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Anatomy and histological evaluation of the reproductive system of marine calanoid copepod *Centropages furcatus* from a mass culture perspective

M. Asrar SHERIFF[1], Vijayaraj RADHA[2], Kareem ALTAFF[1, 2]

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[1] The New College, Department of Zoology, Chennai – 600014, India.

[2] AMET University, Department of Marine Biotechnology, Aquaculture Research Laboratory, Chennai – 603112, India.

ORCID IDs of the author(s):
M.A.S. 0000-0002-3562-2534
V.R. 0000-0002-4170-1685
K.A. 0000-0001-9178-2519

ABSTRACT

Marine finfish culture is a priority area for future human food security. Although many marine finfish species have been domesticated and successful breeding and spawning in captive conditions are achieved, the larval rearing from hatchling to fingerling stage to the desired level is yet to be achieved. One of the essential impediment factors in this process is the availability of suitable live feed for the finfish larvae. The traditional live feeds (*Artemia* nauplii and rotifers) must be more adequate in size spectrum and nutritive value to many marine finfish larvae. In nature, copepod nauplii, copepodite stages, and adults constitute the preferred food of marine finfish larvae. Copepod size spectrum, nutritive value and swimming movements make them ideal live prey items for fish larvae. Nevertheless, the high-density culture of copepods is challenging due to their sexual reproduction and high species-specific variability. Further, adequate knowledge of the candidate species' food and feeding habits, reproductive biology, and life cycle strategies should exist. With this objective present study describes the female and male reproductive system, oogenesis and spermatogenesis, egg production, and reproductive potential of a candidate calanoid copepod species, *Centropages furcatus.* This basic information will help develop mass culture protocol for this species.

Keywords: Copepods, Reproductive system, Anatomy, Histology

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Correspondence:
Kareem ALTAFF
E-mail: kaltaff@gmail.com

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Introduction

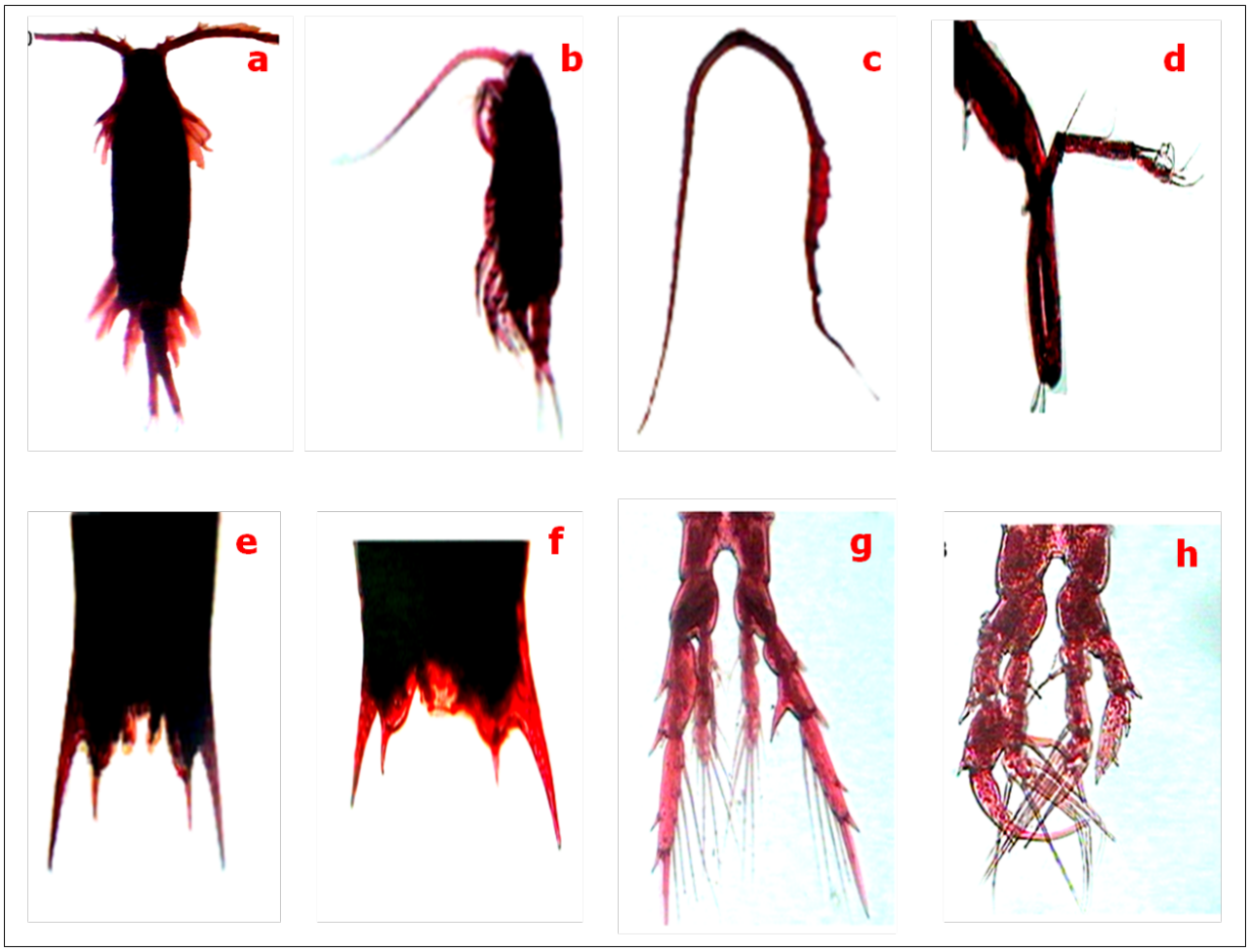
Coastal aquaculture and mariculture are essential in many developing countries' livelihoods, employment, and local economy. Mariculture is practised in areas adjacent to the sea, such as land ponds along the coast and closed lagoons. Advancements in breeding technology, disease management, feeds, and nutrition are vital areas in this field that can improvise and increase efficiency (Araujo et al., 2022). According to FAO (2022) 2020, farmed finfish reached 57.5 million tonnes in 2020, including 49.1 million tonnes from inland aquaculture and 8.3 million tonnes from mariculture in the sea and coastal aquaculture on the shore. Despite the high diversity of marine finfish cultivable species, mariculture contributes far less than inland aquaculture. Although broodstock of many marine finfish is developed and successfully made to spawn in captivity, the desired level of larval rearing for their farming is yet to be achieved (Kailasam et al., 2020). One constraint in the hatchery rearing of finfish larvae is the availability of suitable live feed for different larval stages. The live feed's size spectrum and nutritive value determine the larvae's growth, metamorphosis, and survival. The traditional live feed currently used in finfish larval rearing is rotifers and *Artemia* nauplii, which need improvement to support larval growth and survival. The life cycle of calanoid copepods from tropical marine waters shows six naupliar and six copepodite stages, the sixth being the adult. The body length of the post-embryonic stages from the first nauplius to adult ranges between 95µm to 1680 µm. Further, they contain higher polyunsaturated fatty acids, particularly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), compared to rotifers and *Artemia nauplii*. Copepods constitute the primary food source for finfish larvae in nature, and many reports indicate better performance of finfish larval production in hatcheries when copepods are used as live feed (Rajkumar, 2006; Conceição et al., 2010; Ajiboye et al., 2011; Ma et al., 2013; Rønnestad et al., 2013; Kline and Laidley, 2015; Barroso et al., 2015; Burgess et al., 2020; Vijayaraj et al., 2022). Although many published reports have been on laboratory-scale mass production of copepods, achieving the commercial scale required for commercial finfish larval rearing is still to be accomplished (Imelda et al., 2015; Santhosh et al., 2018). Significant issues in developing copepod high-density culture include species-specific dietary requirements and reproductive patterns (Altaff, 2020). The reproductive potential and survival of copepods may have an unfavourable impact if a suitable diet and physicochemical parameters of the culture medium are not provided. In developing copepod culture, essential aspects of cultivable species required include sex ratio, egg production, egg hatching success, development of nauplii and copepodite stages, and population growth (Altaff and Vijayaraj, 2021). Copepods were the most appropriate live prey for sustainable culture practices of many marine finfish with small mouth sizes and nutritional requirements. The present study reports the reproductive biology of a potential calanoid copepod, *Centropages furcatus*.

Material and Methods

Zooplankton samples were collected offshore of the Kovalam station in Chennai (13.0827° N,80.2707° E) and preserved in 5% buffered formalin. The calanoid copepod *C. furcatus* was separated from the zooplankton sample and identified at the species level (Kasturirangan, 1963; Conway *et al.,* 2003; Lacuna et al., 2013). Live male and female *C. furcatus* were separated and transferred into 2L beakers containing filtered seawater. They were maintained with a mixed algal diet consisting of *Isochrysis* sp.*, Chaetoceros muelleri,* and *Chlorella marina*. To examine the female and male reproductive system of *C. furcatus in situ*, borax–carmine stained and acetic acid differentiated specimens were dissected in glycerol–ethanol mixture under a stereoscopic dissection microscope (Pantin, 1964). The female and male reproductive systems of *C. furcatus* were described using the terminology of Hopkins (1978) and Dussart and Defaye (1995). For histology, the specimens were fixed in aqueous Bouin's fluid, dehydrated in ethanol, cleared in xylene, and embedded in paraffin wax. Serial sections (cross sections and longitudinal sections) of 8 µm were cut and stained with haematoxylin and then counterstained with alcoholic eosin (Jeevaji *et al*., 1983). The different parts of the reproductive system were studied under a compound microscope and photomicrographed at magnifications of 100x and 400x. The live male and female *C. furcatus* collected from the wild were domesticated by raising them in 20L beakers for several generations with a microalgal diet to obtain desirable stock. To study their life span and fecundity, ten batches of males and females were maintained in 2L beakers, and survival and fecundity were followed for 38 days and recorded. Individual adult females and males were maintained in 500 mL beakers and constantly observed for mating and spawning. The number of eggs spawned was counted under a stereoscopic dissection microscope, and the mean of ten spawning was recorded. The body length of the six naupliar and prosome lengths of six copepodite stages of *C. furcatus* was measured under a stereoscopic dissection microscope (Magnus: MSZ-TR with Magcam D series) using ocular micrometre and a mean of ten measurements was recorded.

Results and Discussion

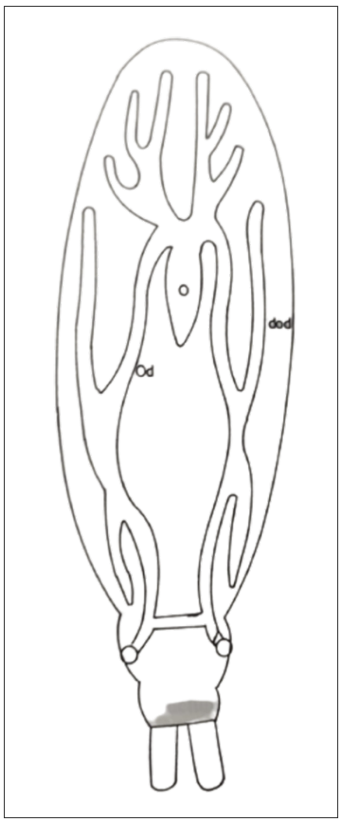
The transparent body of *C. furcatus* has an extended, slender anterior portion. Figures 1a and b show the male and female species' lengths, respectively, at 1.4mm and 1.6mm. This species' antennule, metasome, and P5 exhibit apparent sexual dimorphism in both sexes. According to Figures 1c and d, the male's right antennule is geniculate, with numerous expanded segments and articulation intended to grip the female during copulation. In contrast to the male's, the female's metasomal wings are symmetrical (Figures 1e and 1f). A rounded protrusion is present at the proximal area of the extension on the second exopodite segment of the right P5 of the male. In contrast to the male P5, which is asymmetrical and adapted to deliver spermatophore to the female vaginal pore, the female P5 is symmetrical (Figures 1g and h).



Figures 1. a. Adult female; b. Adult male; c. Antennules of male; d. Distal region of geniculate antennule; e. Metasomal spines of female; f Metasomal spines of male; g P5 of Adult female; h P5 of Adult male

Anatomy of the Female Reproductive System

The median ovary is 460 ±16 µm in length, and the pair of genital ducts make up the female reproductive system of *C. furcatus*. The oviduct takes a posterior route and travels near the ovary before diverting towards the lateral side of the prosome. The genital ducts emerge anterolaterally from the ovary and give rise to the anterior diverticula. They continue till the prosome's tip, at which point they expand into the genital segment and open to the outside. The oviduct produces Diverticulae, which occupy the posterior-lateral portion of the prosome. The genital segment has two seminal receptacles (Figure 2).



(*dod - diverticular of oviduct, o - ovary, od – oviduct*)

Figure 2. Female Reproductive system

Histology of the Female Reproductive System

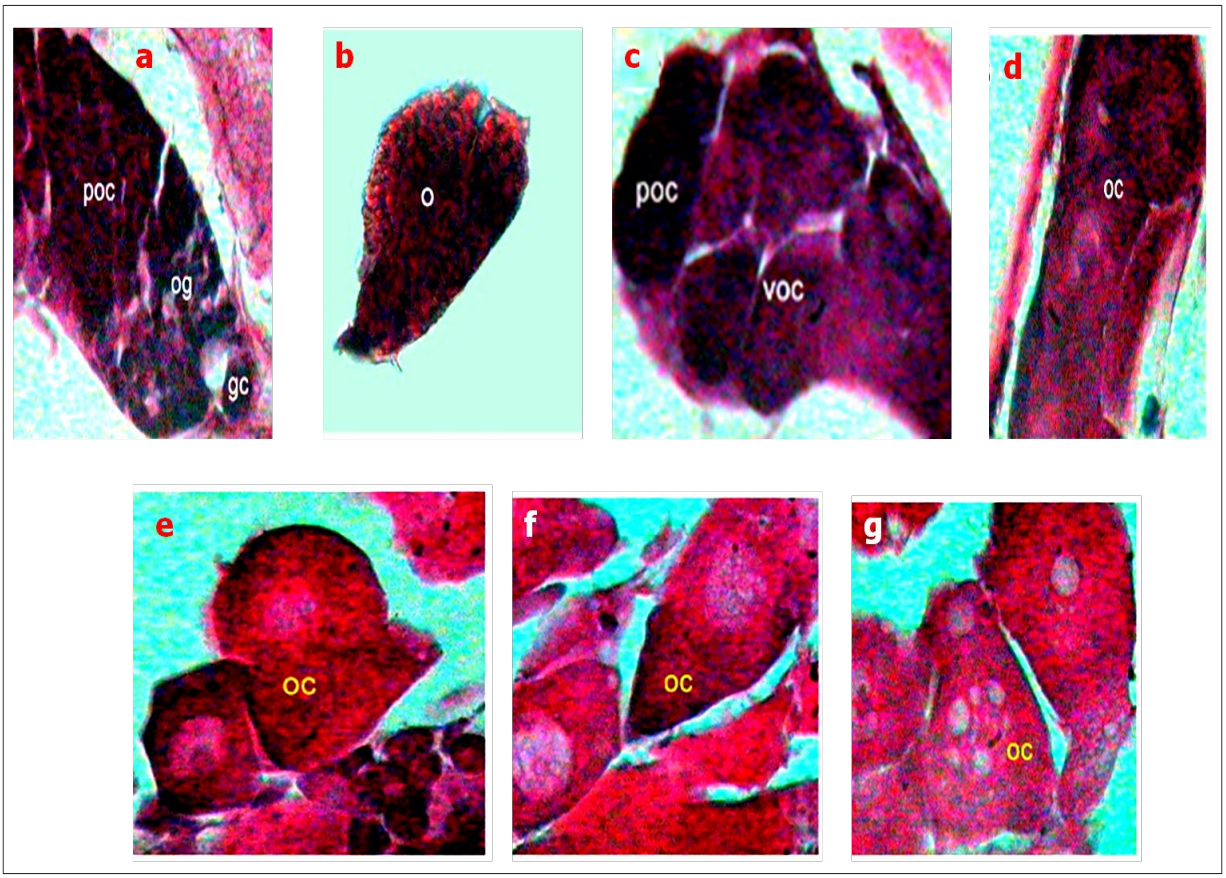
The ovary of the *C. furcatus* has a thin wall with epithelium of rectangular cells and a connective tissue layer. Different phases of oogenesis are grouped in ascending rows up to the front end of the ovary, where the germarium is located (Figures 3a and b). Oocytes in the oviduct go through vitellogenesis. Acidophilic and heavily stained with hematoxylin, the oogonia and primary oocytes are both oocytes. Oogonia develop deeper pinkish with hematoxylin and eosin stain as vitellogenesis progresses and becomes more basophilic. Figures 3c–g show how tightly packed the yolk granules and globules are inside the mature egg. For this species, there are no oocytes in the posterior portion of the oviduct. The posterior section of this species' oviducts is of simple and unaltered construction because the *C. furcatus* releases fertilised eggs straight into the medium. The length and width of a mature oocyte in this species are 112µm and 70µm, respectively.

Anatomy of the Male Reproductive System

A single genital duct and the median testis make up the male reproductive system. The testis is an organ that is long and narrow at the back. From the anterolateral portion of the testis, the vas deferens emerge and travel through the left side of the perivisceral cavity to the last thoracic segment. The mature spermatozoa are subsequently stored in a sizeable seminal vesicle that develops as it ascends towards the body's centre. The ductus ejaculations in the vaginal segment connect the seminal vesicle to the tubular spermatophore sac, which then opens to the outside (Figure 4).

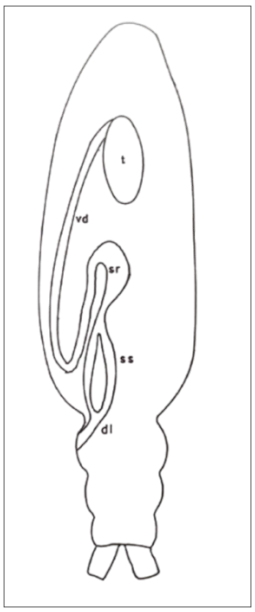
Histology of the Male Reproductive System

The male reproductive system of *C. furcatus* shares several characteristics with *Pseudodiaptomus serricaudatus* and other calanoid copepods in histology. However, the testis and several genital duct locations are very noticeable in this species. The germinal zone's location and the various stages of spermatogenic cells are similar to those of other calanoid copepods, although there are more spermatozoa in the testis' anterior cavity. Other calanoid copepods' spermatozoa are smaller than those of *C. furcatus.* The vas deferens, and seminal vesicle walls are glandular and secrete much material. A substantial amount of core secretion and numerous spermatozoa are tightly packed together in the spermatophore, possibly to retain and nourish the spermatozoa (Figures 5b–l). High fecundity is observed in *C. furcatus* due to the direct broadcasting of fertilised eggs to the medium. As a result, more spermatozoa must be readily available to fertilise the eggs.



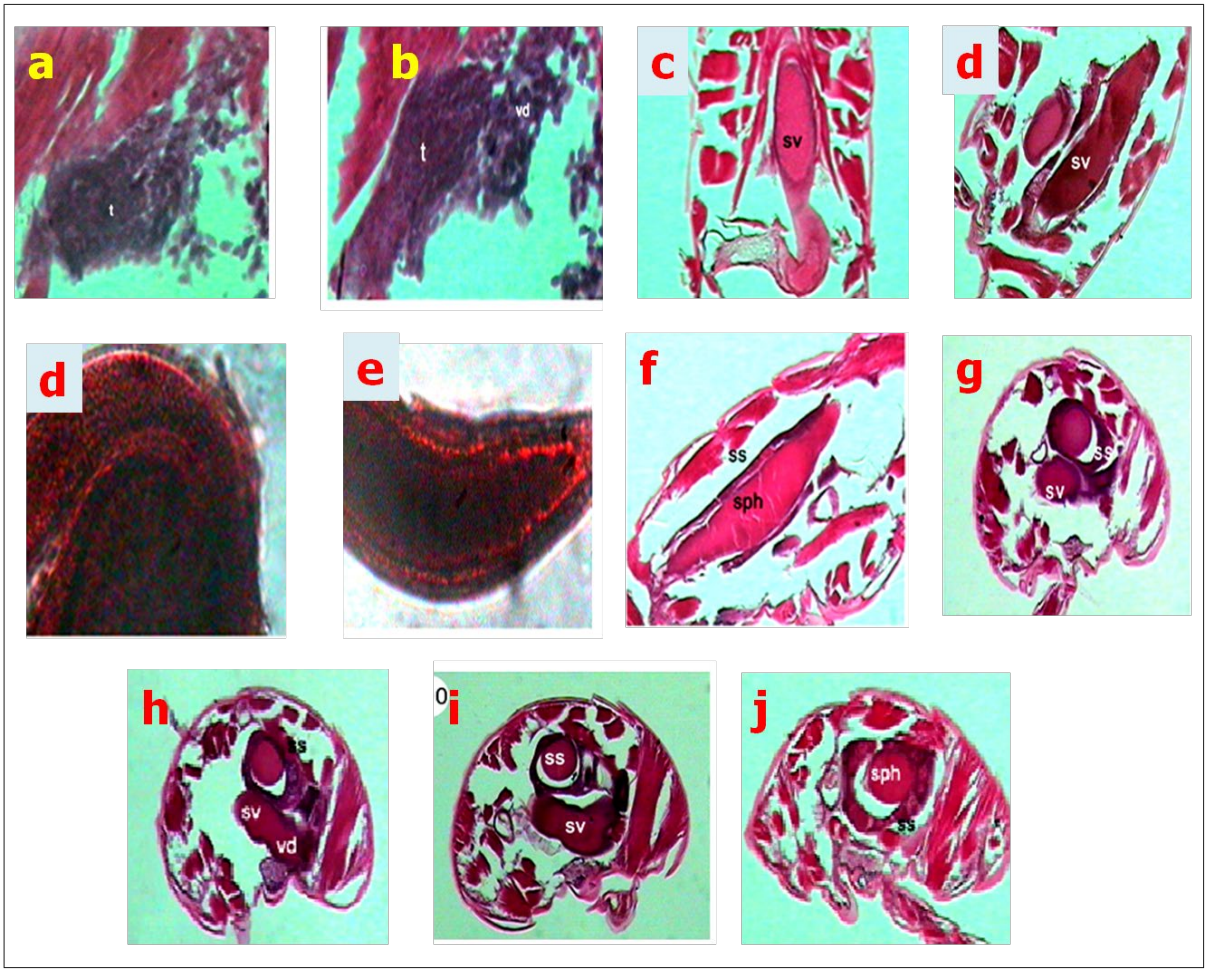
(poc – primary oocytes; voc – vitellogenic oocytes; o – ovary; od – oviduct; odd – oviduct diverticulum; og – oogonial cells; gc – germinal cells)

Figure 3. a and b. Ovary; c and d. Oocytes in the oviduct; e - g. Oocytes in different stages of vitellogenesis



(*t– testis, vs-vas deferens, sv- seminal vesicle, ss- spermatophore sac, de- ductus ejaculations*)

Figure 4. Male Reproductive System



(t – testis; vd- vas deferens; sv – seminal vesicle; ss - spermatophore sac; de - ductus ejaculatoris; sph - spermatophore)

Figure 5. a. Testis; b. Vas deferens; c and d. Seminal vesicle; e and f. Proximal and Distal regions of seminal vesicle; g. Spermatophore sac; h, i and j. C.S. of male showing Vas deferens, Seminal vesicle, Spermatophore sac and Spermatophore

Spermatogenesis and Spermatophore Formation

The spermatogonial cells, which are round and have a prominent, intensely pigmented nucleus, are produced by the testis' germinal cells. The spermatogonial cells change into primary spermatocytes when their volume doubles, subsequently changing into secondary spermatocytes. The smaller secondary spermatocytes, which exhibit a dense nucleus than the earlier stages of spermatogenesis, are reduced to generate the spermatids. The cytoplasm and nucleus of the spermatids alter as they develop into spermatozoa. The nucleus and cytoplasm of the spermatozoa produced by *C. furcatus* are spherical.

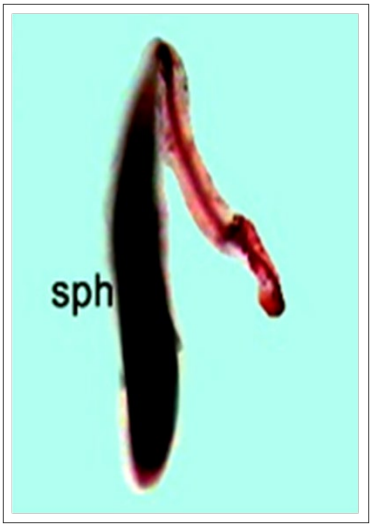


Figure 6: Spermatophore

The secretory material generated by the various genital duct regions is used in the spermatophore sac to form the *C. furcatus* spermatophore. With a broad posterior and a very narrow anterior section, it exhibits an extended tube-like form (Figure 6). During this process, Spermatozoa are moved from the testis to the vas deferens and suspended in the secretory substance. The distal portion of the vas deferens secretes an extra granular secretory material that surrounds the spermatozoa and core secretory material. A very pronounced and lengthy seminal vesicle receives the spermatozoa and the vas deferens secretary material. The spermatophore wall is formed by the secretary material produced by the thick glandular wall of the seminal vesicle. The seminal vesicle includes various spermatophore constituents organised in a spermatophore-like structure. The seminal vesicle's spermatophores material, intended to create a single spermatophore, is released into the spermatophore sac. A tubular spermatophore is created from the spermatophore sac's secretory material, and the spermatophore material is derived from the seminal vesicle. A layer of spermatozoa, an inner layer of secretary material, a layer of core secretary material, and an outside formidable wall make up its structure. Additional secretary material is also produced by the spermatophore sac's wall and functions as an adhesive to secure the spermatophore to the female genitalia. This species has a high reproductive potential and the ability to produce numerous spermatophores, as evidenced by the dense spermatogenic cells in the testis, the highly glandular form of the vas deferens and seminal vesicle, and the vast quantity of spermatozoa, and secretary material in the genital duct. Under laboratory conditions, an adult *C. furcatus* showed a life span of 38 days and produced four batches of eggs (28ºC temperature, 30 PSU salinity, pH 7.8 and dissolved oxygen 5.5 mg/L). The average egg measures 74 ±3 µm in diameter, and 92 ±14 eggs were counted in a batch. This species has six nauplii and six copepodite stages, with a post-embryonic development period of seven (7 ±1) days.

The body length of different naupliar stages and the prosome length of copepodite stages of *C. furcatus* is presented in Table 1. The length of the nauplii and copepodite stages of *C. furcatus* was found to be size-wise suitable live prey for fry to fingerling stages of marine finfish larvae.

Table 1. Body length and prosome length of naupliar and copepodite stages of *C. furcatus* (µm)

	Body Length (µm)		Prosome Length (µm)
Nauplii	NI 108 ±3	Copepodite	CI 326 ±11
	NII 122 ±5		CII 378 ±14
	NIII 167 ±4		CIII 590 ±18
	NIV 212 ±12		CIV 810 ±23
	NV 247 ±6		CV M 1210 ±21
	NVI 281 ±9		CV F 1340 ±17
	--		CVI M 1482 ±26
	--		CVI F 1638 ±18

The calanoid copepods that are free-living exhibit either perennial or seasonal occurrences. While *Centropages congeners* showed a seasonal succession, species like *Acartia clausi*, *Temora longicornis*, and *Pseudocalanus* spp. were reported throughout the year. According to Razouls et al. (2005–2023), the *C. furcatus* is a widely distributed epipelagic species that primarily inhabits equatorial and subtropical regions. According to Kavitha et al. (2018), the East coast of India experiences many *C. furcatus* sightings throughout the year. Most copepod species' yearly reproductive cycles exhibited similar patterns, with maxima in egg production rates occurring between the end of April and June and substantially lower rates occurring in the other months. However, for species like *Centropages typicus*, the highest egg production rates were seen towards the middle of September, during the first week of the species' appearance in the plankton (Halsband and Hirche, 2001). Although many calanoid species from cooler climates have been shown to have a comparable reproductive cycle and egg production (Lindley 1990), species from warmer climates are continuous breeders and actively reproduce all year long (Ianora and Scotto di Carlo, 1988; Ianora and Buttino, 1990). Compared to *Pseudodiaptomus* species from India's east coast, *C. furcatus* has more documented fecundity (Altaff, 2020). Many calanoid copepod species' egg production is significantly impacted by environmental conditions such as temperature, salinity, and phytoplankton content (Dilshad Begum et al., 2912; Dvoretsky and Dvoretsky, 2014). The calanoid copepods exhibited apparent sexual dimorphism, as evidenced by the larger size of the female, the geniculated antennule of the male, the presence of more urosomal somites in the male, the different pattern of the caudal setae in the female, and the modified P5 in both sexes. The male's antennule is asymmetrical and extensively adapted to facilitate copulation and spermatophore transfer to the female during mating, in contrast to the female's simple and symmetrical antennule. Both sexes'

fifth thoracic legs frequently differ from the typical morphology of the other thoracic legs. In the female, they may be diminished or completely absent. Still, in the male, they are typically changed to form a complex prehensile organ that allows them to grasp the female and attach spermatophores to the female vaginal pore. *P. annandalei*, *P. serricaudatus*, and *T. discaudata* had more modified right antennules in the male and P5 in both sexes than *C. furcatus* (Altaff, 2020).

In most calanoid copepod species, the female reproductive system is characterised by a median ovary, two oviducts, and a segment of the genitalia containing the seminal receptacle (Marshall and Orr, 1972; Corkett and McLaren, 1978; Blades-Eckelbarger and Youngbluth, 1984; Razouls et al., 1986, 1987; Norrbin, 1994; Eisfeld and Niehoff, 2007). The size and shape of the ovary, the diverticula in the oviduct's posterior area, the structure of the antrum, and the structure of the seminal receptacles, on the other hand, are all subject to modification (Altaff, 2020). Compared to other calanoids, the female reproductive system of C. furcatus demonstrates greater specialisation, with a conspicuously large ovary and oviducts occupying the anterior to the posterior portion of the prosome. Such large oviducts and their diverticular suggest the ability to generate more eggs.

According to Eckelbarger and Blades-Eckelbarger (2005), the ovarian histology of *C. furcatus* is consistent with the pear-shaped structure previously described for calanoid copepods. Calanoid copepods typically have ovaries in the middle of the prosome, and several stages of ovarian cells arranged spatially from the posterior to the anterior. Similar arrangements are seen in *C. furcatus*, where vitellogenesis and oocyte maturation occurs outside the ovary while the mitotic proliferation of germinal cells and their transformation into previtellogenic oocytes stage through the meiotic process take place inside the ovary. The most mature oocytes form the most ventral layer as some calanoid copepods' previtellogenic oocytes migrate from dorsal to ventral throughout the anterior and posterior diverticula (Niehoff, 2007). However, Ceballos-Vazquez et al. (2009) showed a significant difference regarding the oocyte migration from the ovary in *C. furcatus*. The current study shows this difference in oocyte migration from the ovary to the oviducts and diverticular.

Previtellogenic oocytes in *C. furcatus* were discovered to disperse from the ovary and arrange themselves on the inner wall, forming an outer layer that spans the prosome's entire length and circumference, allowing for a greater area for oocyte maturation and, as a result, favouring continuous egg production. The present study demonstrates such a configuration of vitellogenic oocytes and the vitellogenesis and oocyte maturation processes. Niehoff (2007) classified the *Centropages* species. *C. bradyi, C. hamatus, C. typicus,* and *C. violaceus* as having gonads of the Calanus type, indicating that most oocyte development stages take place simultaneously and that many oocytes typically mature synchronously. The geographical distribution of oocytes is an essential trait that differs from those of the Calanus-type gonad (Ceballos-Vazquez et al. 2009). According to studies done on Calanus-type gonads (Niehoff & Hirche, 1996), the most mature oocytes form the most ventral layer and expand and mature to varying degrees along the lengths of both anterior and posterior diverticula (Niehoff, 2007). The most mature oocytes are found closest to the middle of the body in *C. furcatus,* where the increase in oocyte size and degree of maturation was observed to occur radially towards the centre. From there, the oocytes are carried ventrally through the products to the genital pore. Based on these observations, Ceballos-Vazquez et al. (2009) proposed the furcatus-type gonad as a new gonad morphological type for *C. furcatus.* The current findings support Ceballos-Vazquez et al. (2009)'s hypothesis regarding the kind of *C. furcatus* gonad shape and ongoing egg-laying.

Regarding spawning and embryonic development, calanoid copepods primarily use two primary tactics. In certain species, eggs are released into the environment after fertilisation, and embryonic development occurs in the external medium. In other species, eggs are released into the ovisac following fertilisation. The posterior portion of the oviduct changes into a glandular structure for ovisac production, producing secretory material specifically for ovisac formation. Unlike species whose embryonic development occurs in the ovisac, the *C. furcatus* belongs to the broadcasting form of spawning, which allows for more significant egg production. For *C. typicus*, Carlotti et al. (2007) found that the combination of a temperature-dependent development rate and a food-dependent growth rate in the shelf regions is a more favourable environment than in the offshore regions, as the shelf regions support large females, the production of large numbers of better-quality eggs, and probably better offspring survival. For the *C. furcatus*, a similar observation is applicable.

The male is an active partner in finding and capturing the female in the reproductive biology of calanoid copepods. The right antennule in the male and P5 in both sexes, which play numerous vital roles during copulation and spermatophore transfer, are sexually dimorphic appendages that have evolved. The male first recognises a mate; then, the female is captured. The male transfers and attaches a spermatophore to the female removes the discharged spermatophore, and finally, the female fertilises the eggs and releases them (Ohtsuka and Huys, 2001). *C. furcatus* has also been found to exhibit such sophisticated sexual dimorphism (Maria et al.,

2013). For calculating the male-to-female ratio in the mass culture of this species, *C. furcatus* has an advantageously quick and easy copulation and spermatophore transfer process.

The germinal zone is in the posterior portion of the testis of C. furcatus, similar to the case of other calanoid copepods. According to Marshall and Orr (1972), Park (1966), Corkette and McLaren (1978), Hopkins (1978), and others, all species' germinal cells are spherical in shape, extensively stained with haematoxylin, and have giant nuclei. Nevertheless, there is variety in their size. As the various phases of spermatogenesis occur in ascending rows from the posterior to anterior region, the process of spermatogenesis is completed within the testis in all species (Hopkins, 1978). This spermatogenetic process has been observed in the majority of calanoid copepods. Although the histology of the testis of *C. furcatus* is like that of other calanoid copepods, in this species, the testis is ovoid in shape during the CIV and CV stages and becomes elongated in the CVI stage. The posterior part of the testis contains germinal cells, and other spermatogenic stages occupy the anterior part of the testis in ascending rows. This species' spermatozoa have a spherical form and are not mobile.

The modification of the *C. furcatus* genital duct into the vas deference, seminal vesicle, spermatophore sac, and ductus ejaculators show similarity to other calanoid copepods (Blades-Eckelbarger and Youngbluth, 1991a). All of these regions of the genital duct in *C. furcatus* are extensively developed with ample secretory material. Typically, calanoid copepods use the secretory material produced by the vas deference, seminal vesicle, and spermatophore sac to produce an elongated or flask-shaped spermatophore in the spermatophore sac. However, the variety of secretory materials generated depends on the sort of spermatophore a calanoid species produces. According to Park (1966), Hopkins (1978), Blades and Youngbluth (1981), and Blades-Eckelbarger (1991), the front section of the spermatophore of calanoid copepods can either be a simple tube or a sophisticated coupling plate-like structure. When mating and spermatophore transfer, the spermatophore of *C. furcatus* is of a fundamental kind with a narrow and long tube that may be linked to the female's genital hole simply and precisely. This promotes excellent reproductive potential.

The current study on the *C. furcatus* reproductive system revealed several distinctive characteristics, including year-round natural occurrence, continuous breeding, and high reproductive capacity in both sexes. The high fecundity of this species, which produces more than 100 eggs in each clutch, is also far higher than the fecundity reported for many calanoid copepods that are now being mass-produced. Numerous marine finfish larvae in various developmental stages can be found as live prey on *C. furcatus* nauplii, copepodites, and adults in the abovementioned size range. This species' broadcasting form of spawning also allows mass production of diapausing dry eggs. These qualities make *C. furcatus* a potential top choice for mass cultivation and use in aqua hatcheries to develop finfish larvae.

Conclusions

An Indigenous live feed is essential for rearing marine finfish larvae in hatcheries to enhance their growth and survival rates. Copepods serve as suitable live prey, and the commercial production and utilisation of copepods could have beneficial mariculture. Compared to the free-living planktonic marine calanoid copepod species occurring in nature, only a small number have been mass-cultured and utilised as live prey for rearing marine finfish larvae. The present study reports the reproductive biology of a potential calanoid copepod, *C. furcatus,* and its advantageous features for mass production.

Compliance with Ethical Standards

Conflict of interest: The authors declare that they have no actual, potential, or perceived conflict of interest for this article.

Ethics committee approval: Ethics committee approval is not required for this study.

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Disclosure: -

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