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Tumour-like Anomaly of Copepods - An evaluation of the possible causes in Indian Marine Waters

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Globally, Tumour like Anomalies (TLA) in copepods and the critical assessment of their possible causes are rare. The exact causative factor and ecological consequences of TLA in copepods are still unclear and there is no quantitative data available so far to prove conclusively the mechanism involved in developing TLA in copepods. TLA in copepods are considered as a potential threat to the well-being of the aquatic food web prompted us to assess these abnormalities in Indian marine waters and assessed the possible etiological agents. We carried out a focused study on copepods collected from 10 estuarine inlets and 5 coastal waters of India using a FlowCAM, advanced microscopes and laboratory incubated observations. The analysis confirmed the presence of TLA in copepods with varying percentage of incidence in different environments. TLA was recorded in 24 species of copepods, which constituted ~1- 15% of the community in different environments. TLA was encountered more frequently in dominant copepods and exhibited diverse morphology - ~60% was round, dark and granular, whereas ~20% was round/oval, transparent and non-granular. TLA was mostly found in the dorsal and lateral regions of the prosome of copepods. The three suggested reasons/assumptions about the causes of TLA such as ecto-parasitism (*Ellobiopsis* infection), endo-parasitism (*Blastodinium* infection) and epibiont infections (*Zoothamnium* and *Acineta*) were assessed in the present study. We did find infections of endo-parasite *Blastodinium* , ecto-parasite *Ellobiopsis* and epibiont *Zoothamnium* and *Acineta* in copepods, but these infectious percentages were found <1.5% to the total density and most of them are species specific. Detailed microscopical observations of the samples collected and the results of the incubation experiments of infected copepods revealed that ecto-parasitism, endo-parasitism and epibiont infections have less relevance to the formation of TLA in copepods. On the other hand, these studies corroborated the view that wounds on the exoskeleton caused by partial predation as the potential reason for the TLA of copepods in Indian waters.

Key words: Copepods, Tumour-like Anomalies, *Blastodinium*, *Ellobiopsis*, Partial predation.

1. Introduction

Copepods, the most abundant crustacean plankton, play a crucial role in the aquatic food web in channelling the organic carbon from primary to tertiary trophic levels. They inhabit almost all aquatic ecosystems ranging from freshwater to hypersaline seawater and surface waters to deep ocean ridges. Several past records have reported significant morphological aberrations/anomalies of copepods from many parts of the world, which include freshwater lakes, estuaries, coastal marine waters and deep ocean ridges (Crisafi & Crescenti 1977; Messick et al. 2004; Skovgaard 2004; Bhandare & Ingole 2008; Mantha et al. 2013). Studies evidenced that these abnormalities of copepods differ in size, morphology, structure and location, taking into account which the terminology 'Tumor-like Anomalies' (TLA) was considered apt for representing all kinds of copepod morphological anomalies/abnormalities recorded from different parts of the world (Skovgaard 2004; Bhandare & Ingole 2008; Omair et al. 1999).

TLA in copepods was first reported from the Mediterranean Sea and later from many other parts of the world (Crisafi & Crescenti 1975). However, a focused research on this topic began once the reports from the famous Lake Michigan gained tremendous scientific interest (Omair et al. 1999; Bridgeman et al. 2000). This unprecedented scientific attention focused upon the Lake Michigan studies was due to the worry that TLA in copepods could be an emerging threat to the well-being of the plankton food webs across the world. Subsequently, several attempts were made globally to understand the incidence and causes of TLA in copepods (Bhandare & Ingole 2008; Mantha et al. 2013; Crisafi & Crescenti 1975). The histological studies of TLA evidenced the manifestation of necrotic tissue expelled from the affected copepods through the process of herniation (Omair et al. 2001; Messick et al. 2004). It was found that the base of the TLA was continuous with the copepod body tissue and it protruded through a fissure in the exoskeleton. Though the exact causative factor and ecological consequences of TLA are still unclear, it certainly decreases the longevity of the affected copepod (Bridgeman et al. 2000).

Though considerable literature is available on the distribution and taxonomy of copepods from Indian waters (Madhupratap 1987; Smith & Madhupratap 2005; Jagadeesan et al. 2013; Fernandes & Ramaiah 2014), virtually no record is available yet on TLA, indicating the research gap in this field. Usual zooplankton analysis considers a small percentage of the total stock for identification, during which it is quite likely to overlook TLA incidences. Therefore, in this paper, we present the results of a focused research on TLA of copepods in Indian waters using a FlowCAM and advanced microscopes, including a Scanning Electron Microscope. The study critically examines the

applicability of various possible causes of TLA and infers the most likely factor in Indian waters. The objectives of the present study are (a) to consolidate information on TLA of copepods by integrating background knowledge gathered from various parts of the world (b) to find out the occurrence of TLA of copepods in Indian waters and compare it with the global scenario and (c) to ascertain the most likely causative factor of TLA in Indian waters.

2. Materials and Methods

2.1. Background Information on TLA

TLA incidences in copepods and their possible causes in different parts of the world have been summarized as the background information (Table 1). Initial records from the Atlantic, Pacific and Indian Oceans linked TLA with long-term environmental change as well as aquatic pollution (Crisafi 1974; Crisafi & Crescenti 1975). Even though the actual environmental change and aquatic pollutant responsible for the observed TLA in copepods were unclear in these studies, several later researchers adhered to this theory to explain the morphological abnormalities in copepods they observed (Silina & Khudolei 1994; Vanderploeg et al. 1998; Dias 1999; Bhandare & Ingole 2008; Mantha et al. 2013). A counterview was presented by Skovgaard (2004) who showed that endo-parasites, especially *Blastodinium*, had the potential to develop TLA in copepods. Another opinion pointed towards the infections of ecto-parasites such as *Ellobiposis* as the potential cause of TLA (Messick et al. 2004; Bhandare & Ingole 2008; Bridgeman et al. 2000; Manca et al. 2004). More recently, it was also suggested that infection of epibionts can lead to the development of TLA in copepods (Mantha et al. 2013).

A careful evaluation of the background information shows that the causative factors of TLA proposed from different parts of the world are mostly assumptions based on the generalized understanding that environmental change/pollution and parasitism can cause negative impacts on aquatic organisms. In other words, there is no quantitative or experimental data available so far to prove conclusively that the suggested factors are actually involved in developing TLA in copepods. In contrast to this, there are a few experimental evidences indicating that wounds on copepods can lead to the development of TLA, and such wounds in natural environment are mostly caused by partial predation or parasitic attacks (Messick et al. 2004; Skovgaard 2004). The parasitic and epibiont infections can also cause stress and swimming impairment to copepods, which in turn can make them more vulnerable to predatory attacks (Ianora et al. 1987). Nonetheless, it is amply clear that the actual reasons behind TLA of copepods in varying environments across the world are still vague and uncertain. Individual ecosystems have large differences in their environmental setting,

including the level of pollution and nature of food web, which impacts the parasitic and predation effects.

2.2. TLA Records in India

TLA records of copepods are virtually absent from Indian waters. The historical studies of zooplankton during the International Indian Ocean Expedition (IIOE) has records of copepod parasites from the Indian Ocean, but their role as a potential causative factor of TLA remains unknown (Sewell 1951; Santhakumari & Saraswathy 1979; Santhakumari 1985). Though there are several records of parasites and TLA of copepods from other parts of the world, including a few from the Central Indian Ocean ridges (Table 1), there is virtually no information available from Indian waters. This background made us suspect the possibility that routine copepod analysis from the Indian waters might have overlooked the TLA incidences, as this subject has never been the focus of research in this part of the world. Therefore, we considered the relevance of the subject and investigated the incidence of TLA in copepods in Indian waters with a careful and critical assessment of its possible causes.

2.3. Methods

Zooplankton samples collected from 10 estuaries, five each along the west and east coasts of India were considered for the present study. Zooplankton collected from selected coastal marine environments along the southwest coast of India (off Kochi), northwest coast of India (off Mumbai), southeast coast of India (Gulf of Mannar and Palk Bay), and central east coast of India (off Kakinada) were also considered for the present analysis. The details of sampling have been provided in Table 2 and the geographical positions of these environments in the Indian subcontinent are represented in Fig.1. During the sampling, surface zooplankton (~0.5m) was collected by horizontal hauls using a standard WP net (200 µM mesh size). The samples were carefully preserved in 4% formalin for further analysis, which include the basic separation of copepods from other taxonomic groups as per a standard protocol (Postel et al. 2000).

During the analysis of copepods in the laboratory, the utmost care was taken not to miss out any incidence of TLA, and parasitic and/or epibionts infection in copepods. A FlowCAM (Fluid Imaging, USA) was used for the preliminary imaging and analysis of copepods for detecting TLA and other anomalies. In the present analysis, five samples of zooplankton representing each environment (50% of subsamples or whole samples) were analysed using the FlowCAM. For practical convenience in imaging copepods in FlowCAM, samples were considered in two size classes (< 500 µM and > 500 µM) separated using a suitable mesh (Hydrobios). For the analysis of the two size classes of

copepods, 600 µm and 1000µm flow cells were utilized for 200 - 500 and >500 size classes, respectively. Damaged or partially imaged specimens of copepods were omitted, and only those with a clear image of prosome and urosome were considered for the present analysis. Copepods with TLA and/or parasitic, epibiont infections detected in the FlowCAM were analysed in detail with a combination of light (Lynx stereo zoom), fluorescence (Olympus IX 51) and Scanning Electron Microscopy (Neoscope – JCM 5000).

2.3.1. Seasonal Variations of the TLAs in Copepods

A few earlier studies conducted elsewhere had showed that TLAs of copepods exhibit seasonal variation in their percentage of occurrence (Crisafi and Crescenti 1977; Manca et al. 2004). These observations represent a completely different geographical setting as compared to the present study domain. Therefore, in the present study, attempts were also made to understand the seasonality of TLA in Indian waters by considering specific cases of one monsoonal estuary (Cochin estuary) and two coastal waters of India (the Gulf of Mannar and the Palk Bay) (Fig.1). Zooplankton samples representing the Spring Intermonsoon (March - May) and the Southwest Monsoon (June - September) were considered for the Cochin backwaters, whereas samples representing the Southwest Monsoon and the Northeast Monsoons were considered for the Gulf of Mannar and the Palk Bay.

The frequency of parasitic and epibiont infection was determined by examining the copepods under a stereo-zoom microscope. The infected individuals were separated and their detailed structures were analysed under inverted and scanning electron microscopes. The endo-parasite *Blastodinium* infection was identified by the dark-coloured parasite trophonts inside the alimentary tract of copepods (Chatton 1920; Sewell 1951; Shields 1994; Skovgaard 2005; Skovgaard et al. 2012). Ecto-parasite *Ellobiopsis* infection was identified based on available literature (Sewell 1951; Shields 1994; Skovgaard & Saiz 2006). Standard publications were utilized for identifying epibionts associated with copepods (Curds 1985; Fernandez-Leborans & Tato-Porto 2000; Utz & Coats 2005).

2.3.1. Life cycle Studies of Ecto- and Endo-parasites and their Role in TLA

Several sets of laboratory experiments were carried out to study the possible role of endo-parasites, ecto-parasites and epibionts in causing TLA in copepods. For experimental studies, live zooplankton samples were collected several times from the Cochin estuarine inlet in 20 L containers and transported to the laboratory. Ecto- and endo-parasite infections were randomly checked for in the collected samples. 5-8 copepod specimens with *Blastodinium* infection in the mid-gut position were selected with a wide mouth pipette using a stereo-zoom microscope and placed individually in 6-well culture plates (6 ml) containing 0.2 µm filtered *in-situ* water. In the case of *Ellobiopsis,* 5-8

individuals of copepods with later stage of infection were isolated and placed in culture well plates. In both cases, they were incubated at room temperature for one week and examined in every 24 hrs to check for development of TLA. For studying epibionts and their relevance to TLA, epibiont-infected copepods were isolated from live samples collected from the Cochin estuarine inlet and incubated in the laboratory for 15 to 30 days. In the present approach, two species of the epibionts (*Acineta* and *Zoothamnium*) were found on the exoskeleton of the copepods. 5-10 individuals with epibiont infections were isolated and maintained in 6-well culture for 10-15 days. The copepods were examined every 24 hrs during the incubation period to inspect for any symptom of TLA development.

2.3.2. Predatory Experiments

Predatory experiments were conducted to assess the role of partial predation in developing TLAs in copepods. Small hydromedusae were used as the predators in the laboratory experiments. In general, the process of predation consists of discrete stages that include encounter, contact, capture, ingestion and digestion. The escape response of copepods after the encounter with the predator leads to partial predation-related wounds in the exoskeleton (Fields et al. 2012). In order to confirm this possibility, an experiment was conducted to monitor the escape responses of copepods exposed to the attack of hydromedusae, which furnished compelling evidence that partial predation can cause TLA in copepods. Thereafter, detailed experiments were conducted to quantify the role of partial predation of a typical carnivore (hydromedusae) in developing TLA in copepods. In the experimental set up, 100 - 250 ml beakers were used for the trial monitoring while the quantification experiments employed 2 litre beakers. In both cases, live copepods and hydromedusae collected from Cochin estuarine inlet were used. Samples were collected in 20 litre polythene cans and brought to the laboratory on the same day. Copepods and hydromedusae were isolated and acclimatized in small acrylic tanks for 24 - 48 hrs. Healthy and active copepods without any signs of damages on the appendages and exhibiting active escape responses against pipette siphoning were used in the experiments. During the experiments, copepods were fed with laboratory-cultured mixed algal cells.

During the trail experiments, five copepods and two small hydromedusae were maintained in each of the five experimental bottles with estuarine inlet water. These bottles were visually monitored for the contact of hydromedusae tentacle with the copepods. Once the contact with the nematocysts occurred, the escape responses of copepods were video recorded using the Progress Pro software of the Olympus microscope (IX91). In the remaining beakers, the surface morphology of the copepods were analysed at the end of the incubation period. The hydromedusae were removed once the

copepods successfully escaped from the contact of the nematocyst. The hydromedusae were removed after the incubation from the non-video graphed beakers also. In both cases, copepods were maintained for 24 hours further and examined for any changes in swimming behaviour and/or development of TLA due to injury from the predatory attack of the hydromedusae. We observed subsequently that copepods with nematocysts attached on their body indeed developed TLAs.

Altogether, 9 experimental bottles were incubated for quantifying the role of predation in developing TLA of copepods. 6 of these had both prey and predator individuals while the remaining 3 had only the prey (control bottles). 180 copepods were placed in the experimental and control vessels (20 each) half an hour before the experiment to ensure steadiness in their swimming. Single hydromedusae per experimental vessel was introduced and incubated for 6 hrs. Later, the incubated hydromedusae were removed from the experimental bottles and the copepods were stained with neutral red as per the standard method of Elliot and Tang (2009). Neutral red staining helps to differentiate the live and dead copepods at the end of the experiment. Live organisms absorb the neutral red stain while the dead do not. Remarkably, most of the copepods displayed no sign of predatory action (damages in body parts), signifying the successful escape of copepods before the predator's contact. Copepod individuals bearing evidences of damage in the appendages or body parts were considered for studying their post-contact escape responses against hydromedusae predation. The differences in the total counts from initial and final (sum of all, including all the cases) counts were considered as the result of hydromedusae consumption.

3. Results

3.1. Occurrence of TLA in Various Copepod Species

FlowCAM analysis during the present study evidenced the occurrence of TLA in all the Indian estuaries and coastal environments studied (Supplementary material 1), with varying percentage of incidence in different environments (Table 2). TLA was recorded in 24 species of copepods (Fig. 2 & Table 3) in which 20 belonged to the order Calanoida, 2 to Poecilostomatoida and 1 each to Harpacticoida and Cyclopoida. The percentage of TLA was generally high in the dominant copepods in the respective study regions. In general, the copepods *Paracalanus parvus, Temora turbinata, Acartia danae, A. erythraea, Undinula vulgaris, Clausocalanus arcuicornis, Corycaeus danae, Oncaea venusta*, *Pseudodiaptomus serricaudatus, Acrocalanus gracilis* were the dominant TLA-bearing species in the coastal waters. On the other hand, *Acrocalanus gracilis, Paracalanus parvus, P. aculeatus Pseudodiaptomus serricaudatus, Temora turbinata, Acartiella gracilis, Heliodiaptomus cincuatus*, and *Allodiaptomus* sp., were the dominant TLA-bearing copepods in the estuarine waters.

The prevalence of TLA was generally higher in small-sized copepods (<500 µm) as compared to the larger-sized fractions (>500 µm). The overall frequency of TLA ranged between 3.6 and 12.4 % in different environments; a higher percentage was found in shallow, enclosed environments such as the Cochin estuary (av. 11.7%), Chilka Lake (av. 8.9%) and Palk Bay (av.12.4%) as compared with the rest of the study regions (Table 2).

3.2. Position and Types of TLA

TLAs were mostly located on the dorsal and lateral surfaces of the prosome and urosome of the copepods (Figs 2 - 6). The occurrence of TLA was significantly higher on the dorsal surface of the prosome (70 - 90 %) than on the urosome (10 - 30%).TLA in urosome was recorded mostly in *Acartia erythraea*, *A. danae*, *Temora turbinata*, *Paracalanus parvus*, and *Pseudodiaptomus serricaudatus*. Two kinds of TLAs were common (Fig.3) on the prosomal region (a) round, dark and granular and (b) round, non-granular and transparent. Between the two, the former was dominant and more widespread (55 -70%). The TLAs recorded in the urosome showed several morphological structures; the commonest was round and granular while the others included multi-lobed, elongated, transparent with small granules, elongated with appendages and with a black clot-like appearance (Figs. 4 and 5).

3.3. Seasonality of TLA in Coastal Marine Waters (Gulf of Mannar and the Palk Bay)

TLA of copepods showed marked differences in frequency of occurrence between the Gulf of Mannar and the Palk Bay, the two enclosed seas along the southeast coast of India. TLA incidences varied from 4.4 - 7.3% in the Gulf of Mannar and the Palk Bay. The percentage of TLA was always higher in the Palk Bay as compared to the Gulf of Mannar (Table 2). 15 out of 81 species of copepods identified from the Gulf of Mannar during a seasonal study of 2012 had reported TLAs (Jagadeesan et al., 2013). Out of the 67 species of copepods identified from the Palk Bay, 12 had TLAs. The percentage of TLA was generally high in the dominant copepods in the respective study regions (Fig.7). In both the Gulf of Mannar and the Palk Bay, TLA was common in *Paracalanus parvus*, *Acrocalanus* sp., *Acartia erythraea*, *A. danae*, *Temora turbinata*, *Oncaea venusta*, *Coryacaeus danae*, *Pseudodiaptomus serricaudatus* and *Undinula vulgaris*. TLA in *Paracalanus parvus* was found during both the seasons studied, but their infestation was higher during the Northeast Monsoon (38.69%) when they contributed ~60% of the total copepod community abundance (Fig.7f). During the Southwest Monsoon, TLA was high in *Temora turbinata* (anal and lateral), *Undinula vulgaris* and *Pseudodiaptomus* sp. The highest incidence of TLA (35.26 %) was noticed in *Temora turbinata* in the Gulf of Mannar during the Southwest Monsoon (Fig.7e).

Irrespective of seasons and sampling regions, ~10% of the total density of *Acartia erythrae*a and *A. danae* showed TLA.

3.4. Seasonality of TLA in a Monsoonal Estuary (Cochin backwaters)

TLA in copepods was found entire observations in the Cochin estuary. TLA incidence varied from 8 % to 15 % of the total copepod community, showing a significant difference in the seasonal occurrence as observed in the Gulf of Mannar and the Palk Bay. TLA incidence was high during the Pre-Southwest Monsoon period during which copepods belonging to the genera *Acrocalanus, Acartia*, *Paracalanus* and *Pseudodiaptomus* were the dominant ones and they together contributed 80% of the total copepod abundance. The TLA incidence was found to be high in *Acartia, Acrocalanus* and *Paracalanus,* which contributed 95% of the total TLAs. During the Southwest Monsoon, the copepod total abundance decreased in the Kochi estuary with the dominant copepods now being *Diaptomus* and *Acartiella;* these showed a TLA occurrence percentage of 8-14%. The TLA prevalence was found to be relatively lower during the Southwest Monsoon than during the Pre-Southwest Monsoon (Fig. 7).

3.5. Endo-parasites and TLA

Blastodinium was found in the alimentary tract of several species of copepods (Table 3), though the percentage occurrence was infinitesimal as compared to the TLAs (Table 4). The *Blastodinium* infection in copepods was recorded from the Kochi backwaters, Ashtamudi estuary, Coastal waters – Cochin, Coastal waters – Mumbai, Coastal waters – Kakinada, Gulf of Mannar and Palk Bay. The presence of *Blastodinium* was recorded in the digestive tracts of *Undinula vulgaris, Clausocalanus arcuicornis, Paracalanus parvus, P.aculeatus, Oithona* sp., and *Corycaeus danae* (Fig. 8). The prevalence of *Blastodinium* infection was the highest in *Undinula vulgaris* and *Corycaeus danae* and the infection ranged from 0.14 to 1.49 % of the total density of the copepods and 0.2 - 4% of the individual species density (Table 4). The infection showed a single trophont of the parasite in the digestive tract of calanoid copepods and in rare cases, two trophonts were also found. In laboratory-incubated *Blastodinium*, the release of the endo-parasite was through the anus of the host, after maturation within 4 days and causing no morphological aberration to the host. Similar to the laboratory experiments, TLA-like morphological aberrations were completely absent from ~500 *Blastodinium*-infected copepods observed in the field samples.

3.6. Ecto-parasites and TLA

The ecto-parasite *Ellobiopsis* sp. was found in *Undinula vulgaris* collected from the Cochin estuary, Chilka Lake, and coastal waters of Cochin and Mumbai (Table 4). The *Ellobiopsis* parasite was attached to the cephalic appendages of *Undinula vulgaris* by their stalk (Figs. 9 & 10), and their infection ranged from 0.08 – 0.31% of the total density of *Undinula vulgaris* (Table 4). Mostly one or two parasite attachments were found in a single host (Figs. 9 &10), though rare occurrence of four parasites' attachment in a single host was also found and the size of the parasite ranged from 30 -110 µm. interestingly, none of the *Ellobiopsis* sp., infected copepods collected from the field displayed TLA. Similarly, the laboratory-incubated copepod individuals with *Ellobiopsis* sp infection did not show any symptom of TLA development on the host. It was amply clear from the experiments that *Ellobiopsis* release their spores from the gonomere within 2 – 4 days of incubation, without causing any morphological damage to the host and the *Ellobiopsis* attachments were found only in the cephalic appendages.

3.7. Epibionts and TLA

The infection of the peritrich ciliate (*Zoothamnium* sp.) was observed on the surface of *Acartia erythraea* and *A. danae,* and their infestation percentages ranged from 0.28 to 1.14% of the total copepod population (Table 4). The number of the attachments in a single host ranged from 0 – 60, and this was found mostly on the lateral surfaces of the prosome (Figs.11a & b). *Acineta* infection was observed on the lateral sides of the entire body (prosome and urosomal) (Figs.11c & d) of Harpacticoid copepods, *Microsetella norvegica, M. rosea*, *Macrosetella gracilis, M. oculata*, and *Euterpina acutifrons*, and the infestation percentage ranged from 0.15 to 0.46 (Table 4). The infestation density in a single host ranged from 0 to 140. *Zoothamnium* infection was observed in the estuary like Cochin, Ashtamudi, Tejaswini, Zuari, Uppanar and Chilka Lake. The *Acineta* infestation was observed in Cochin estuary, Ashtamudi estuary, Tejaswini estuary and Chilka Lake. In experimental studies using epibionts, the host copepods did not show any symptom of TLA development due to epibiont attachment. The epibionts apparently use the copepod body only as substratum for their attachment, without harming the host.

3.8. Predation Experiments

The post-contact predator avoidance behaviour of the copepods was evidenced in the video (supplementary video 1). One observation from the given video depicts two copepod individuals that got simultaneously entangled with the nematocyst of the hydromedusae and tried to escape (Fig.12). Both showed vigorous movements to escape the predator, finally managing to do so but with damages to their urosome (caudal remi), an injury that caused unusual swimming pattern in

both (Supplementary video). In another observation given in the video, nematocysts of the hydromedusae got detached from the copepod body due to the latter's escape movements and later, the development of TLA was noticed on the same specimen, at the point where the nematocysts had struck (Fig.12).

Based on neutral red live staining technique, five forms of copepod specimens were obtained from the predatory experiments (a) copepods without any damage (stained - live), copepods with damages on their body (stained - live), copepods with TLA (stained - live), copepods (unstained - dead), copepods with TLA (unstained - dead). Altogether 20-40 % of the copepods in the experimental beakers escaped from any contact of the hydromedusae due to their active escape responses. 60- 80% of the copepods could not escape the contact of the hydromedusae, and in this group, 15-25 % were consumed completely by the predators, 10 - 15% died due to nematocyst penetration, 8-10% escaped but developed TLA and 6-10 % died after developing TLA.

4. Discussion

The present study reports that TLAs in copepods are present in the Indian waters as well, suggesting that this anomaly has a circumglobal occurrence and common reasons. In the present study, shallow environments TLA (av. 10%) were high compared to the coastal and offshore environments (av. 7%). This characteristic feature was reported earlier from many parts of the world where it was noticed that copepods inhabiting shallow regions displayed a higher percentage of TLA incidences as compared to the deeper waters (Omair et al. 1999; Silina & Khudolei 1994; Manca et al. 2004). On one hand, the percentage of TLA incidences recorded in the present study was significantly lower than earlier records from the temperate waters (41.7 - 80 % and 10 – 50% respectively) while on the other, it was comparable with the earlier records from Indian Ocean ridges (Omair et al. 1999; Silina & Khudolei 1994; Bhandare & Ingole 2008). This difference in the percentage of occurrence of TLA recorded in the present study with the temperate region can be attributed to the geographical difference between the study regions.

The percentage contribution of each copepod species to the total TLA incidences varied significantly from one environment to the other and, in general, a high percentage of incidences were noticed among the dominant species (Table 3). The highest TLA incidences were noticed in Calanoids, the most dominant copepods in Indian waters. Species with a high percentage of TLA include *Paracalanus parvus*, *Acrocalanus* sp., *Acartia erytharaea*, *Acartia danae*, *Temora turbinata*, *Coryacaeus danae* and *Undinula vulgaris*. This feature was more clear in two convincing cases observed in the present study such as (a) *Temora turbinata* presented the highest percentage of TLA

in the Gulf of Mannar (av. 35.26%) and the Palk Bay (av. 18.3 %) during the Southwest Monsoon (Figs. 7 c & d), when their swarms were observed in the regions associated with the intrusions of upwelled waters from the South-eastern Arabian Sea (Jagadeesan et al., 2013) and (b) *Paracalanus parvus* exhibited the highest percentage contribution to TLA in the Palk Bay (38.7 %), when their contribution was ~60% of the total community (Fig. 7f). Messick et al. (2004) reported a high percentage of TLA in *Diacyclops* sp. (33%) when they dominated the community. Similar observations were reported by Omair et al. (1999) who found the highest percentage of TLA (71.4 %) in *Epischurala custis* when they were predominant in the environment. Other similar observations (Bhandare & Ingole 2008; Dias 1999) strongly suggest that the formation of TLA in a copepod species has a direct linkage with the proportionate abundance of that species in the environment and the factors that cause the TLA have a random effect on the copepods.

The appearance of TLA varies largely and has a heterogeneous nature (Omair et al. 1999; Bridgeman et al. 2000). It was found in the present study that among the two common kinds of TLAs, the round and granular type (Fig. 3) was more prominent (70 - 90% of cases), as noticed earlier by several researchers (Bhandare & Ingole 2008; Omair et al. 1999; Bridgeman et al. 2000; Manca et al. 2004), than the non-granular type. It was also found in the present study that most of the TLA were positioned on the dorsal lateral surfaces of the prosome of copepods, especially in the intersomite region between the last prosome and the first metasomal segments (Figs. 3, 5 and 6), which corroborated various earlier reports (Messick et al. 2004; Manca et al. 2004). The intersomite regions of copepods are more vulnerable to wounds and rupture than the other body parts (Messick et al. 2004). In the present study, though such histological observations were not attempted on the formalin preserved samples, most cases of TLA showed internal connection with body muscle tissues, and the digestive and reproductive systems. The histological studies of TLA carried out earlier showed necrotic tissues, granular materials, herniated tissues, host muscle projection, homocytes and pycochromatic tissues of the host (Messick et al. 2004).

4.1. Assessment of the Possible Causes of TLA

The contrasting views of earlier researchers on the possible causes of TLA from different parts of the world itself indicate the complexity and limitations in identifying the actual causes. Many of the earlier suggestions such as long-term environmental change and aquatic pollution (Crisafi 1974; Silina & Khudolei 1994; Manca et al. 2004) are rather ambiguous and difficult to prove scientifically as there is no prior data available on such aspects. As proved in histological studies conducted elsewhere (Crisafi 1974; Silina & Khudolei 1994; Manca et al. 2004), TLA in copepods seems to be

the manifestation of herniated body tissue rather than ‘malignant growth’ as considered in the case of humans. Therefore, suggestions such as the influence of carcinogenic agents behind TLA in specialized environments (Bhandare & Ingole 2008; Mantha et al. 2013; Silina & Khudolei 1994) is not supported with robust scientific reasoning as there is no direct evidence so far to consider so.

The present study forms the baseline information on TLA from Indian waters. Moreover, the present study shows that TLA in copepods is present in all the environments including estuaries, coastal and offshore waters and, therefore, claiming environmental pollution to be a potential reason of the anomaly in Indian waters seems to be unrealistic as these environments are considered to possess large variations in hydrographical settings. On the other hand, as indicated in some of the earlier studies (Messick et al. 2004; Omair et al. 2001), we consider wounds, puncture or damage in the external carapace of the copepods as the key reason for TLA formation in copepods. We confirmed this possibility by prickling/rupturing the copepod exoskeleton using a sterilized needle and noticed the development of TLA in the damaged body part within a day. We also attempted predation experiment of copepods using medusae as the predator in controlled lab experiments and noticed TLA development in the region where medusae tentacle attachment produced damage (Supplementary material 1). We critically assessed various possible means by which copepods get wounds on their body and also the proportionate role of each of these means to produce TLAs in Indian waters. As evident in earlier studies, ecto-parasitism, endo-parasitism, epibionts and predation are the potential factors that cause wounds/injury on copepods (Skovgaard 2004; Bhandare & Ingole 2008; Mantha et al. 2013; Bridgeman et al. 2000; Omair et al. 2001; Manca et al. 2004). The possibility of diatom puncture on copepods during the zooplankton sample collection is also suggested but seems to be of low magnitude as the net tow is usually for short durations (Bridgeman et al. 2000).

4.1.1. Ecto- and Endo-parasites, Epibiont and other Causes

Endo-parasites (*Blastodinium*) and ecto-parasites (*Ellobiposis* sp.) can cause TLA infection in copepods (Messick et al. 2004; Skovgaard 2004; Bhandare & Ingole 2008; Bridgeman et al. 2000; Manca et al. 2004). The life cycle of *Blastodinium* explained by several authors showed that the parasite zoospores mature inside the digestive tract of the copepods and get eliminated through the anus of the host (Chatton 1920; Shields 1994; Jepps 1937) and, therefore, in usual cases, there is no need for the parasite to create physical damage to the host. However, in some rare cases, as recorded by Skovgaard (2005), due to some abnormal development, the endo-parasite split open the alimentary tract and muscle tissue of the copepod; such cases can potentially lead to TLA. In the

present study, though we recorded *Blastodinium* infection in several copepods, the expected TLA was not found in the infected copepods in the laboratory-incubated live organisms and preserved specimens from the natural environment. Hence, the possible role of endo-parasites as a causative factor of TLAs may have minor relevance in Indian waters.

Earlier studies suspected that the ecto-parasite *Ellobiopsis* infection causes TLA in copepods (Bridgeman et al. 2000). However, the life history studies of *Ellobiopsis* (Sewell 1951; Shields 1994; Jepps 1937; Gomez et al. 2009) and the present observation do not support the above view. The life cycle of *Ellobiopsis* includes various stages; initially, the free motile spores get attached to the setae of the copepod's buccal appendages where they later metamorphose into the trophomere. When the parasite's body reaches a certain size, it becomes transversally septate and forms the gonomere in the distal part. The distal gonomeres become granulated and progressively develop into small groups of pre-spores that fall from the segregating mass (Fig. 9). As discussed in the above sections, most of the TLAs occur on the dorsal and lateral surfaces of the copepods, whereas *Ellobiopsis* infection mostly occurs on the appendages of copepods (Table 5) (Sewell 1951; Santhakumari 1985; Santhakumari & Saraswathy 1979; Shields 1994; Gomez et al. 2009). Furthermore, out of ~100 incidences of *Ellobiopsis* infection on copepods noticed from various environments and the laboratory-incubated live organisms during the present study, none of the host copepods exhibited TLA incidence. This indicates that *Ellobiopsis* has less potential to cause TLA in copepods in Indian waters. Similar observation was made by a few other researchers as well from elsewhere in the world (Bridgeman et al. 2000; Manca et al. 2004). The puncturing of copepod exoskeleton by diatoms has been suggested as a potential cause of TLA in copepods (Bridgeman et al. 2000), but its applicability in the natural environment is insufficiently assessed.

The observation of anal TLA in the present study forms only the second record after Crisafi (1974), who assumed that such anomaly was due to intestine prolapse induced by aquatic pollution. A recent study (Skovgaard & Daugbjerg 2008) showed that endo-parasites (plasmodial parasite and *Paradinium* sp.) infecting copepods carry unstructured or structural residual materials in their anus through which the gonospores of the parasite is expelled out. It is evident in some cases Figs 4f & 4h that the extended/protruded TLA structures carry the dark-coloured continuity of the intestine of the host that may represent incomplete expulsion of gonospores by the endo-parasites.

4.1.2. Role of Predation

It is fundamental that copepods are preyed upon by almost every invertebrate and vertebrate predator in aquatic ecosystems like chaetognaths, medusa, siphonophores, fish larvae and fishes (Mills 1981

& 1995; Suchman & Sullivan 2000; Omori et al. 1995; Verity & Smetacek 1996; Purcell 1992; Hansson et al. 1990). Copepod predators exhibit diverse feeding adaptations and behavioural responses to prey upon copepods (Buskey et al. 2011). The predators locate the prey by random encounter or detect their presence through vision or by the hydrodynamic disturbances created by the prey (Greene 1985). As mentioned before, the predation process was divided into four quantifiable components: encounter, contact, capture and ingestion, and digestion. The process of the attack, capture and ingestion of the prey may follow an encounter and their relative outcome determines the success of the predation (Ohman 1988; Lima and Dill 1990). However, copepods have numerous adaptations to avoid the risk of predation and facilitate their survival (Buskey 1984; Haury et al. 1980; Ohman 1988; Kiørboe et al. 1999; Kiørboe & Visser 1999). The behavioural interaction of the prey and predator can affect the predation risk through enhancing or limiting the encounter rates and the success of the subsequent events (Greene 1985).

It was observed during the present study that more than 50% of the nematocyst contacts of hydromedusae on copepods were in the regions between the prosomal and first metasomal segments. Earlier, Beyer (1992) & Ohman (1984) presented similar observations that predators attack copepods mostly between the prosomal and the first metasomal segment and cause injuries/damages. Similarly, several studies on copepod carcasses have showed that most of the injuries to the dead specimens are in the dorsal side between the prosomal and metasomal regions (Beyer 1992; Ohman 1984; Terazaki & Wada 1988). Other studies have demonstrated that euphasids injure copepods between the prosomal and metasomal regions (Ohman 1984; Beyer 1992). All these observations support the results obtained through our experimental and field studies.

In general, copepods are efficient in performing rapid and directed escapes in response to fluid signals created by their predators (Fields et al. 2012). The escape responses of the copepods and the timing and magnitude of an escape reaction often act as the determining factors governing the success of a copepod in avoiding predation (Fields et al. 2012). However, delayed (post-encounter / post-capture refuse) escape responses cause injury to the copepod's body, leading to unsuccessful or partial predation (Fig.12). The attack by the predators and the predatory avoidance/escaping behaviour of copepods causes injuries/ partial predation/ damages in the copepods' external exoskeleton (Fig.12). Based on the severity and the depth of the injury, the responses of the copepods vary. If the damage is minimal, the body initially undergoes herniation between the somites, which later develop as transparent TLAs. This was experimentally proved – when copepods were slightly punctured by a sharp needle they produced transparent TLAs (Bridgeman et al. 2000). If the injury is moderately deep into the copepod body, it initially causes the swelling or extrusion of

the body tissues while if it is more severe, internal organs (digestive tract and the reproductive structures are burst out) are herniated or extruded from the body (Fig.12) due to variations in hydrostatic pressure or turgor pressure (Bridgeman et al. 2000). The argument that partial predation is the most likely cause of TLAs in copepods has been proved in the present study. In the laboratory experiments, it was evident that copepod individuals exposed to firm sting cells of the predator but managing subsequent escape developed TLAs on their bodies (Supplementary material 1).

In hydromedusae predation, the success rate of prey escape after nematocyst contact depends on several factors, which include the ability of the nematocysts to penetrate the carapace of the prey, the susceptibility of the prey to cnidarian toxins and the strength of the prey's escape response (Suchman & Sullivan 1998; Purcell & Mills 1988; Costello & Colin 1994). As evident in the supplementary video presented, copepods exhibit vigorous movements to escape from the contact of the nematocyst and in many cases, they execute successful escape with wounds/ damages in the body surfaces. If the damage is minimal it may lead to a transparent TLA, otherwise causing dark granular TLA.

The predation success primarily depends upon numerous factors (Fig.13), which are both biological as well as physicochemical. Biological factors include food availability, chemical interaction between the prey and predator, size and crowding of the prey and differences in swimming velocity between the prey and predator (Gerritsen & Strickler 1977; Poulet & Marsot 1978; Purcell 1992; Sullivan et al. 1994). Physicochemical variables mainly affect the predation through altering the ability of the predator to locate prey or the chances of escape of the prey or defence of the prey from an approaching predator (Gerritsen & Strickler 1977; Sullivan et al. 1994). This include variations in water clarity (Benefield & Minello 1996), turbulence (Gilbert & Buskey 2005) and concentration of gases in aquatic systems (Brietberg et al. 1994). The high abundances of TLAs in shallow waters can be the result of high turbidity, shallowness, size-selective feeding, escape responses of the prey against the predation and feeding behaviour of the predator. Future experiments can target the elucidation of these factors.

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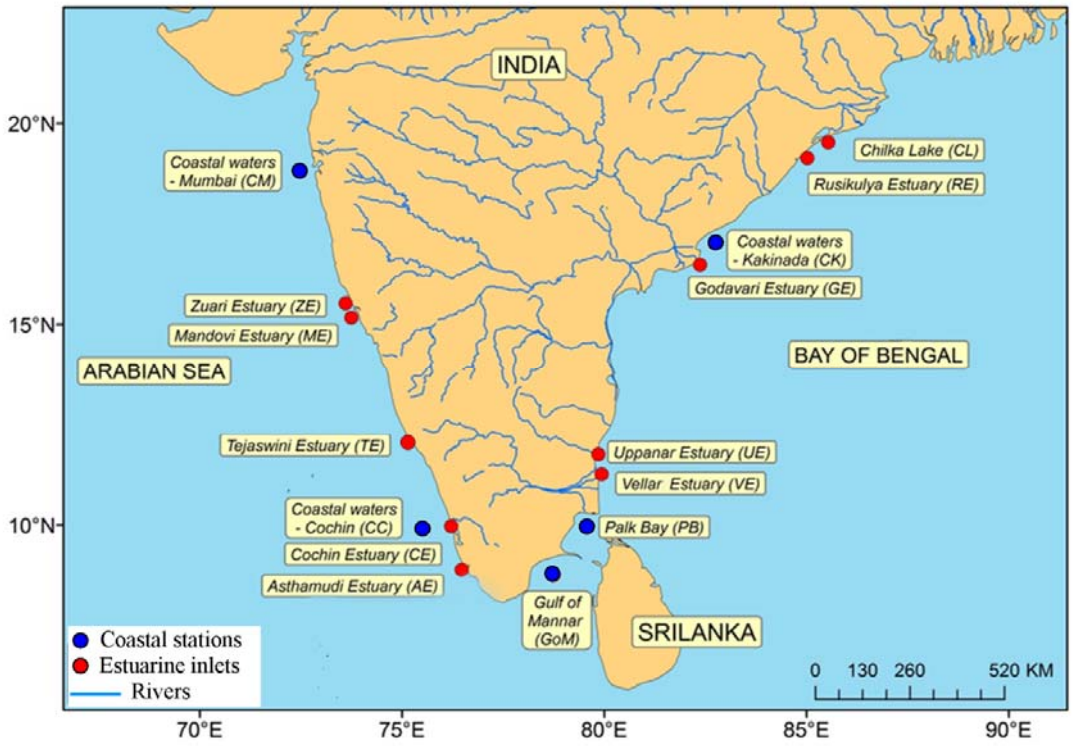


Figure 1. Sampling locations. Samples were collected from 10 estuarine inlets (red circles) and 5 coastal waters (Blue circles) of India.

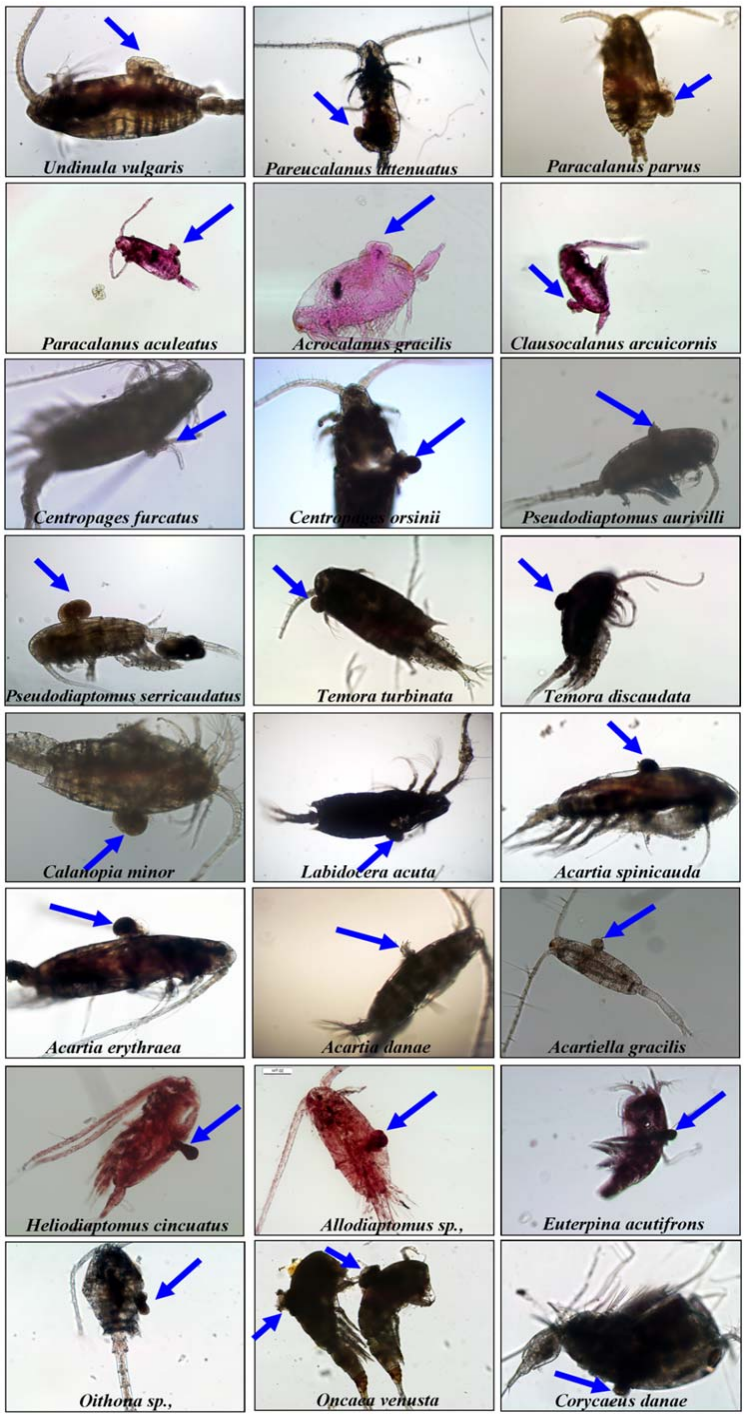


Figure 2 - TLA observed in the prosome of copepods. The dark granular TLA mostly occurred between the cephalosome and metasome of copepods. The neutral red stained (Red colored) specimens of *Paracalanus aculetus, Acrocalanus gracilis, Clausocalanus arcuicornis, Heliodiaptomus cincuatus, Allodiaptomus sp.,* indicated that TLA bearing copepods were live and the anomaly was viable somatic tissue of the host.

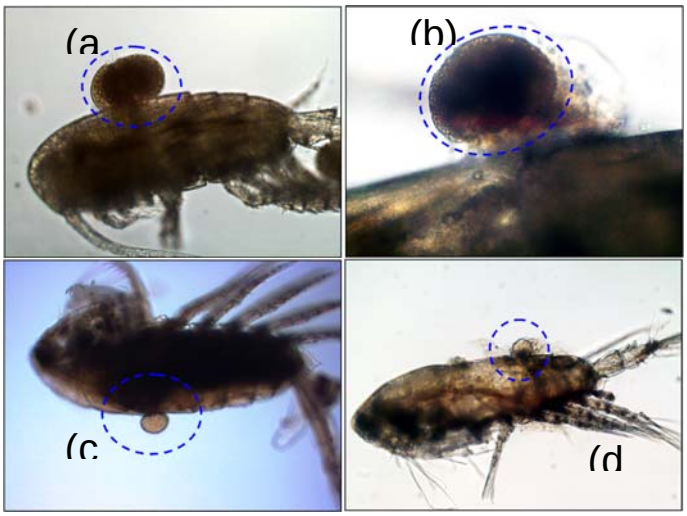


Figure 3 - Two types of TLA in the prosome of copepods (a & b) round, granular and dark and (c &d) round, non-granular and transparent.

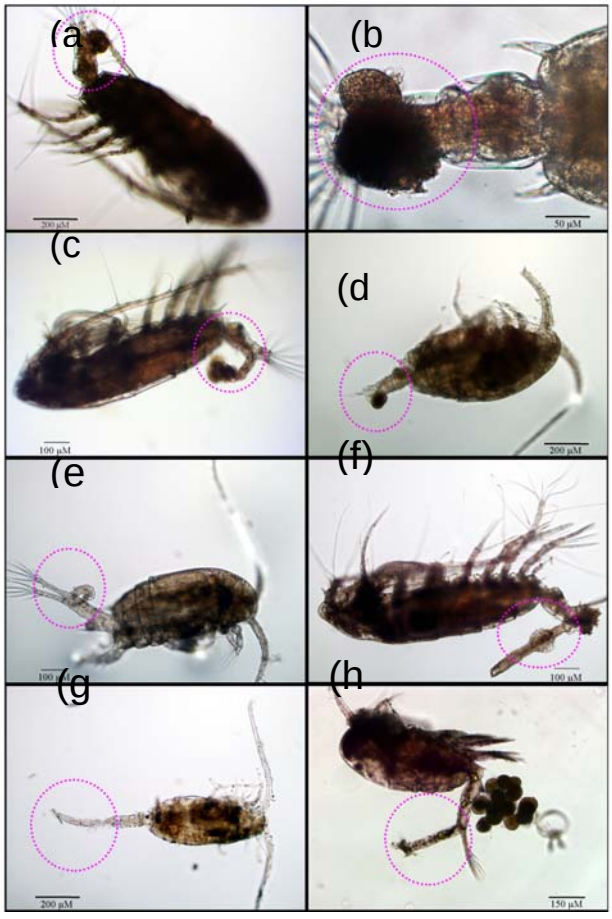


Figure 4 - Various types of TLAs in the urosome of copepods - (a) *Acartia erythrae*, (b) *Acartia danae*, (c) *Temora turbinata*, (d) *Paracalanus parvus* and (e) *Pseudodiaptomus serricaudatus*. The anal TLA varied in appearance (a & d) dark granular, (b) multi-lobed (c & f) muff shaped with a stalk, (e) small and slightly curled, (g) transparent and elongated and (h) stalk-like extension. All these anal TLA showed continuity with the digestive tract of the affected copepod.

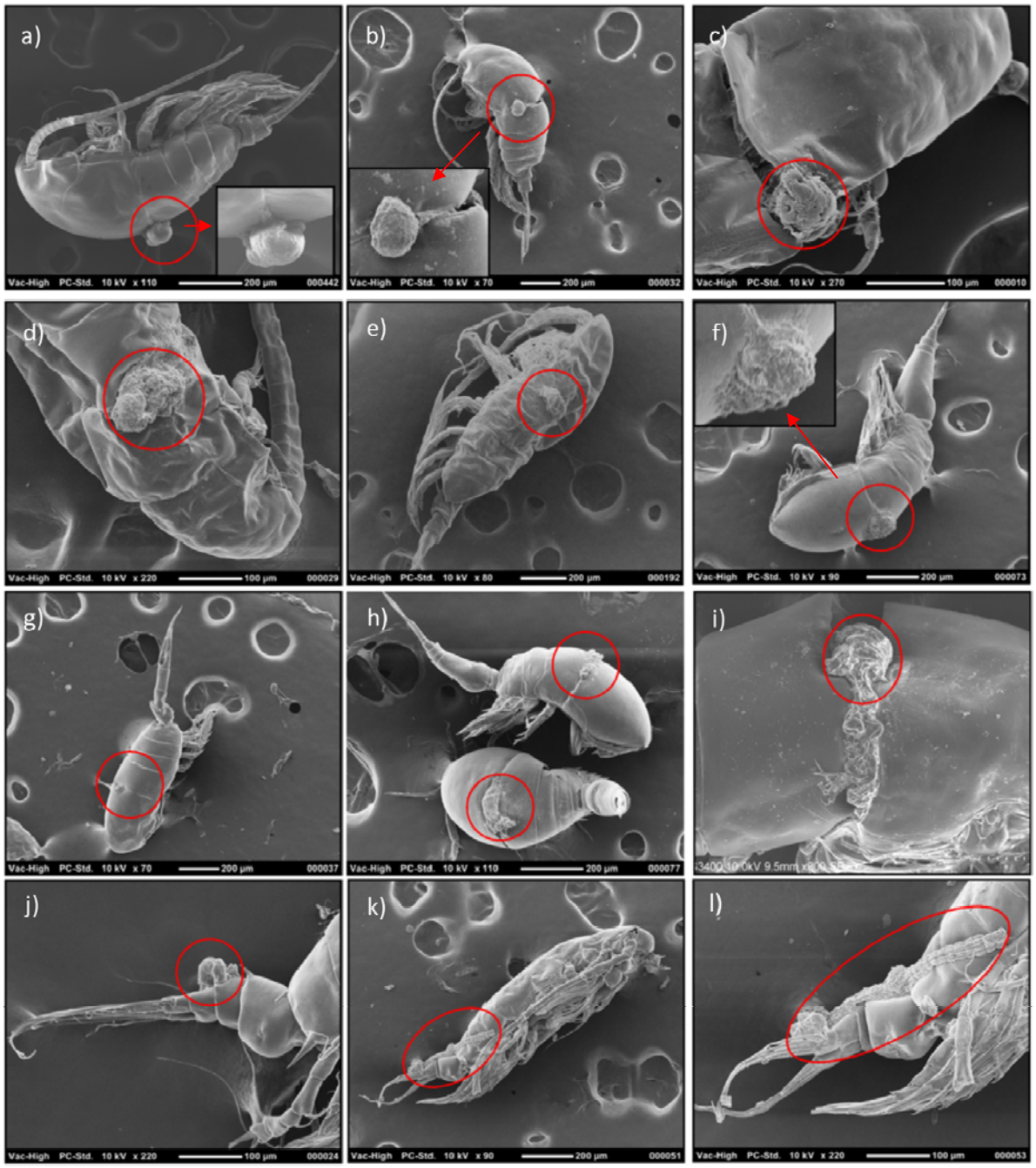


Figure 5 - SEM images of TLA in copepods. Red circles in panels (a - i) represent TLA on the prosome and (j - l) on the urosome of copepods. The inserts showed the close view of the tumour.

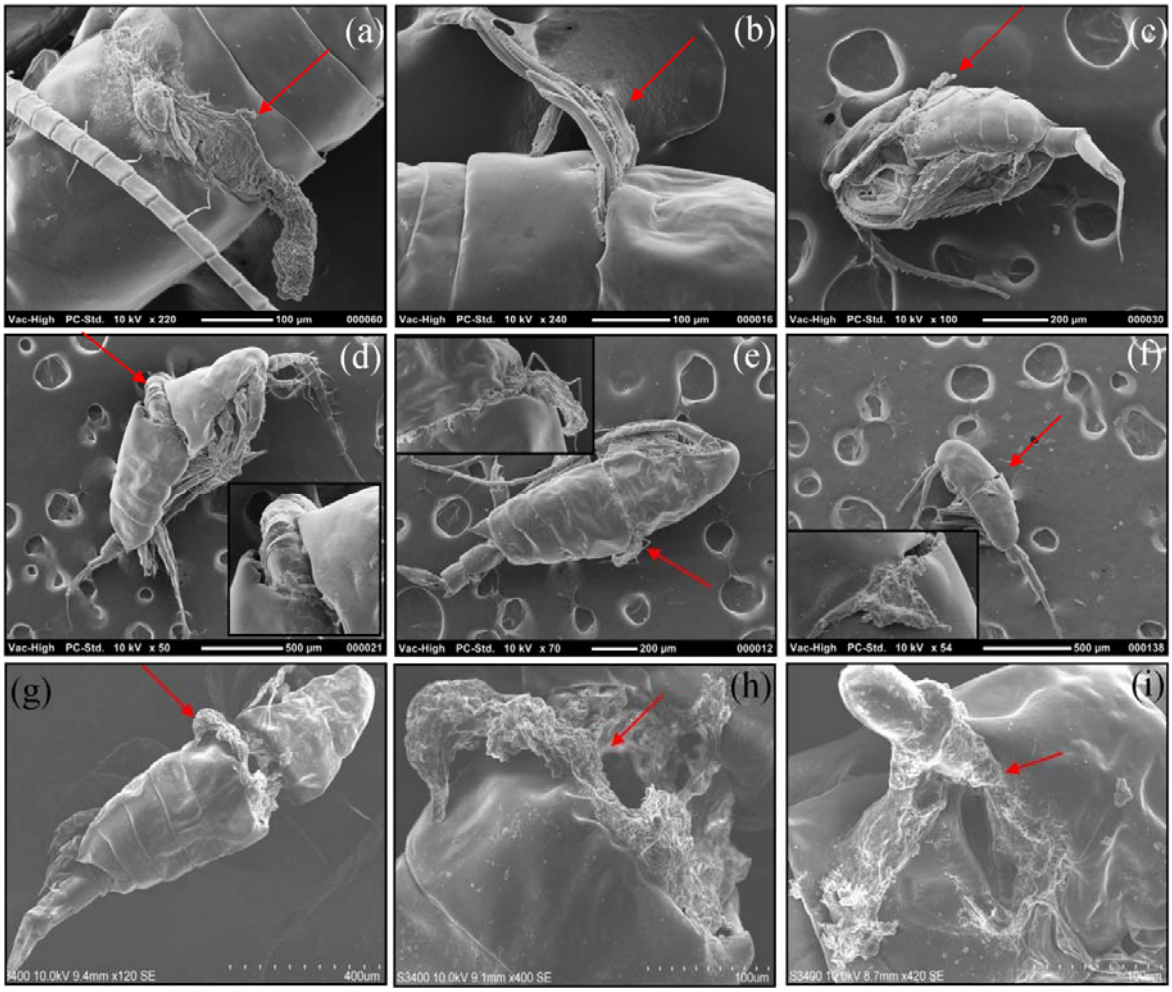


Figure 6 - SEM images of TLA associated expulsion of internal body parts in copepods. The expulsion happens mostly between the inter somital regions between the last prosome and the first metasomal segment.

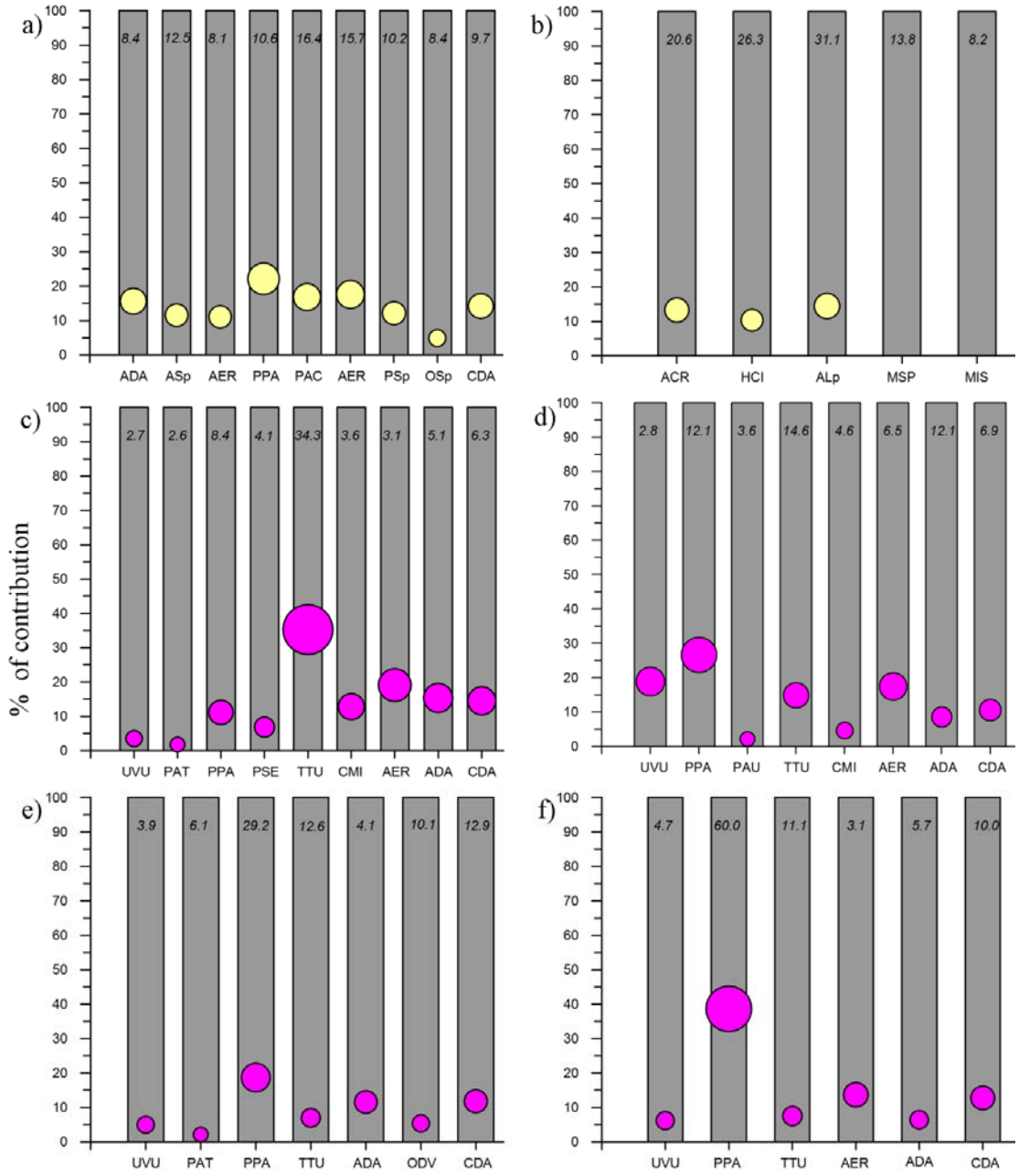


Figure 7 - Seasonal variations in the percentage of TLA incidences in a typical (a & b) Monsoonal estuarine inlet (Cochin estuarine inlet) and (b - e) coastal waters. Panels (a) and (b) represents the TLA percentage in dominant copepods in the Cochin estuarine inlet during the pre-monsoon and Southwest Monsoon respectively. Panels (c) and (d) show the TLA percentage in the Gulf of Mannar and (e) and (f) in the Palk Bay during the Southwest Monsoon and Northeast Monsoon respectively. The copepod species abbreviations are same as in Table 3. Numbers in the bars represents the contribution of a particular species to the total copepod abundance. The highest incidence of TLA was found in dominant copepods *Temora turbinata* in the Gulf of Mannar during the Southwest Monsoon and *Paracalanus parvus* in the Palk Bay during the Northeast Monsoon. Irrespective of seasons *Acartia danae*, *Acartia erythrae* and *Corycaeus danae* have >10% of the TLA.

Figure 8 - Endoparasite *Blastodinum* infection in the digestive tract of copepods indicated by thin violet arrows (a-e) *Undinula vulgaris*, (f - i) *Paracalanus parvus*, (j) *Oithona sp.*, (k) *Corycaeus danae*, (l) *Corycaeus catus*. The thick blue arrow in panel (g) indicates the internal parasite structure after the dissection and in panel (h) indicates the route of the expulsion of the mature gonocyte of *Blastodinium* (blue circle) through the anus.

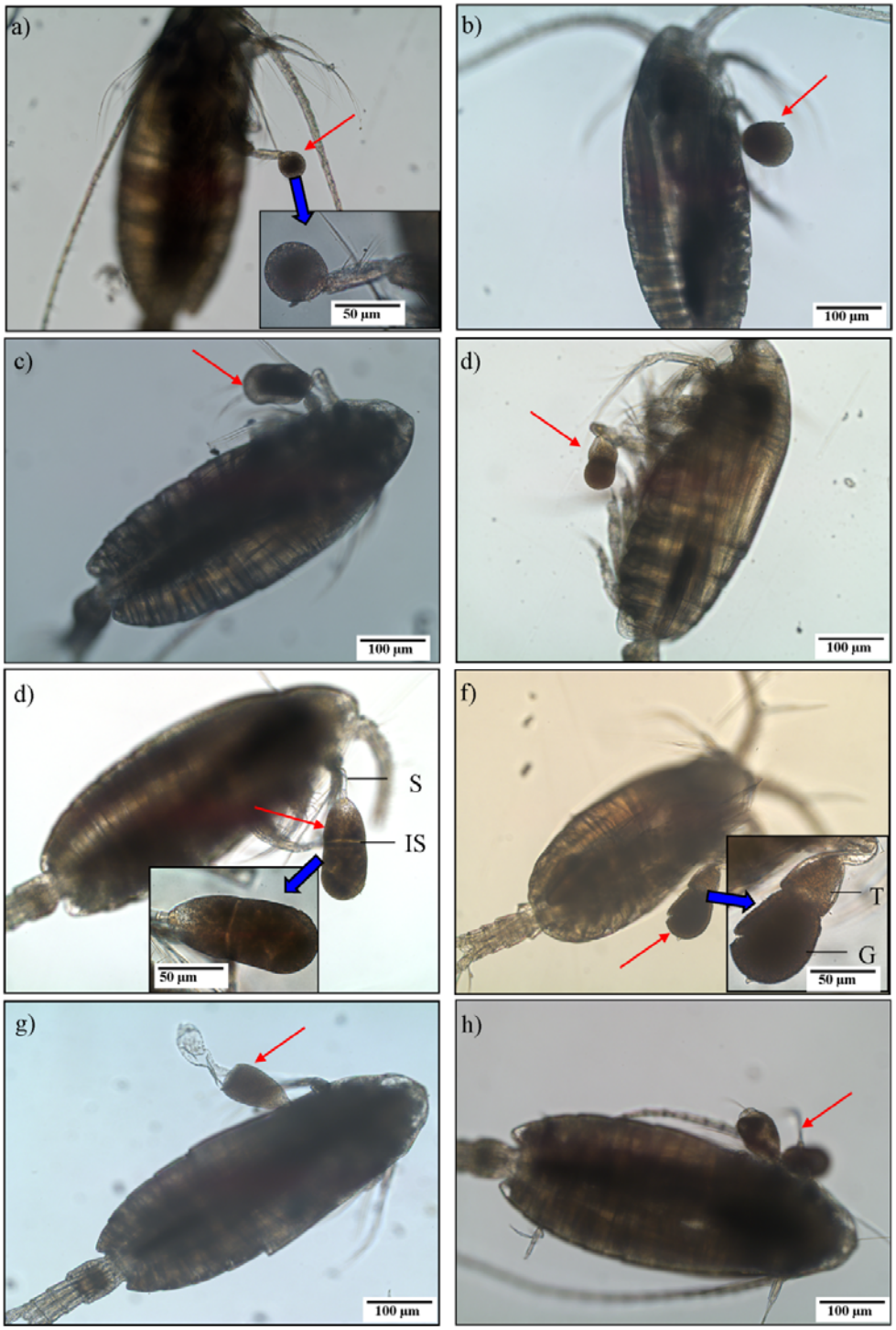


Figure 9 - Ectoparasite *Ellobiopsis* infection in *Undinula vulgaris.* (a – g) show different life stages of *Ellobioposis* infection in copepods (a) initial stage, (b-c) development of trophomere, (d) development of internal septa, (e) clear demarcation of trophomere and gonomere (f) maturation of gonomere (g) post-spore release, (h) two parasites attached to the host. Abbreviations: S- Stalk , T –Trophomere, G- gonomere.

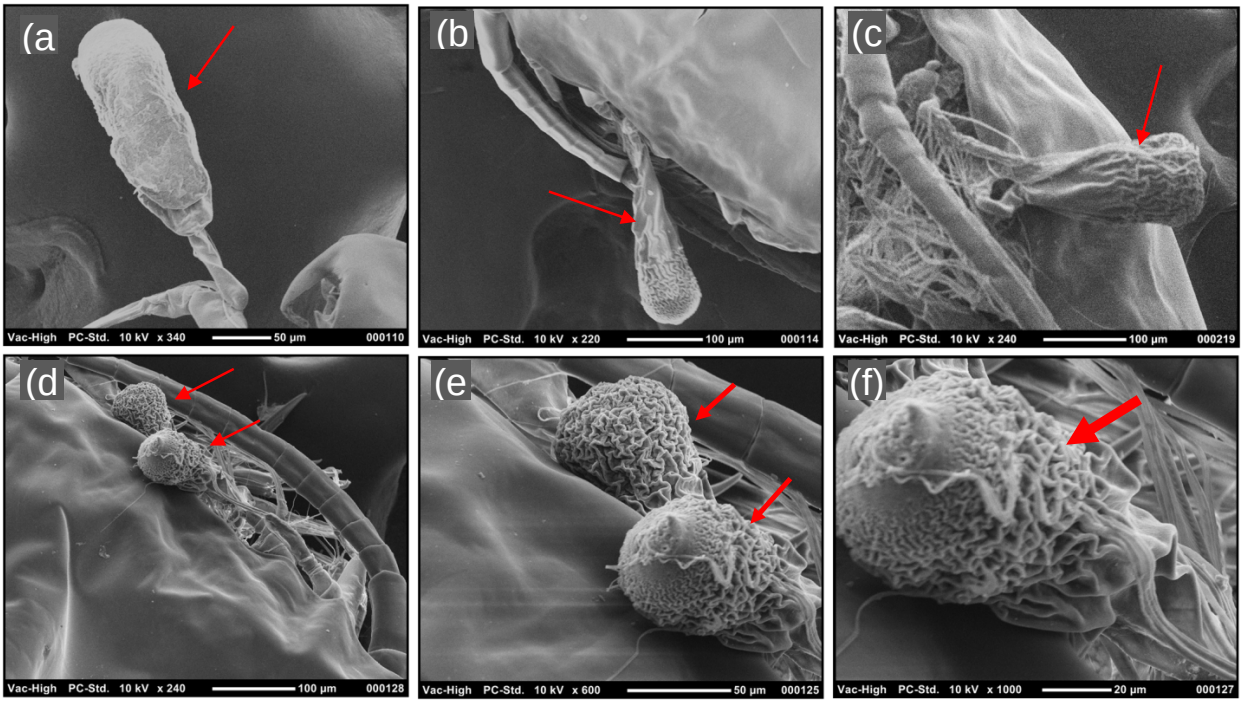


Figure 10 - SEM images of *Ellobiopsis* infection in copepods, (a-c) single *Ellobiopsis* attached to the feeding appendages, (d) show two *Ellobiopsis* attached to the copepod and (e –f) their detailed morphology in higher magnifications.

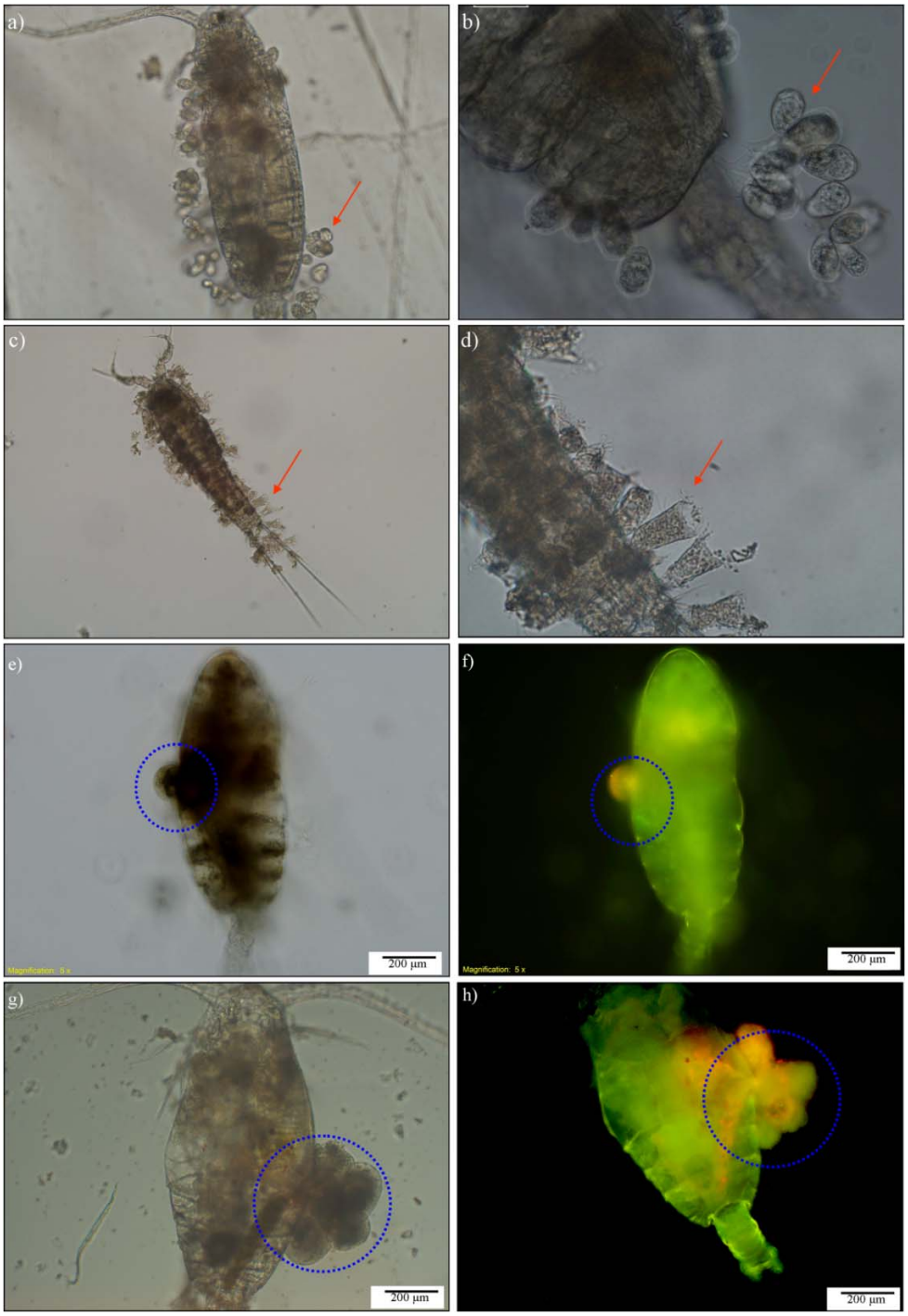


Figure 11 - Epibionts attachments on copepods, *Zoothamium* attachment on *Acartia danae* (a & b) and *Acineta tuberso* attachments on *Macrosetella gracilis* (c - d). Photomicrographs of light (e and g) and florescence microscopes (f and h) showing the TLA as a protrusion of the digestive tract.

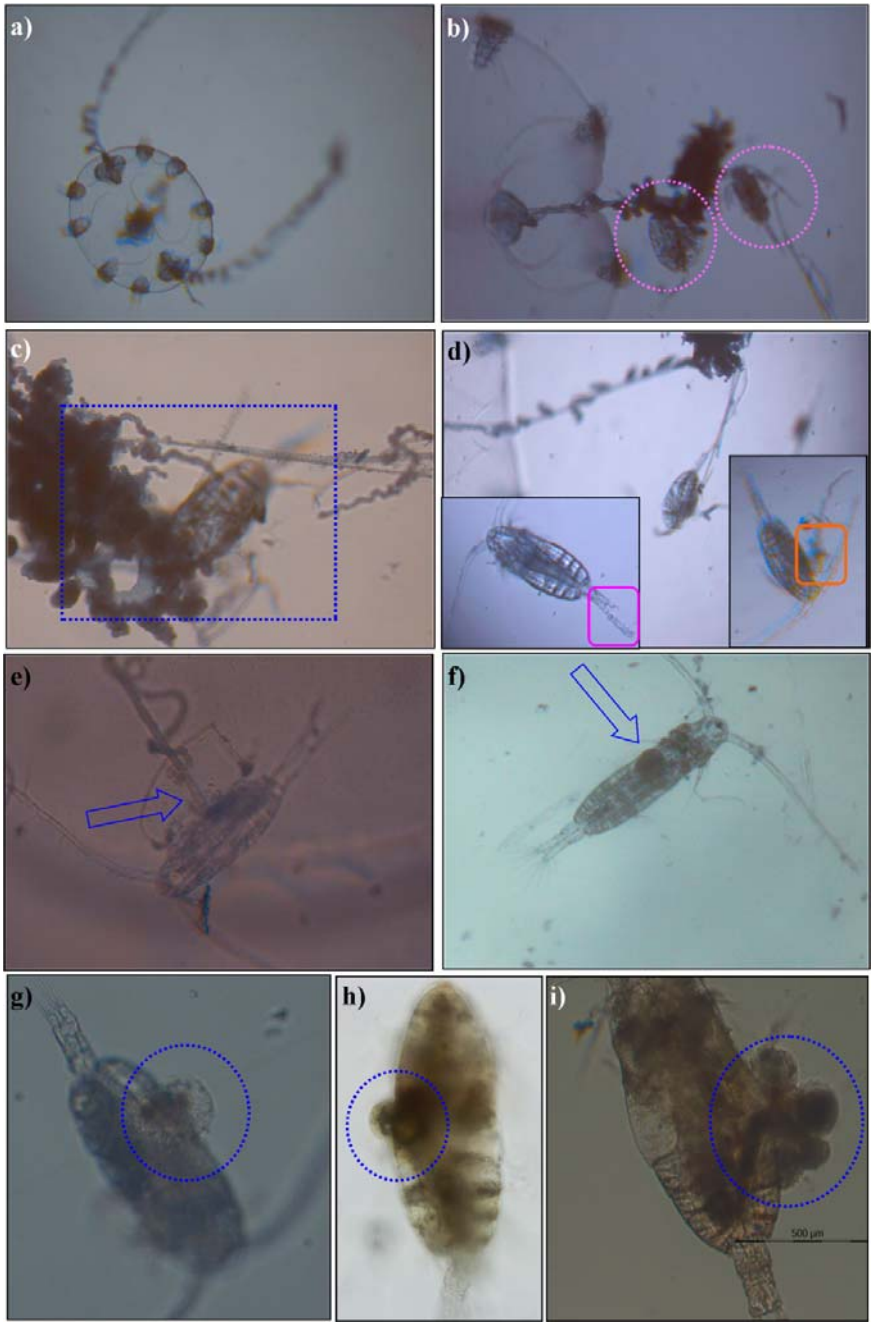


Figure 12 - Sequential snap shots of predation of hydromedusae on copepods. (a) indicate the hydromedusae before predation attempts on copepods, (b) copepods entangled with nematocysts of hydromedusae highlighted in pink circle, (c) closer view of the entanglement indicated in blue rectangle (d) copepods stretched to escape from the contact of the nematocyst; insets show escaped copepods with wounds/damages on their body (e) Nematocysts attached to the dorsal side of the copepods body observed in another experiment, (f) copepods escaped from the nematocyst contact and develop TLA in regions of nematocysts attachment, (g, h and i) other TLAs observed during the predation experiment using hydromedusae highlighted with blue circles. The events involved in the formation of TLAs described above are available as a movie clip (Supplementary video 1).

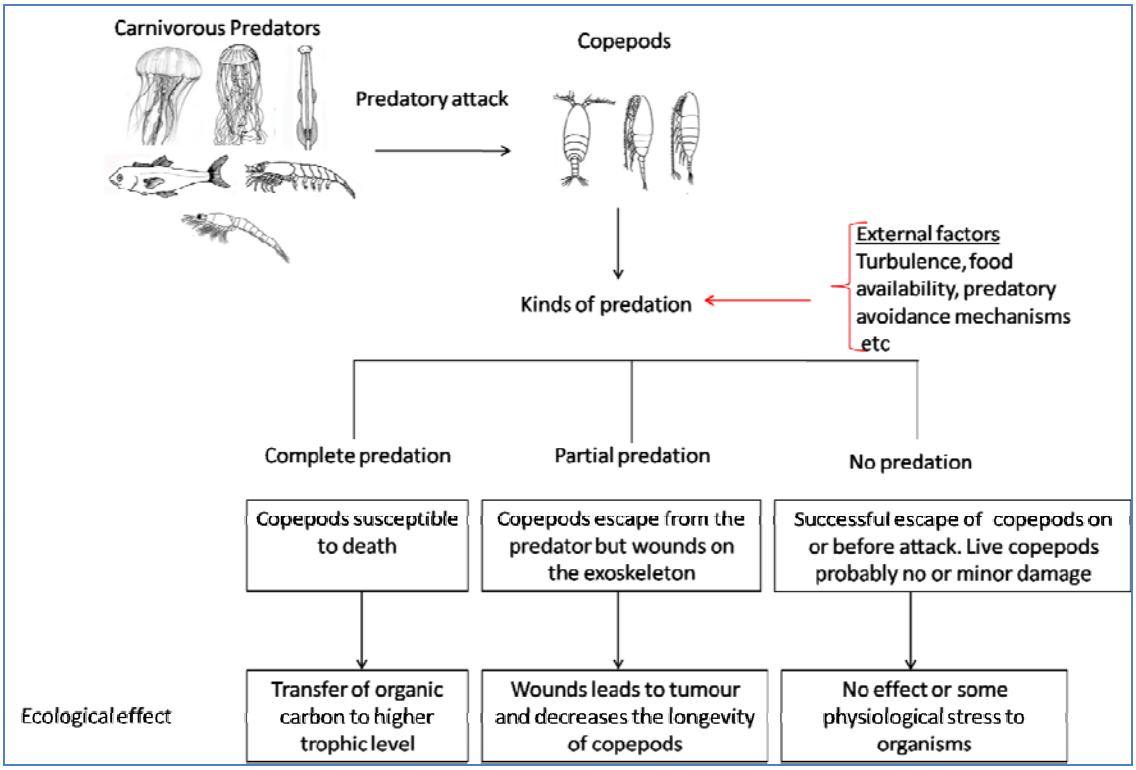


Figure 13 - Diagrammatic representation of the copepods predation and their results.

Table 1 Earlier records of morphological anomalies in copepods from various environments across the globe and their causative agents. Most of the suggested causative factors are assumptions based on the morphological and histological observations of the affected copepods but the etiological agents that cause TLA still remains as a mystery. * indicate histological studies.

No.	Source	Geographical area	Terminology used	Suggested causative agents
1	Crisafi, 1974	Mediterranean, Atlantic and Pacific coastal seas.	Muff shaped formations	Environmental change
2	Crisafi & Crescenti, 1975	Coastal waters – Italy	Extruded protoplasmic mass /TLA	Environmental Change
3	Crisafi & Crescenti, 1977	Black Sea, Mediterranean Sea, Coastal Atlantic, Pacific, off Singapore and New Zealand	Tumour like appendages/ Tumour out growths	Environmental Change
4	Silina & Khudolei, 1994	Estuary and lakes nearby of Baltic Sea	TLA	Pollution, Hydrocarbons, Toxins
5	Manca et al., 1996	Lago Maggiore Lake (Italy)	Cysts	Oligotrophication
6	Vanderploeg, 1999	Lake Michigan (USA)	Tumours	Endocrine disruption - pollutants from laundry detergents
7	Omair et al., 1999	Lake Michigan (USA)	TLA/ Exophytic lesions	Chemicals, Ultraviolet radiation, Viral or bacterial infections/ Injury
8	Dias, 1999	Espirito Santo Bay (Brazil)	Morphological abnormalities	Industrial/Domestic Pollution
9	Bridgeman et al., 2000	Lake Michigan (USA)	Cyst / hernia	Ectoparasite - *Ellobiopsis*
10	Omair et al., 2001*	Laurentian Great Lakes (USA - Canada)	Protrusions/ Herinations	Wounds caused by Predators/ Parasites
11	Messick et al., 2004*	Lake Michigan (USA)	Protrusions/ TLA	Wounds by predators/ parasites/ Diatom puncture
12	Manca et al., 2004	Lago Maggiore (Italy)	Exotopic protrusions	Ectoparasite - *Ellobiposis*
13	Skovgaard, 2004	North west Mediterranean	TLA	*Blastodinium* infection, Predator attack
14	Bhandare & Ingole, 2008	Central Indian Ocean ridges	Cysts/ TLA	Toxic chemicals from hydrothermal vents, *Ellobiopsis*
15	Mantha & Hswang, 2011, 2013	Kueishantao Island, off Northeastern Taiwan coast	TLA	Toxic chemicals from hydrothermal vents, infections by epibionts

Table 2 TLA in copepods recorded from various Indian waters. The percentage abundance and mean salinity are presented. The approximate geographical location and suitable acronyms for environments are given.

SN.	Position	Environments (acronyms)	Percentage	Mean salinity
1	09.96° N, 76.26°E	Cochin Estuary (CE)	11.7	30.14
2	08.93° N,76.54° E	Ashtamudi Estuary (AE)	5.1	30.24
3	12.23° N, 75.12°E	Tejaswini Estuary (TE)	6.2	30.56
4	15.40° N, 73.89°E	Zuari Estuary (ZE)	4.4	31.32
5	15.52° N, 73.81°E	Mandovi Estuary (ME)	5.2	31.14
6	11.50° N, 79.78°E	Vellar Estuary (VE)	5.8	32.16
7	11.70° N, 79.77°E	Uppanar Estuary (UE)	4.2	31.54
8	16.71° N, 82.32°E	Godavari Estuary (GE)	3.7	31.24
9	19.37° N, 85.05°E	Rusikula Estuary (RE)	4.9	31.18
10	19.73° N, 85.36°E	Chilka Lake (CL)	8.9	31.56
11	09.95° N, 76.16°E	Coastal waters -off Kochi (CC)	3.6	33.56
12	18.83° N, 72.75°E	Coastal waters -off Mumbai (CM)	4.1	34.32
13	17.07° N, 82.46°E	Coastal waters -off Kakinada (CK)	5.8	34.21
14	8.76° N, 78.74°E	Gulf of Mannar (GoM)	5.7	34.14
15	9.95° N, 79.55°E	Palk Bay (PB)	12.4	33.02

Table 3 List of dominant copepods (>1% in any one of the samples) found in the estuaries and coastal waters of India considered in the present study. TLA were found in 24 species of copepods, which was high in Calanoids and Poecilostomatoids. Endo-parasite was reported inside the digestive tract of 6 species of copepods. Similarly, ecto-parasite (EPS) and epibiont (EPB) were found in one and seven species of copepods respectively. + represents presence and – represents absence. ABR – Abbreviations and TLA - Tumour like anomalies.

Order/Family			ABR	TLA	ENP	EPS	EPB
Order: Calanoida	SN.	Species					
Family - Calanidae	1	*Undinula vulgaris*	*UVU*	+	+	+	-
Eucalanidae	2	*Eucalanus elongatus*	*EEL*	-	-	-	-
	3	*Pareucalanus attenuatus*	*PAT*	+	-	-	-
Paracalanidae	4	*Acrocalanus gracilis*	*AGR*	+	-	-	-
	5	*Clausocalanus arcuicornis*	*CAR*	+	+	-	-
	6	*Paracalanus parvus*	*PPA*	+	+	-	-
	7	*P.aculeatus*	*PAC*	+	+	-	-
Centropagidae	8	*Centropages orsinii*	*COR*	+	-	-	-
	9	*C.furcatus*	*CFU*	+	-	-	-
Pseudodiaptomidae	10	*Pseudodiaptomus aurivilli*	*PAU*	+	-	-	-
	11	*P.serricaudatus*	*PSE*	+	-	-	-
Temoridae	12	*Temora turbinata*	*TTU*	+	-	-	-
	13	*T.discaudata*	*TDI*	+	-	-	-
Pontellidae	14	*Calanopia minor*	*CMI*	+	-	-	-
	15	*Labidocera acuta*	*LAC*	+	-	-	-
	16	*Pontella danae*	*PDA*	-	-	-	-
Acartiidae.	17	*Acartia spinicauda*	ASP	+	-	-	-
	18	*A. erythraea*	AER	+	-	-	+
	19	*A.danae*	ADA	+	-	-	+
	20	*Acartiella gracilis*	*AGR*	+	-	-	-
Diaptomidae	21	*Heliodiaptomus cincuatus*	*HCI*	+	-	-	-
	22	*Allodiaptomus sp.,*	ASP	+	-	-	-
Order: Harpacticoida							
Ectinosomatidae	23	*Microsetella norvegica*	*MNO*	-	-	-	+
	24	*M. rosea*	*MRO*	-	-	-	+
Miraciidae	25	*Macrosetella gracilis*	*MGR*	-	-	-	+
	26	*M. oculata*	*MOC*	-	-	-	+
Euterpinidae	27	*Euterpina acutifrons*	*EAC*	+	-	-	+
Order: Cyclopoida							
Oithonidae	28	*Oithona rigida*	ORI	-	-	-	-
	29	*O.brevicornis*	OBR	-	-	-	-
	30	*O.similis*	OIS	+	+	-	-
Order: Poecilostomatatoida							
Oncaeidae	31	*Oncaea venusta*	OVE	+	-	-	-
Corycaeidae	32	*Corycaeus danae*	CDA	+	+	-	-
	33	*Farranula gibbula*	FGI	-	-	-	-
		Total		24	6	1	7

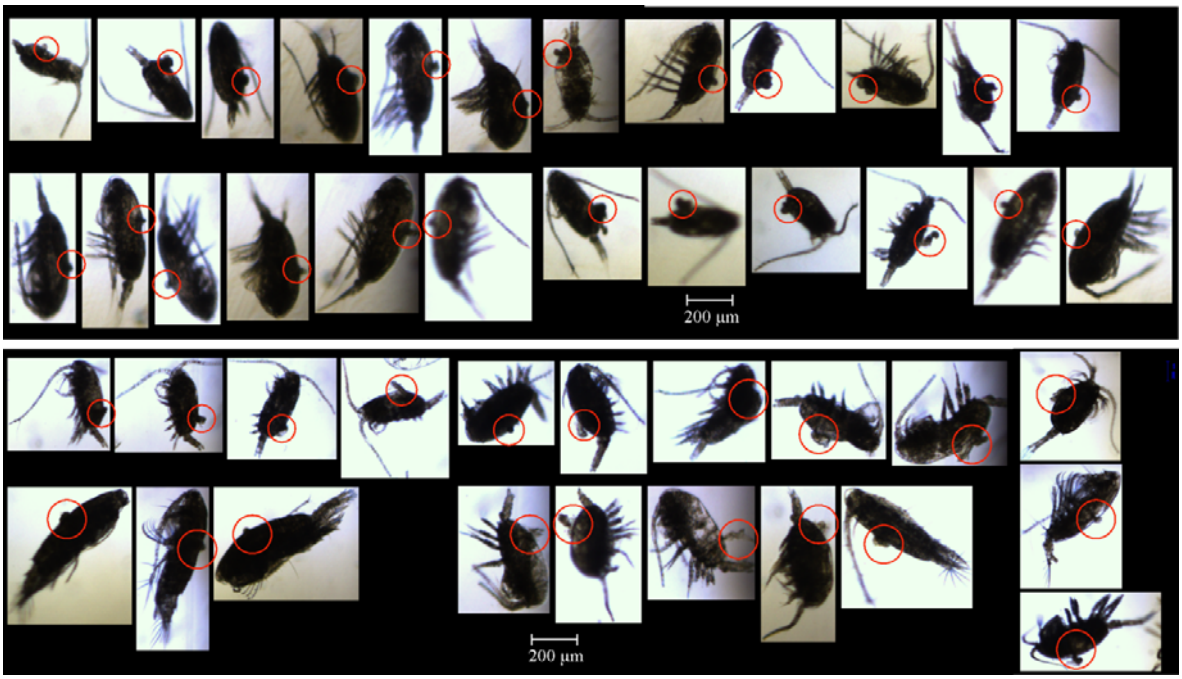
Table 4 Distribution of epibionts, endo (*Blastodinium* sp.,), ecto (*Ellobiopsis* sp.,) infection in the waters around India and their percentage of the occurrence to the total copepod population was presented in parenthesis.

		Endoparasite	*Ectoparasite*	*Epibionts*	
SN.	Environments (acronyms)	*Blastodinium*	*Ellobiopsis*	*Zoothamnium*	*Acineta*
1	Cochin Estuarine inlet (CE)	+ (0.14)	+ (0.08)	+ (0.32)	+ (0.46)
2	Ashtamudi Estuarine inlet (AE)	+ (0.17)	-	+ (1.12)	+ (0.24)
3	Tejaswini Estuarine inlet (TE)	-	-	+ (0.94)	+ (0.31)
4	Zuari Estuarine inlet (ZE)	-	-	+ (0.41)	-
5	Mandovi Estuarine inlet (ME)	-	-	+ (0.51)	-
6	Vellar Estuarine inlet (VE)	-	-	-	-
7	Uppanar Estuarine inlet (UE)	-	-	+ (0.28)	-
8	Godavari Estuarine inlet (GE)	-	-	-	-
9	Rusikula Estuarine inlet (RE)	-	-	-	-
10	Chilka Lake (CL)	-	+ (0.07)	+ (1.14)	+ (0.15)
11	Coastal waters – Cochin (CC)	+ (0.66)	+ (0.23)	-	-
12	Coastal waters – Mumbai (CM)	+ (0.94)	+ (0.31)	-	-
13	Coastal waters - Kakinada (CK)	+ (0.28)	-	-	-
14	Gulf of Mannar (GoM)	+ (0.24)	-	-	-
15	Palk bay (PB)	+ (1.49)	-	-	-

Table 5 *Ellobiopsis* insertion point to the host body and their comparison with previous study from Indian waters (Santhakumari and Saraswathy, 1979) and Bay of Biscay (Albaina and Irigoien, 2006). Present study and earlier study from the Indian waters *Ellobiopsis* infection was found only in *Undinula vulgaris*. In Bay of Biscay the *Ellobiopsis* infection was found in *Calanus helgolandicus*. All these studies showed that *Ellobioposis* insertions occur on the appendages of the copepods rather than the body parts.

SN.	Point of attachment	Present study	Indian waters	Bay of Biscay, Italy
1	Rostrum	-	-	1 (0.31)
2	Antennule	11 (10.29)	12 (16.92)	112 (34.25)
3	Antenna	53 (49.53)	27 (38.02)	29 (8.87)
4	Mandible	5 (4.67)	11 (15.49)	14 (4.28)
5	Maxillule	4 (3.73)	6 (8.45)	70 (21.41)
6	Maxilla	7 (6.54)	3(4.22)	35 (10.7)
7	Maxilliped	26 (24.30)	4 (5.63)	63 (19.27)
8	1st swimming legs	1 (0.93)	-	3 (0.92)
9	2nd swimming legs	-	-	-
10	3rdswimming legs	-	-	-
11	4thswimming legs	-	-	-
12	5th swimming legs	-	8 (11.27)	-
13	Prosome	-	-	-
14	Urosome	-	-	-
	Total	105	71	327
	% prevalence	4.64	8.3	6.8

a)



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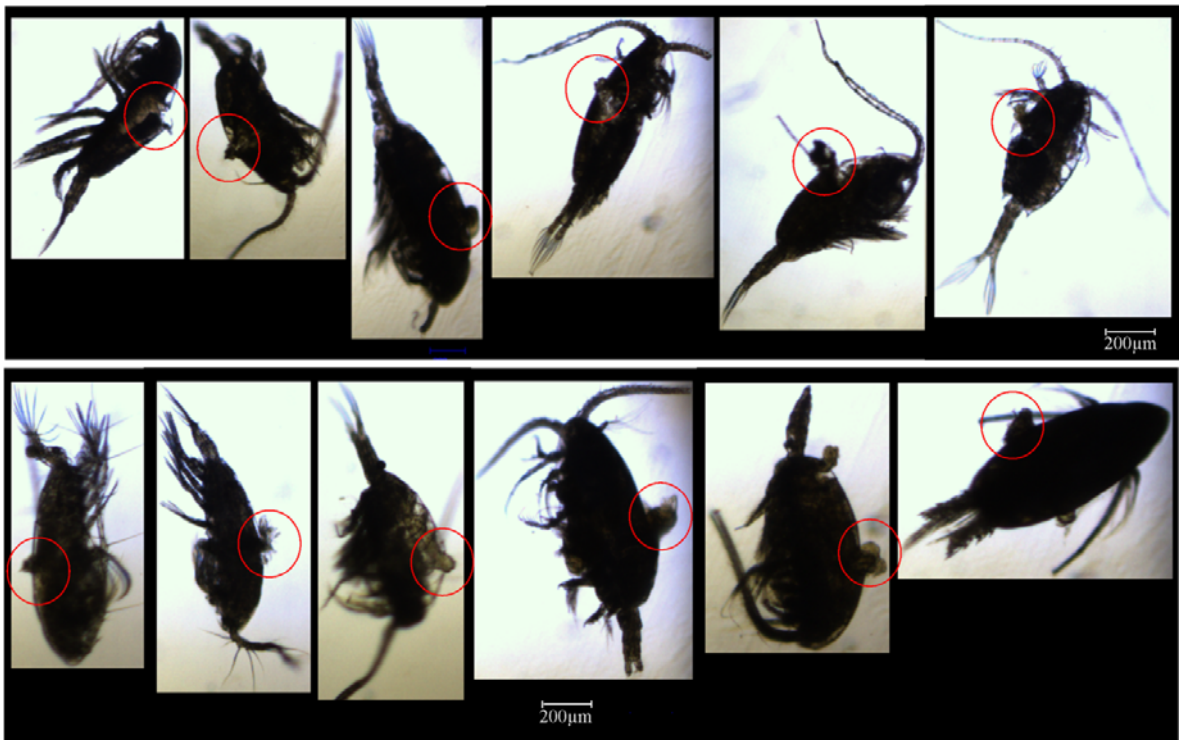

Supplementary figure 1 - Snapshot of FlowCAM images of TLAs in copepods. The red circles denotes the location of TLA. Panels represent (a) sorted images of <500 µm size fraction and (b) >500 µm size fraction of copepods.