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Original Article

Reproductive potential of male calanoid copepod *Sinodiaptomus* (*Rhinediaptomus*) *indicus* Kiefer: A potential live prey for fish larvae

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Abstract: Copepods are a morphologically, physiologically, and ecologically highly diverse group with different reproductive characteristics and have evolved structural specialties of sperm form and spermatophore structure. These reproductive peculiarities are important for their implications in applied biology. Copepods are the preferred food for finfish and shellfish larvae due to their optimal size range and nutritive value. Although many copepod species are successfully mass-cultured, limited success is still reported in commercial-level production for use in aquaculture hatcheries. Unlike rotifers and *Artemia*, which reproduce by parthenogenesis and attain high-density production easily, copepods reproduce sexually, involving mate selection, a copulatory process, and leading to the transfer of spermatophores and fertilization of eggs. This necessitates an understanding of the reproductive biology of cultivable copepod species to establish effective culture protocols. The freshwater calanoid copepod *Sinodiaptomus* (*Rhinediaptomus*) *indicus* is a promising candidate for mass culture, and this article describes the reproductive biology of the male using histological and electron microscopic studies. The ultrastructure of the spermatozoon of this species is reported for the first time. Laboratory experiments showed long lifespan and high spermatophore production (34 in 25 days) in this animal, which is a key factor in deciding male-female ratio formulation in the inoculum for mass culture. It is suggested that the *S.* (*R.*) *indicus* could be an ideal species for mass culture and use as live feed in fish and prawn larval rearing.

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Introduction

In the reproductive process of calanoid copepods, males are typically the active partners, playing a crucial role in identifying potential mates and participating actively in copulation and sperm transfer. This has led to sexual dimorphism, with the geniculate antennule modified in males and the P5 in both sexes. These appendages facilitate the precise attachment of the spermatophore to the female reproductive pore by the male and removal after the discharge of the spermatophore and release of the fertilized eggs by the female. Considerable variations in modifying geniculate appendages based on environmental conditions and mating behavior of calanoid copepod families have been reported (Ohtsuka and Huys, 2001). Higher asymmetrical modifications of appendages involved in the reproductive process are observed in freshwater calanoid copepods than in marine ones (Altaff, 2003). Though there are many contributions to the reproductive biology of calanoid copepods (Park, 1966; Hopkins, 1978; Blades-Eckelbarger, 1986; Ershova and Kosobokova, 2012; Ali et al., 2014), further investigations are still required to comprehend their different mechanisms due to high diversity and species-specific variability.

There is considerable variation in the reproductive potentials of different species of calanoid males; some produce single or few spermatophores in short life periods, while some produce many spermatophores (Blades-Eckelbarger and Youngbluth, 1991). In several parasitic families of Copepoda, dwarf males

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are often reported to be significantly smaller than their females (Østergaard and Boxshall, 2004). The success in the hatchery production of fish fingerlings for stocking in the grow-out production system largely depends on the availability of suitable live food for feeding fish larvae, fry, and fingerlings. Some qualities that make copepods a good candidate species for mass culture are their small size, acceptability by marine and freshwater fish, tolerance to high densities, and short generation time. In recent times, applications of marine copepods in various fields, like a live feed for marine fish larval rearing and as a source of biomolecules for medical applications have drawn the attention of many investigators towards their mass culture and use (Fernandez and Ingber, 2013; Santhosh et al., 2018; Santhanam et al., 2019; Altaff, 2020; Altaff and Vijayaraj, 2021).

However, relatively few attempts have been made to culture freshwater species. In the mass culture of copepods, besides diet and environmental conditions, knowledge of the reproductive potential of the culture species is essential. Copepods are sexually reproducing organisms; therefore, assessing the reproductive potential of males and females is essential for high-density culture to achieve the desired density in the culture system. In this regard, knowledge of male reproductive potential is essential for determining the male-female ratio in the inoculum of the culture protocol. Toward this objective, this article describes the reproductive biology of the male of a common freshwater calanoid copepod, *Sinodiaptomus* (*Rhinediaptomus*) *indicus*.

Materials and Methods

Zooplankton samples were collected from the Chetpet pond, Chennai (13.0827°N, 80.2707°E), using a plankton net made up of Bolten silk (mesh size 120 µm) and preserved in 5% neutral buffered formalin. Morphological observation of the whole animal and its appendages was made by mounting it in a lactophenol medium, and the calanoid copepod *S.* (*R.*) *indicus* was identified based on the taxonomic key characters described by Reddy (1994). A live plankton sample was collected, and *S.* (*R.*) *indicus* were separated under a binocular stereoscopic dissection microscope using wide-mouthed droppers and maintained in pre-treated water. They were fed baker's yeast and mixed algae at a concentration of 10^5 cells on alternate days.

For studying the intact male reproductive system *in situ,* specimens were stained with Borax carmine overnight and destained with acetic acid (Pantin, 1964). For histology, Bouin's fixed male *S.* (*R.*) *indicus* were dehydrated in an ascending series of ethyl alcohol, cleared in xylene, and embedded in paraffin wax. Serial sections were taken at 8 µm thickness and stained in Ehrlich's hematoxylin with aqueous eosin as a counter-stain (Patki et al., 1987). For the scanning electron microscopic study, animals were fixed in 4% glutaraldehyde for 12 hours at 4ºC, and post-fixation was carried out in 1% osmium tetroxide. The specimens were then treated with propylene oxide and air-dried below room temperature. The specimens were glued to self-sticking carbon tape, coated with palladium gold (JEOL JFC110E) ion sputtering device, and scanned in a scanning electron microscope (JEOL JSM 5300) at 15 KV. For transmission electron microscopy, specimens were fixed in 2% glutaraldehyde and post-fixed in 1% osmium tetroxide. Ultra-thin sections of 60 nm were taken, stained with uranyl acetate and lead acetate, and observed under a transmission electron microscope (Philips CM 10).

To study life span, laboratory-reared copepodid six stages, male and female, were maintained in 250 ml containers and fed mixed algae (*Chlorella, Ankistrodesmus,* and *Selenastrum*) and Baker's yeast in the ratio of 1:1 on alternate days. To ascertain the reproductive potential of males and females in different combinations (1 male + 1 female, 1 male + 2 females, and 1 male + 3 females), they were maintained in 250 ml beakers and fed mixed algae and Baker's yeast. The number of ovisacs formed by the female in a day was taken as an index of the number of spermatophores formed by the male. The number of ovisacs formed per day was recorded after meticulous observations. Experiments were conducted over 25 days, with 10 trials performed for each combination.

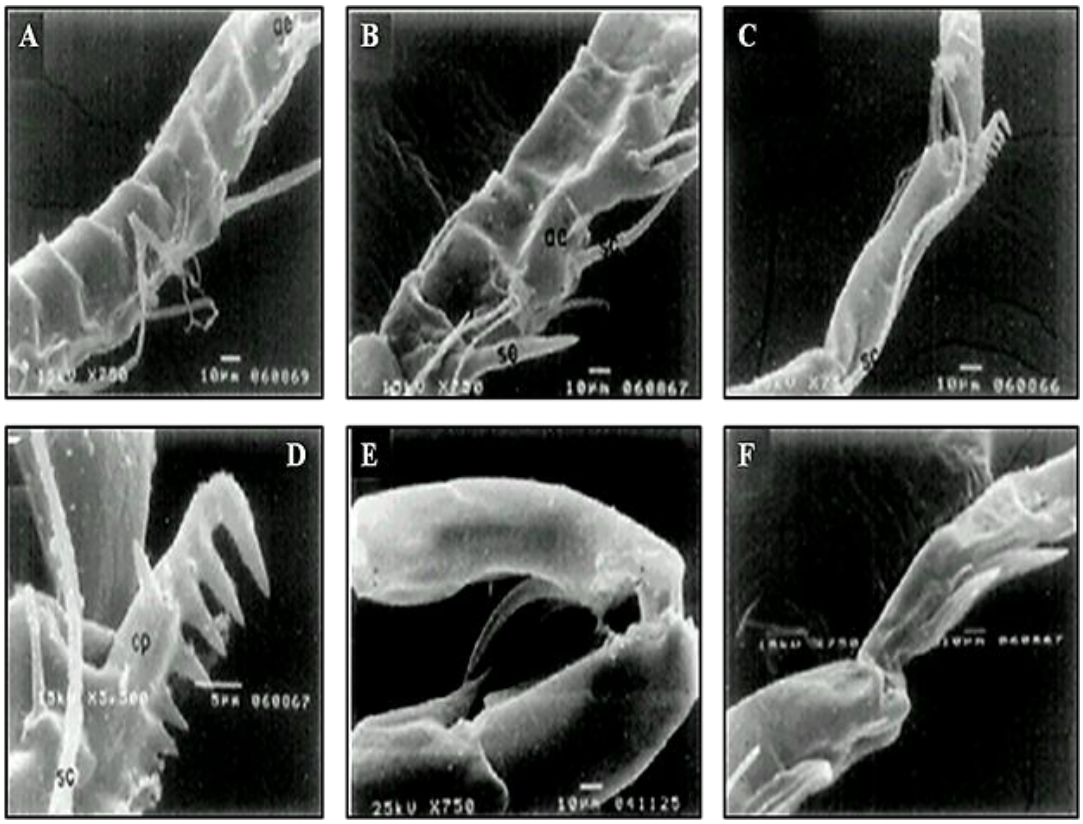

Figure 1. SEM of male *Sinodiaptomus* (*Rhinediaptomus*) *indicus*. (A-F) Different regions of the right antennule.

Results

Geniculate appendages: In males of *S*. (*R*.) *indicus*, the antennules are asymmetrical, and the right antennule is 22-segmented, while the left one is 25-segmented. The fifth legs (P5) of both sexes are modified; the female P5 is symmetrical, and that of the male is asymmetrical. Segments thirteen and eighteen of the right antennule of the males are enlarged, and a movable articulation occurs between the eighteenth and nineteenth segments. The segments from the nineteenth to the twenty-third fused to form a compound segment. The segments ten, eleven, fifteen, and seventeen carry a spine. Thick processes occur in the seventeenth to nineteenth segments. Segments seven, nine, fourteen, and nineteen have a small club-shaped aesthetasc proximally. The distal end of the twenty-third segment bears a comb-like hyaline process with nine linearly arranged teeth and a laterally directed tooth. Long setae occur in the last three segments (Fig. 1).

The P5 of the female is symmetrical, small, and differs from other thoracic legs markedly. The coxopodites are large and bear a spine, while basipodites are short and carry a long seta. The endopods are reduced, pillar-like structures. Of the two exopod segments, the first one is large and cylindrical, while the second is tapering and forms a strong conical claw, whose apex is curved and whose margins carry hair-like spines (Fig. 2A).

The P5 of the male is asymmetrical and highly modified. The right leg is much longer than the left. The coxopodite of the right leg is significantly broader than that of the left leg. The basipodite of the right leg is short, while that of the left leg is cylindrical. The endopodite of the right leg is short and fused with the basipodite. Regarding the exopodite of the right P5, the first segment is small, and the second segment is comparatively large and laterally compressed. The terminal segment is modified into a strong ‘S’ shaped claw. The base of the claw is rounded and articulated with the second segment. The exopodite segments bear spines and setae. The endopodite of the left leg is two-segmented. The exopod of this leg has a long segment with an arch-like, sharp curve, possessing a

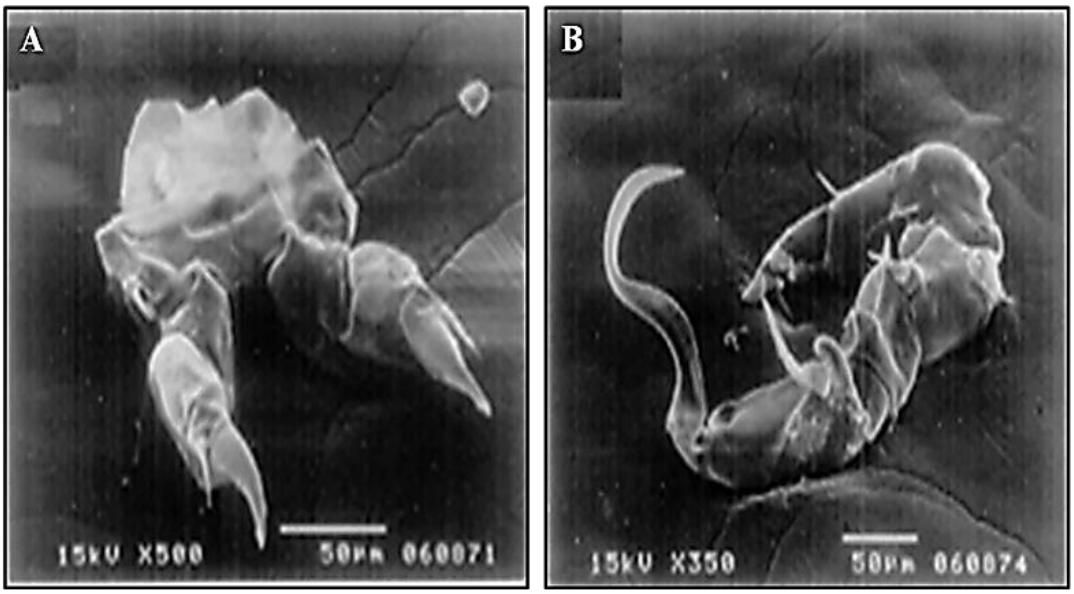

Figure 2. SEM of male *Sinodiaptomus* (*Rhinediaptomus*) *indicus*. (A) P5 of female and (B) P5 of male.

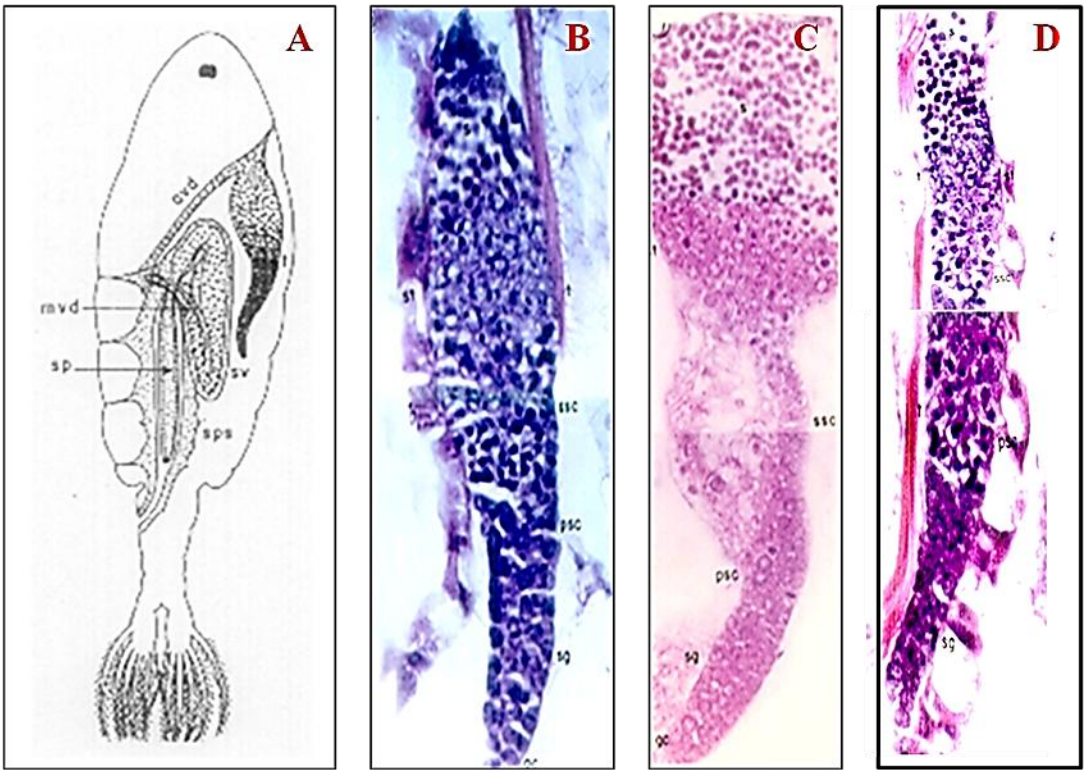

Figure 3. Male *Sinodiaptomus* (*Rhinediaptomus*) *indicus*: (A) Adult reproductive system, (B) histology of testis of immature, (C) Mature, and (D) Spent animal.

bunch of short and long hairs. The terminal part of this segment has a thump-like structure with lamellar processes (Fig. 2B).

Male reproductive system: The male reproductive system of *S.* (*R.*) *indicus* consists of a median testis and a genital duct demarcated into anterior vas deferens, mid vas deferens, seminal vesicle, spermatophore sac, and ductus ejaculatorius (Fig. 3A). The germinal cells occur at the posterior region of the testis. The spermatogonial cells, primary spermatocytes, secondary spermatocytes, and spermatids are arranged in ascending series from the posterior to the anterior region of the testis. The spermatids transform into spherical, immotile mature

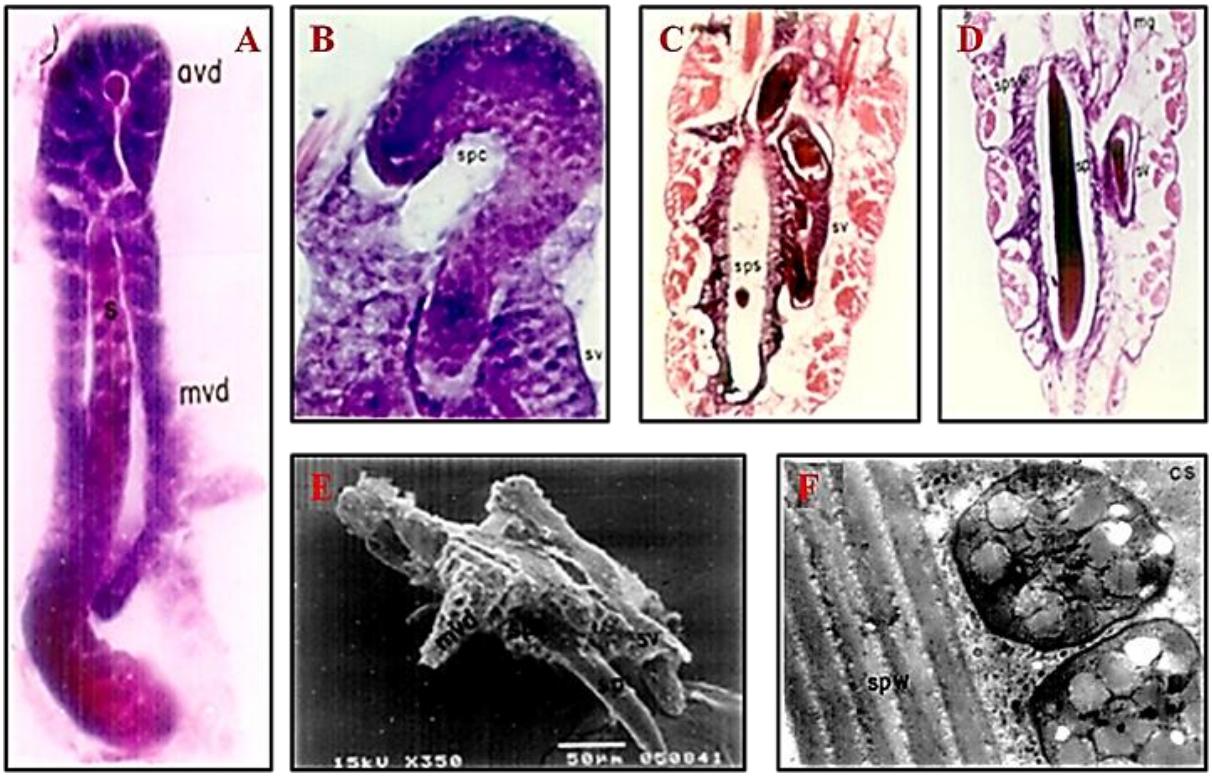

Figure 4. Genital duct of male *Sinodiaptomus* (*Rhinediaptomus*) *indicus*: (A) Histology of anterior and mid vasdeference, (B) Seminal vesicle and spermatophore content, (C) Seminal vesicle and Spermatophoric sac, (D) Spermatophoric sac with Spermatophore, (E) SEM of Genital duct with spermatophoric content and fully formed spermatophore, (F) TEM of Spermatophore wall and spermatozoa.

sperm at the anterior region of the testis. The immature testis shows mostly pre-spermatogenic cells (Fig. 3B); the testis of this animal in the peak phase of reproduction contains, apart from spermatogenic cells, numerous sperms in the anterior cavity of the testis (Fig. 3C). The testis of the spent animals contains reproductive cells sparsely distributed (Fig. 3D).

Spermatophore formation: Spermatogenesis is completed in the testis, and sperm are packed into a tubular spermatophore in the genital duct. The spermatozoa glued in the secretory material secreted by the anterior vas deferens pass into the mid vas deferens (Fig. 4A). The spermatozoa and secretory material of the vas deferens are then transferred to the seminal vesicle for storage. The seminal vesicle also produces secretory material that is used in the formation of the spermatophore's wall. From the seminal vesicle, spermatophoric contents form a single spermatophore, which is transferred to the spermatophore sac, where a tubular spermatophore is molded (Fig. 4B, C). The tubular spermatophore has an elongate body and a narrow neck (Fig. 4D). The spermatophore sac produces adhesive material for the attachment of the spermatophore to the female genitalia. From the histology of the different regions of the male reproductive system and scanning electron microscopic observation of the seminal vesicle and spermatophoric sac, the availability of five spermatophore-forming materials is inferred in the genital duct of this animal (Fig. 4E). The mean length and width of the spermatophore of *S*. (*R*.) *indicus* are 396±22 and 36±7 µm, respectively. The sperm of *S*. (*R*.) *indicus* is immotile and spherical (Fig. 4F).

Mating and spermatophore transfer: During mating, male *S*. (*R*.) *indicus* transfers a spermatophore to the female and precisely attaches it to the female genital pore. The initial approach is made by the male grasping the female's caudal setae with its geniculate right antennule, then suddenly pulling the female downwards, followed by a somersault to hold the female with the claw of its right P5. After establishing such contact, the male and female face ventrally opposite each other in the form of a 'V'. The male and

female ascend to a height and then passively descend. During one such descending movement, the male extrudes the spermatophore and transfers it to the female.

Male reproductive potential: Laboratory experiments on the reproductive potential of male *S*. (*R*.) *indicus* maintained with different combinations of females (1 male + 1 female, 1 male + 2 females, 1 male + 3 females) showed interesting results. In the first combination (1 male + 1 female), at the end of 25 days, the female had produced 13 ovisacs. In the second combination (1 male + 2female), a total of 22 ovisacs were produced. In the third combination (1 male + 3 females), a total of 34 ovisacs were produced. It could be taken as an indirect indication of the reproductive capacity of the male to remate with the female and also produce a large number of spermatophores during its life span.

Discussions

The reproductive process of copepods commences at the end of the sixth copepodid stage, beginning with a chemically induced, mate-seeking behavior. Usually, in most of the calanoid copepods, it is a species-specific, precise, and habitual activity followed through various morphologically and physiologically directed movements and orientations of the male with the female and ends with the attachment of a spermatophore to a precise location on the female genital segment (Blades-Eckelbarger and Youngbluth, 1980). Such a species-specific mating ritual is observed in *S*. (*R*.) *indicus* by the male, leading to the attachment of a spermatophore precisely to the female reproductive pore, where a temporary spermatheca is formed and the spermatozoa are discharged into it. The structure and functioning of the geniculate appendages in this diaptomid are unique modifications for precise mate recognition, mating, the transfer of spermatophores, and the effective fertilization of eggs. In another calanoid copepod, *Heliodiaptomus viduus*, also species-specific geniculate appendages, performing copulation and transfer of spermatophore are reported earlier (Altaff, 2003). In general, freshwater diaptomid copepod females require remating to produce every clutch of eggs due to the absence of sperm-storing seminal vesicles (Sheriff and Altaff, 1993). Probably, such a requirement necessitated a high reproductive potential in males, leading to an increased number of spermatophores produced during their lifespan.

From a mass culture point of view, the S. (R.) indicus possesses many advantageous characteristics, including omnivorous feeding, a short embryonic development period, and a lifespan of about 75 days under laboratory conditions. Further, with mixed algae and Baker's yeast diet, *S*. (*R*.) *indicus* is capable of producing high fecundity, enabling high-density culture. The female *S*. (*R*.) *indicus* from maturity (end of sixth Copepodid stage) produces an ovisac on alternate days till the end of lifespan. It produces 23±3 ovisacs carrying 32±5 eggs per clutch. Nevertheless, a higher number of eggs is produced in the first three quarters of the reproductive phase than in the last quarter (Dharani and Altaff, 2004). Unlike marine calanoid copepods, in the case of male *S*. (*R*.) *indicus,* higher longevity is recorded compared to females. The histology of the testis of immature, mature, and spent males indicates a longer active reproductive phase followed by a short declining phase of reproduction. The 34 spermatophores produced by the males during the 25-day experimental period indicated a high reproductive potential. Such a high rate of spermatophore production has been reported for the first time.

Studies on the growth and survival of the larval snakehead, *Channa striatus*, yielded results comparable to those with *Artemia* nauplii and live zooplankton (Mehrajuddin War et al., 2011). Furthermore, the biochemical profile of S (R) indicus contains the desired levels of polyunsaturated fatty acids and essential amino acids in the post-larvae of *Macrobrachi*um *resenbergii* (Aman and Altaff, 2004). The *S*. (*R*) *indicus* produces subitaneous eggs in normal conditions and diapausing eggs during unfavourable environmental conditions. Diapausing eggs were observed in both sediment samples from wild and laboratory culture systems (Dharani and Altaff, 2004). The diapausing eggs give the advantage

of storing them dry and hatching them to nauplii when required as live prey. Thus, copepods are a natural and ideal food source for many finfish and shellfish larvae, and the availability of this live feed through mass culture will promote faster growth and higher survival rates of larval rearing in hatcheries. In this regard, the present study suggests *S*. (*R*.) *indicus* as an ideal species of calanoid copepod for mass production.

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