99.2 ± 1.6% success in hatchability. In comparison with treatments receiving Cd recorded organogenesis and just before hatching showed malformations and decreased hatching percentage. As reported by Sen [21], total hatching and hatching success rates were calculated as 98.8-98.9% and 84-86%, respectively, and the hatching period of the paralarvae occurred between the days 5-7 at 18 ± 1°C. The morphology of paralarvae has also been described during observation of embryonic development. Watanabe et al. [37] showed that hatchlings have only one or two suckers near the base of arms I and II, and that in 4-5-day-old paralarvae the distal portion of tentacles terminates in acute tips. In this study, paralarvae treated with Cd had stunted growth of arms and tentacles with acute tips. Also, they calculated total hatching and hatching successes as 98.2 ± 1.6% and 94 ± 2.3%, respectively, and the hatching period of the paralarvae lasted 4 days at 19.8 ± 1.6°C [22].

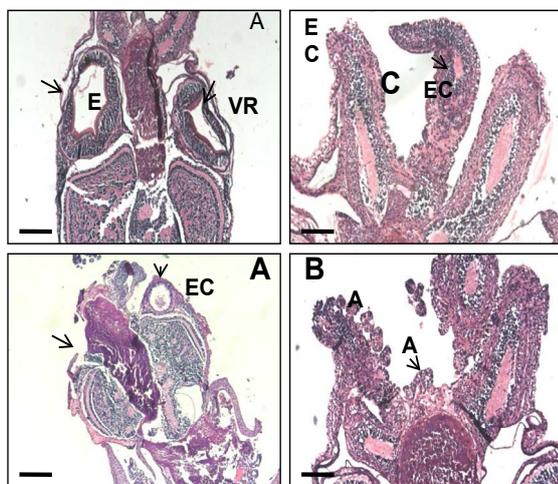


Figure 4A-D: Section through the paralarvae of *Sepioteuthis lessoniana*. 4A and 4B: Photomicrographs of control paralarvae showing normal aspect of eye cup (EC), lengthy arms (A). scale bar: 50 µm. 4C and 4D: Photomicrographs of cadmium treated paralarvae showing partial eye cup on the right side (EC) and stunted growth of arm tips (A). scale bar: 50 µm.

When compared to those data with the current results, it is prominent that the total hatching was nearly the same proportion in the control group, while, hatching successes was significantly lower in Cd treated groups. The embryo mortality rates of 100% occurred within 1 day of cadmium treatment. However in control, the embryos were survived more than 3 days. So far, numberless studies were carried out about adverse effects of cadmium on accumulation, behaviour, biochemistry, cell(s), development, enzyme(s), growth, histology, mortality, physiology and reproduction in molluscs [38].

Post-hatch conditions were similar for all hatchlings, despite being raised at different developmental temperatures. This may have changed the physiological consumption of the yolk [39]. Yolk consumption was studied in crabs (*Chasmagnathus granulata*), with salinity as the variable factor, and although salinity stress resulting in an increase in egg size [40]. At the time of hatching, the young squids possess a large internal yolk supply and appear, on morphological grounds (no apparent mouth, suckers without acetabulae, and so forth), to be incapable of feeding. The treated group shows the presence of vitellin reserves on the head. In control, all of the hatchling types had distinctive chromatophore patterns. Newly hatched individuals showed expanded chromatophores of dark brown colour on a yellowish background along the whole body, and they often assumed a “flamboyant” arm display [41]. The chromatophore arrangements in the paralarvae of *S. lessoniana* are similar to those reported for *L. opalescens* [42], *L. vulgaris* [25,43] and *L. reynaudii* [44,45]. The present observations on cadmium treated paralarvae showed the absence of chromatophores on the body. Variability in hatchlings from the same egg capsule can be associated with intrinsic factors like multiple paternities [46], or with extrinsic factors such as the possible differences in the oxygen availability to embryos. Afterwards, they moved quickly towards the prey in order to attain appropriate attacking distance [47].

Histological analyses demonstrated that the eye stalks are very conspicuous projections from the sides of the dorsal end of the embryo. The ridges of the head divided into three arms, upon each side of the body, about half-way between the mantle and the opposite pole of the egg. Eye primordial was not clear as in control. The pair of arms makes their appearance as buds upon the next pair and appeared as elevations of the integument upon the surface of the body. Rodrigues et al. [48] had described the histological aspect of the paralarvae of Atlantic bobtail squid *Sepioida atlantica*.

The developmental stages of arm suckers can vary with arm position in ommastrephid and onychoteuthid paralarvae, in *T. rhombus* paralarvae arm sucker development clearly occurs in two separate stages [49]. The proximal suckers of *T. rhombus* develop with chitinous rings, whereas the middle suckers are bud-like in shape and have no chitinous rings. In this study, the presence of arms and suckers were absolutely normal in control groups compared to Cd treated group. Wakabayashi, et al. [50], studied the detailed morphological aspects of the paralarvae and its arm and tentacle arrangements in the diamondback squid *Thysanoteuthis rhombus* Troschel (1857). Similarly, the present study also corroborates the finding of Wakabayashi, et al. [50] in control group. Other than perhaps, cadmium treated group exhibited the stunted growth of arms and tentacles as described in scanning electron microscopy. Hence it was confirmed that, cadmium adversely affected development, survival and hatching of *S. lessoniana* eggs and paralarvae.

Conclusion

In conclusion, the present concentration exhibited toxic effects

to both eggs and the paralarvae. Therefore, the Cd level as high as we used in the current study could give good information on dangerous Cd levels on *S. lessoniana*. Consequently, hatching is an extremely sensitive period in early cephalopod ontogenesis. Taking that into consideration in *S. lessoniana*, Cd adversely affected not only one mechanism of development, but also whole developmental and hatching process. Finally, we recommended that more detailed studies should be examined investigating the metal kinetics of penetration and the properties of the *S. lessoniana* egg capsule towards the metals etc. Moreover, the current results indicate future areas of research in squids, which may be fruitful towards understanding the effect of Cd on the innate behavioral response and metabolism.

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