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Research Article

Real Time PCR detection of *Macrobrachium rosenbergii* (de Man, 1879) larvae with emphasis to their ecology

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Abstract – Species specific identification of early larval stages of many decapod crustaceans sampled from plankton collections remains cumbersome owing to lack of distinguishable characteristics, where DNA based molecular methods provide accurate results without taxonomic ambiguities. In the present study, an attempt was made to detect temporal occurrence of early zoea of freshwater prawn *Macrobrachium rosenbergii* (de Man) using real-time PCR assays in polyhaline, mesohaline and oligohaline areas of a tropical positive estuary, the Vembanad lake (S. India). High caridean larval abundance could be recorded in polyhaline areas in all seasons while it could be recorded in monsoon season in mesohaline and oligohaline areas. 113 DNA isolations were successfully made from morphologically identified taxonomic units (MOTU) and SYBR Green based RT-PCR amplifications using designed primer for *M. rosenbergii* yielded positive detections in 38 samples (34%) representing all seasons in all three zones. Positive detections could be recorded in all months except May in mesohaline areas and differed significantly ($F = 17.2$ $p < 0.01$) with the same in polyhaline and oligohaline areas. The present results of molecular detection of *M. rosenbergii* larvae extend confirmation of its breeding ground in Vembanad lake where appropriate management strategies could be enforced for stock conservation of this species.

Keywords: Plankton samples / *M. rosenbergii* larvae / COI / absolute quantification / SYBR green

1 Introduction

Members of family Palaemonidae (Infraorder Caridea; Order Decapoda) are known to be amphidromous with females releasing their ripe eggs in freshwater stream flow and embryos hatching out to meroplanktonic larvae that get passively dispersed to low saline regions where they complete metamorphosis before returning to freshwater conditions at advanced life stages (Bauer, 2011). The plankton trophic larval phase in their early development facilitates them in achieving wider spatial dispersion and thereby, in confronting with ecological challenges including food availability, favourable environmental conditions and predation (Rumrill, 1990; Morgan, 1995; Pechenik, 1999; Anger, 2006; Pineda et al., 2007). Some palaemonid larvae get dispersed to limited spatial distances which support stable genetic exchange and population connectivity through regular recruitment. In many species of *Macrobrachium*, females undertake downstream breeding migrations to low saline areas in estuaries during seasonal stream flows for facilitating larval release and metamorphosis (Hartmann, 1958; Ling, 1969; Ibrahim, 1962; Bauer and Delahoussaye, 2008). Extensive breeding migration has also been reported in *Macrobrachium rosenbergii* (de Man) inhabiting Indian waters (Rao, 1967; Raman, 1967; Kurup et al., 1992; Harikrishnan and Kurup, 1996). Females of this species undertake extensive differential breeding migration to downstream regions of Vembanad lake, South India and release their larvae in areas of congenial environmental conditions which return to upstream rivers after metamorphosing to post larval stages (Raman, 1967; Kurup et al., 1992; Harikrishnan, 1997). However, closure of a barrage for arresting salinity intrusion into oligohaline regions of the lake has been reported to have seriously affected its larval ecology and stock conditions (Kurup et al., 1992; Kurup and Harikrishnan, 2000: Harikrishnan and Kurup, 2001).

Information on larval ecology of estuarine fauna is important as they yield precise insights into ecological processes that influence their populations (Schwamborn et al., 1999; Queiroga and Blanton, 2015; Dos Santos et al., 2008). Information on life history, population abundance, distribution, role to play in ecosystem etc. are

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vital for aquatic fauna of fishery importance since these conditions seriously determine exploitation of their adults in their ecosystems. However, species specific identification of larval forms of many crustaceans is cumbersome owing to their complex life cycle with multiple stages and that most larvae do not resemble their adult forms (Anger, 2001). Phenotypic plasticity in larval forms often results in uncertain inferences (McManus and Katz, 2009). Knowledge on larval morphology of many caridean species are well described (Gurney, 1942; Williamson, 1982; Anger, 2001), but differentiating larval forms of closely related species becomes difficult, even for experienced taxonomists. Morphological examination on palaemonid larval forms for species identification also often ends up unsuccessful (Harikrishnan, 1997). Identification of *Macrobrachium* larval forms using traditional morphological methods is hampered by high morphological similarity and lack of unique characters differentiating species. Patterns of chromatophores of their third abdominal segment (Menon, 1938; Ling, 1969; Uno and Kwon, 1969; Pillai and Mohamed, 1973) help in species identification, which, however, are most unreliable owing to their vulnerability to preservatives used for storing larval collections. Taxonomic identification using molecular methods including DNA barcoding (Hebert et al., 2003; Deepak et al., 2021) have extensively been used for identifying yeast (Fell, 1995), phytoplankton (DeLong et al., 1989), copepods (Bucklin et al., 1999) and invertebrate larvae (Olson et al., 1991; Dixon et al., 1995; Medeiros-Bergen et al., 1995; Goffredi et al., 2005; Chow et al., 2006). Recently, real time PCR based methods were also used for detection of aquatic invertebrates including decapod crustacean larvae (Bilodeau et al., 1999; Vadopalas et al., 2006; Pan et al., 2008, Wight et al., 2008; Jensen et al., 2012). Fernandes et al., 2018). Mitochondrial cytochrome c oxidase I (mtCOI) is considered as a major molecular marker for bio-identification of crustaceans as its sequence divergence differentiates even closely allied species (Jose and Harikrishnan, 2016; Kobayashi, 2020). The present account encompasses results of real time PCR based assays for detecting *M. rosenbergii* larvae among caridean larval collections from polyhaline, mesohaline and oligohaline areas of Vembanad lake and thereby, delineating their temporal and spatial occurrence in the lake which will help in developing sustainable management strategies for the species.

2 Materials and methods

2.1 Sampling and morphological identification

Vembanad lake (Lat. 9^0 29′ and 10^0 10′ N; Long. 76^0 13′ and 76^0 31′ E) (Fig. 1), encompasses largest estuarine habitat in south west coast of India and supports a diversified aquatic fauna that depend on this aquatic system for food, spawning and nursery. The lake is separated from the Arabian sea by an extensive land barrier which runs parallel to its long axis until it joins the sea by a permanent bar mouth at Cochin. It receives freshwater discharge from five rivers originating from the Western Ghats, rivers Pamba, Achankovil, Manimala, Meenachil and Muvattupuzha. However, the northern part of this lake retains typical estuarine environment with highly variable physico-chemical features owing to tidal inundation. Being a typical tropical positive estuary, this lake provides polyhaline, mesohaline and oligohaline ecological conditions influenced by premonsoon, monsoon and post monsoon seasons.

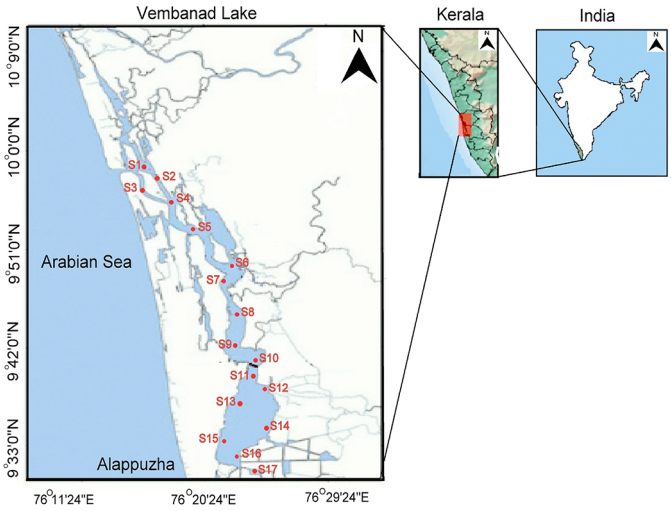

Fig. 1. Map of Vembanad lake (S. India) showing sampling stations.

Plankton and surface water samples were collected from 17 sampling stations (Fig. 1) in Vembanad lake on monthly intervals with the help of M.B. Indfish (School of Industrial Fisheries, Cochin University of Science and Technology). Hydrographic parameters (salinity, temperature, pH and dissolved oxygen) were recorded onboard using handheld refractometer (model) and Eutech PD650 water quality analyser. Surface plankton samples were collected by towing a 60 cm diameter plankton net having mesh size 200 μm horizontally for 10 min duration. The volume of water filtered was calculated with a calibrated flow meter (General Oceanics model number- 2030 R). Immediately after collection, the samples were preserved using absolute alcohol and 4% formalin. Preserved samples were taken to the laboratory for detailed examination. The samples were made to standard volume (100 ml) and sub samples were prepared using Folsom plankton splitter. An aliquot of 5 ml was taken in a counting chamber, observed under a stereo microscope with micro-photography attachment (Leica – D300) and caridean larvae were sorted and identified based on standard literature (Dos Santos and González-Gordillo, 2004; Guerao and Cuesta, 2014). The counting process was repeated 5 times for availing more accurate representation. Total volume of water filtered through the net was calculated from flowmeter readings and caridean larval abundance was expressed in number of organisms per 100 cubic meters of water. The monthly data collected are grouped based on seasons as premonsoon (February to May), monsoon (June to September) and post monsoon (October to January). The caridean larval samples preserved in alcohol were subjected to detailed morphological examination under stereomicroscopes. Morphologically taxonomic units (MOTU) which resembled mostly to *Macrobrachium* species by morphological features were identified following Ling (1969), Ngoc-Ho (1976), Pillai and Mohamed (1973), Pillai (1990, 1991). Such 'MOTU's were selected from every month and were subjected to molecular analysis for detection.

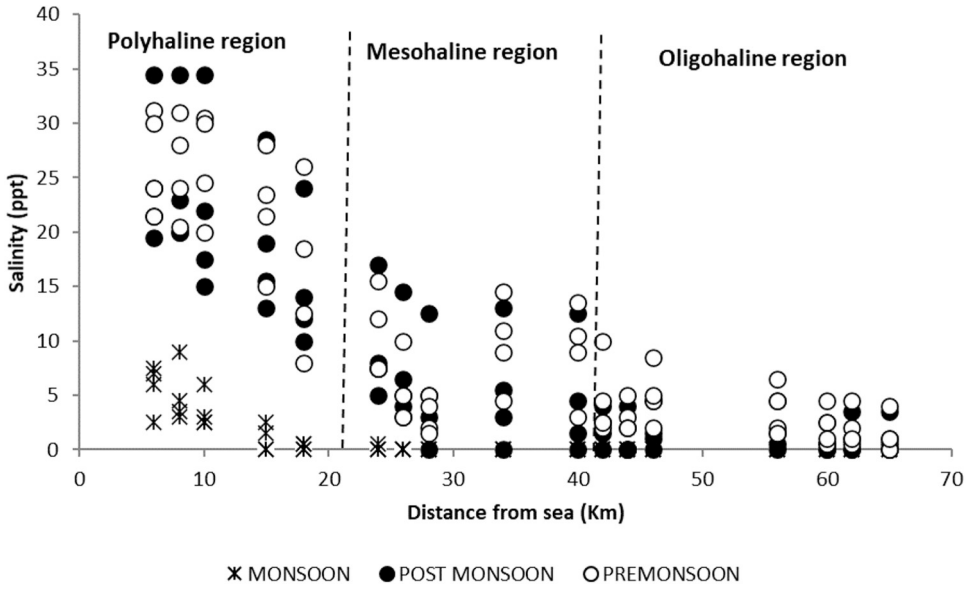

Fig. 2. Salinity recorded in various stations of Vembanad lake and their distance from sea.

2.2 Quantification of *Macrobrachium rosenbergii* larvae by real time PCR

2.2.1 DNA extraction and spectrophotometric quantification

Total genomic DNA of adult *Macrobrachium rosenbergii* individuals were extracted from pleopods preserved in 95% ethanol, using DNeasy Blood and Tissue Kit (Qiagen) following spin column protocol for purification of total DNA. Larval samples were sorted as individual units and incubated overnight in demineralized water and DNA extraction was done using the same protocol. DNA was isolated from individual caridean larvae (without mixing larval samples) and all the extracts were stored at 4 °C for further molecular analysis. Quantity and quality of DNA extracts were analyzed using UV spectrophotometer (Nanodrop 2000). Ratio of absorbance between 260 and 280 nm was measured to analyze the quality of DNA isolates. Isolates having quantification value(s) between 1.7 and 2.0 were considered as good quality for molecular analysis.

2.2.2 Designing of species specific mitochondrial cytochrome oxidase subunit I gene specific primers

"Palumbi" region of *Macrobrachium rosenbergii* mtCOI gene (ranging from 721 to 1320 bp) was selected for species identification from plankton samples (Palumbi and Benzie 1991; Jose and Harikrishnan, 2016, 2019). Two sets of species-specific DNA primers with amplicon target length of approximately 150 base pairs (bp) was designed using Primer3web version 4.1.0 (http://primer3.ut.ee/). Specificity of newly designed primer pairs were checked using DNA extracts of target (*M. rosenbergii*) and non-target congeneric species (data not shown) as template and the best performing primer pair was selected for subsequent use. The forward and reverse primers, 1F-5' GGT TTT GTA TGG GCC CA 3' and 1R-5' TTG ACT TCC ATG GAG TGT GC 3' respectively, amplify 144 base pair of COI gene of *M. rosenbergii* larvae among the collected plankton samples.

2.2.3 SYBR green based real time PCR amplification

In order to generate a 10-fold standard curve (6 point), serial dilution of standard DNA from adult *M. rosenbergii* (15.1 ng/μl) was done and all the extracts were stored at 4 °C. For detecting the presence of *M. rosenbergii* larval forms among the collected plankton samples, species specific primers were used for amplification. Real-time PCR was performed using a 20 μl reaction mix containing 10 μl of iTaq universal SYBR® Green reaction mix (2x), 1 μl concentration of each primers (0.5 μM), 4 μl of genomic DNA (approx. 60 ng) and 4 μl of Nuclease free H_2O. Positive and negative controls were also included. Reactions were analysed with Biorad MJ Mini Real time thermal cycler with temperature profile consisting of an initial denaturation step of 2 min at 95 °C followed by 35 cycles of 40 s at 94 °C, 40 s at 54 °C and 1 min 10 s at 72 °C followed in turn by a final extension of 10 min at 72 °C (8).

3 Results

3.1 Ecology of the study area

Salinity values recorded in three seasons in various sampling stations in Vembanad lake are depicted in Figure 2 which distinctly demarcated polyhaline, mesohaline and oilgohaline regions.

It could be noted that salinity values were perceptibly low in monsoon season in all stations. Stations 1 to 5 were located within 20 km from bar mouth at Cochin and constituted a polyhaline region. Polyhaline regions registered higher salinity levels (35 ppt post monsoon; 31.5 ppt, premonsoon). Stations 6 to 10 located 20 to 40 km from bar mouth could be recognized as mesohaline region where maximum salinity recorded were 17 and 10 ppt in post monsoon and premonsoon respectively.

Stations 11 to 17 were located beyond 40 km from bar mouth constituted an oligohaline region where maximum salinity recorded did not exceed 10 ppt in any season. The seasonal averages of (mean ± standard deviation) of surface water temperature, pH, salinity and dissolved oxygen recorded in three ecological zones of the lake during the study period are depicted in Figure 3. The surface water temperature recorded in three seasons differed significantly between zones ($F = 37.47$ $p < 0.001$). Significantly higher temperature was recorded in pre monsoon season in polyhaline zone (30.86 ± 0.91 °C), mesohaline zone (32.73 ± 1.25 °C) and oligohaline zone (31.89 ± 1.06 °C). Water pH values recorded in three seasons differed significantly between the zones ($F = 14.61$ $p < 0.001$). pH values recorded in polyhaline zone in premonsoon (7.61 ± 0.10), monsoon (7.47 ± 0.33) and post monsoon (7.61 ± 0.24) were significantly higher than that in other zones. pH values were also perceptibly higher in mesohaline (7.13 ± 0.21) and oligohaline (7.23 ± 0.23) zones during premonsoon season. Mean salinity values in three zones differed significantly ($F = 68.98$ $p < 0.001$) and significantly low salinity could be recorded in monsoon season in all zones. The mean salinity values worked out in polyhaline zone during premonsoon and post monsoon were 23.41 ± 6.43 ppt and 23.41 ± 7.45 ppt respectively while the same recorded in mesohaline zones were 7.65 ± 4.28 ppt and 7.41 ± 4.71 ppt respectively. The dissolved oxygen values recorded in three seasons differed significantly between zones ($F = 15.01$ $p < 0.001$). In polyhaline and mesohaline zones, significantly higher dissolved oxygen values were recorded in monsoon months (5.09 ± 0.60 mg/L and 5.73 ± 0.59 mg/L respectively) whereas in oligohaline zone dissolved oxygen values were high in post monsoon (5.67 ± 0.73).

3.2 Caridean larval distribution and abundance

The seasonal mean ± standard error of caridean larval abundance in three ecological zones is given in Figure 3e. In polyhaline zone, high larval abundance was recorded in all seasons, increasing from monsoon (25.42 ± 6.4 × 1000 per 100 m^3) to post monsoon (36.6 ± 5.5 × 1000 per 100 m^3) and to premonsoon (40.05 ± 12.9 × 1000 per 100 m^3). However, in both mesohaline and oligohaline zones, larval abundance during premonsoon was lowest (5.1± 1.5 × 1000 per 100 m^3 and 4.03 ± 1.1 × 1000 per 100 m^3 respectively), highest being recorded in monsoon season (16.5 ± 9.9 × 1000 per 100 m^3 and 14.2 ± 3.4 × 1000 per 100 m^3 respectively). On comparing seasonal abundance by analysis of variance revealed significant difference ($F = 5.52$, $p < 0.01$). The results of pair wise comparison by Tukey analyses indicated no significant difference in larval abundance in polyhaline region between three seasons. Further, the larval abundance in monsoon season did not register significant variation between three ecological zones. However, larval abundance in mesosaline and oligohaline zones during premonsoon and post monsoon were significantly lower than that of polyhaline region ($p < 0.01$).

3.3 Real Time PCR based detection of *M. rosenbergii* larvae

Larval samples collected from 17 stations in polyhaline, mesohaline and oligohaline zones in premonsoon, monsoon and post monsoon seasons were examined morphologically. A total of 113 caridean larvae (morphologically taxonomic units (MOTUs)) were identified and DNA was successfully isolated from them. From the 113 isolates, SYBR Green based amplification in Real Time PCR using designed primers for *M. rosenbergii* yielded 38 positive detections (representing *M. rosenbergii* larvae). Presence of *M. rosenebrgii* larvae from all three zones in all seasons was accounted up to 33.63%. However, the number of positive detections (representing *M. rosenbergii* larvae) were significantly different between the zones ($F = 17.2$ $p < 0.01$) and the same in mesohaline zone (25 positive detections) was significantly higher than polyhaline (7 positive detections) and oligohaline zones (6 positive detections) (Fig. 4). Positive larval detections were recorded in all months except May in mesohaline zone while in polyhaline zone, positive detections were encountered in December, January, February, May and August only. Similarly, in oligohaline zone, positive detections could be encountered in February, August, October and November only.

Absolute quantification based on SYBR Green based RT-PCR using designed primer pair for *M. rosenbergii* was performed. Standard prepared using adult *M. rosenbergii* genomic DNA produced a slope according to its dilution. The COI standard curve exhibited linearity in regression followed by a descending quantification at the lowest dilution with Ct value 30.95. Hence, Ct values not exceeding 30 were considered as positive detections and exceeding values were considered as false positives or nonspecific amplifications. Efficiency (E) of the reaction was 101.0% with considerable R^2 (0.993) and slope (−3.142) value. Melt curve was also devoid of shoulders and multiple peaks (figure not included). Threshold cycles for positive amplifications were recorded within a range of 24–30. Total, positive detections were accounted in 38 samples, contributing up to approx. 34% (representing all seasons in all three zones (Fig. 5)) of the total samples.

Positive larval detections were recorded in all months except May in mesohaline zone while in polyhaline zone, positive detections were encountered in December, January, February, May and August only. Similarly, in oligohaline zone, positive detections could be encountered in February, August, October and November only.

4 Discussion

The study area is located in southern part of Cochin estuary which forms a typical tropical microtidal positive estuarine system greatly controlled by strong monsoonal river discharges (Qasim, 2003). The estuary experiences three distinct seasonal hydrographic features during monsoon (June to September), post monsoon (October to January) and premonsoon (February to May). During monsoon, most part of estuary turns into freshwaters with less dense freshwater at surface and more dense seawater at bottom resulting in high stratification and salinity wedging (Joseph and Kurup, 1989; Lakshmanan et al., 1982; Menon et al., 2000). Jacob et al. (2013) have reported that this halocline extends up to station 8 (mesohaline zone in the present study) during monsoon. It has also been reported that salinity intrudes in the lake from a lowest of 10km in monsoon to more than 40 km in other

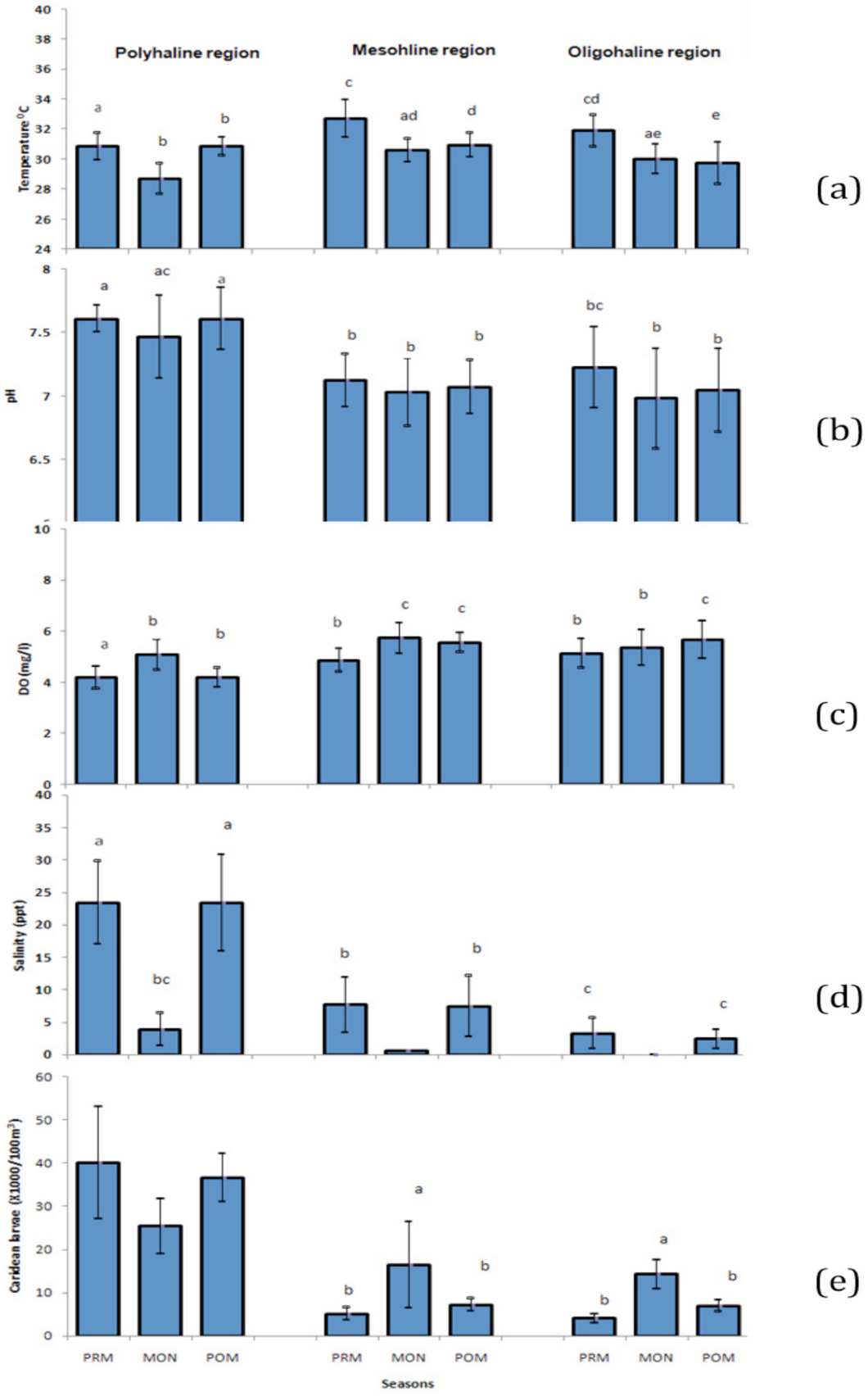

Fig. 3. Mean ± standard error of a: surface water temperature; b: pH; c: salinity; d: dissolved oxygen; and e: caridean larval abundance recorded in premonsoon (PRM), monsoon (MON) and post monsoon (POM) in polyhaline, mesohaline and oligohaline zones in Vembanad lake.

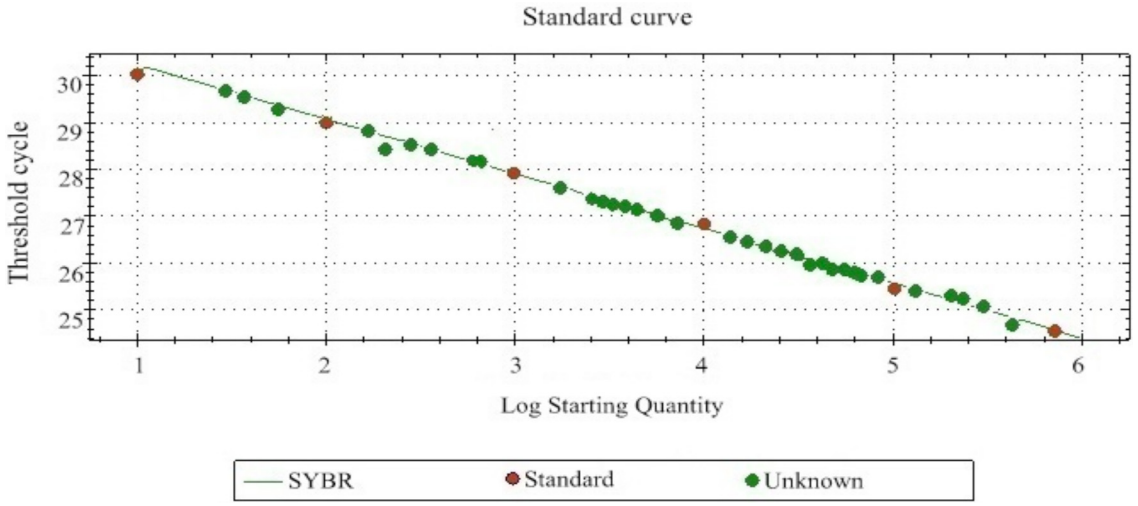

Fig. 4. COI standard curve for across a six-fold dilution series of gDNA from *M. rosenbergii*.

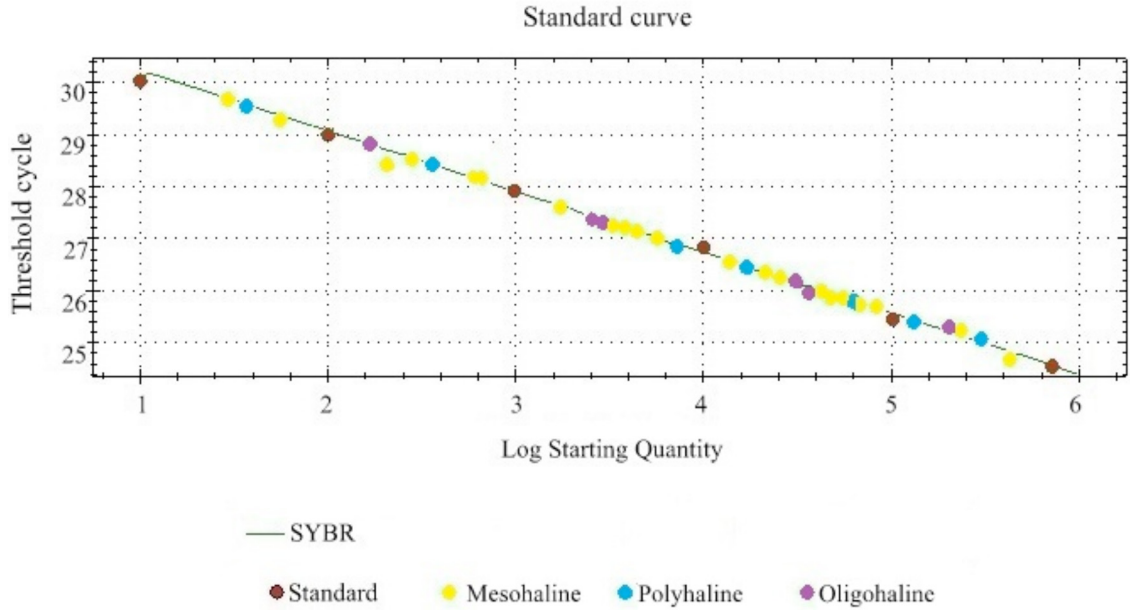

Fig. 5. Standard curve showing zone wise detections of *M. rosenbergii* larvae.

seasons. The surface salinity and temperature recorded in three zones in the present study corroborated with previous reports. Comparatively higher temperature was noted in southern (mesohaline and oligohaline zones in present study) in contrast to polyhaline north end (Menon et al., 2000), which, however, could be observed mainly in premonsoon season in the present study. In the present study, pH values were perceptibly higher in polyhaline zone especially in post monsoon and were comparable to previous results (Thasneem et al., 2018). Relatively higher dissolved oxygen recorded in mesohaline and oligohaline zones were noteworthy and highest mean dissolved oxygen was registered in mesohaline zone during monsoon season. Present results also indicated higher preponderance of caridean larvae in monsoon season in mesohaline and oligohaline zones in contrast to other seasons. In polyhaline zone, larval abundance was glaringly high in all seasons, gradually increasing from monsoon to premonsoon. The downstream part of Vembanad lake is known to provide natural abode to a large number of invertebrate species and about 21 species belonging to caridean families Alpheidae, Atyidae, Palaemonidae and Ogyrididae have been reported (Thomas, 1976; Sureshkumar, 1998; Roy et al., 2009). Breeding season of alpheids (Harikrishnan et al., 2010) and some palaemonids (Pillai, 1990; Sureshkumar, 1998) coincided with post monsoon and premonsoon season and this could be attributed to higher occurrence of caridean larvae in downstream part of lake when increasing salinity conditions favoured their metamorphosis.

Among carideans, giant freshwater prawn, *M. rosenbergii* (Family Palaemonidae) is contributing considerably to the lucrative fishery landings in Vembanad lake and it commands excellent demand in both domestic and export markets. However, remarkable dwindling in its exploited fishery has been reported from an annual average landing of 400 tonnes in sixties (Raman, 1967) to 89 tonnes in eighties (Kurup et al., 1992), 121 tonnes in nineties (Harikrishanan and Kurup, 2001) and 29 tonnes in 2000-01 (Harikrishnan and Kurup, 2006). The decline in stock of this species has largely been attributed to severe anthropogenic interventions in the lake ecosystem by way of extensive reclamation, mangrove destruction, pollution, overfishing and construction of a salinity barrier across the lake that transformed it to two ecological zones (Kurup et al., 1992). The fishery was mainly constituted by differentially migrating adult males and females from adjoining rivers to the lake proper (Raman, 1967; Kurup et al., 1992; Harikrishnan, 1997) where they are prone to hostile ecological conditions and heavy fishing pressure. Males predominated in catches from March to June while females outnumbered males in landings during July to February period (Harikrishnan and Kurup, 2001).

As the magnitude of *M. rosenbergii* fishery in Vembanad lake depends mainly on recruitment of post larvae from estuarine area to upstream rivers (Kurup et al., 1992), adequate information on abundance and distribution of its larval forms are important. Adults of *M. rosenbergii* are reported to inhabit upstream river bottom (Raman, 1964). Being euryhaline and amphidromous (Goodwin and Hanson, 1975; Anger, 2001; Bauer, 2011), its females assist larvae in reaching congenial conditions in distantly located downstream, as newly hatched lecithotrophic larva could not survive drifting passively along current before reaching low saline waters where they moult to next larval stage (Bauer and Delahoussaye, 2008; Bauer, 2011). Females carrying early-stage eggs (orange-coloured eggs) in their brood pouches were observed in upstream rivers which moved to mesohaline region (maximum 18 ppt) in southern part of Vembanad lake (Raman, 1967). However, a salinity barrier was constructed across the lake during seventies for favouring agriculture and its operation changed the salinity profile of the lake causing drastic salinity decline to 6 ppt (Kurup et al., 1992). Therefore, the egg bearing females were compelled to move about 40 km northwards (mesohaline zone in the present study) for providing congenial ecological conditions to their larvae (Raman, 1967; Kurup et al., 1992; Kurup and Harikrishnan, 2000). Such egg bearing females were reported to appear in exploited fishery landings from July to January period with peak occurrence in September to November period (Raman, 1967; Kurup et al., 1992; Harikrishnan and Kurup, 1997). It has been reported that these females are heavily exploited and only 30.2% of their stock could manage to reach downstream (Harikrishnan and Kurup, 1997). It may be pointed out that the mesohaline zone identified in the present study conforms to the breeding grounds reported by previous studies (Kurup et al., 1992; Harikrishnan and Kurup, 1996; Harikrishnan and Kurup, 1997).

Accurate identification of *M. rosenbergii* larvae using traditional methods are very difficult to perform. As these larvae are tiny and fragile to identify, its abundance and distribution data are also limited. In such cases, RT-PCR along with fluorescent reagents like SYBR Green I, TaqMan probe, and molecular beacon represents a valuable research tool applicable over environmental samples (Zhang and Fang, 2006). Among these, SYBR Green based assay based on intercalation of double-stranded DNA-binding dyes are the simplest and cheapest method for species detection (Odero et al., 2018; Wang et al., 2015). DNA extracted from samples provide template to screen for species-specific DNA sequence via quantitative PCR (QPCR) technology. Since the dye can bind with all double-stranded DNA producing both specific and nonspecific PCR products, species specific primers are to be designed to ensure the amplification of target specimens. In this study, two primer pairs for amplifying the "Palumbi" region of *M. rosenbergii* COI gene with an amplicon length of approximately 150 bp was designed and tested using conventional PCR (Wang et al., 2015). Only one primer pair (please refer materials and methods) was selected and used for absolute quantification using RT-PCR assay because of its specificity and sensitivity, thus avoiding non-specific amplifications. In this work, positive results of samples after absolute quantification were randomly selected and verified by sanger sequencing (data not shown). Nucleotide BLAST analysis revealed that all of the amplicons were the expected *M. rosenebrgii* COI fragment (144 bp), and there was no amplification of either the negative or NTC samples, confirming the specificity of designed primers of *M. rosenbergii*. Standard curve was also generated using adult *M. rosenbergii* genomic DNA to carry out conversions of RT-PCR Ct values and number of *M. rosenbergii* larvae. Wang et al. (2015) developed a real-time PCR assay based on SYBR Green I for rapid identification and quantification of *Aurelia sp.* using species specific primers designed for amplifying mitochondrial 16S rDNA. A similar approach was carried out by Vadopalas et al. (2006) and Bouma et al. (2006) for identification and quantification of pinto abalone (*Haliotis kamtschatkana*) larvae in seawater. Jensen et al. (2012) also developed a real time PCR assay using COI gene for detection of planktonic red king crab (*Paralithodes camtschaticus*) larvae. This indicates that the RT-PCR assay developed here could be used in rapid and absolute quantification of *M. rosenebrgii* in field samples.

Results obtained from this study indicated that highest positive detections could be made in *M. rosenbergii* larvae collected from mesohaline zone were positive detections could be made in all months except May. Further, high preponderances of positive detections during monsoon (11 positives), post monsoon (7 positives) and premonsoon (7 positives) could also be recorded. Prolonged occurrence of females carrying eggs in advanced embryonic stages were reported from July to January in these areas before (Harikrishnan and Kurup, 1997). It has been reported that females mature in upstream rivers and move down to estuarine areas during February to and April and during May to August, heavy monsoonal river discharges transport faster to downstream estuarine areas (Raman, 1967) and this could be attributed to high preponderance of *M. rosenbergii* larvae in meso saline zone during monsoon and premonsoon. Though surface water in this zone during monsoon is purely freshwater, bottom water becomes brackish owing to salt wedge from inundating tidal waters (Joseph et al., 1996). In this zone, bottom salinity was reported to range between near 5 ppt in

monsoon to 25 ppt in premonsoon (Menon et al., 2000). It may also be noted that river Muvattupuzha joins Vembanad lake very near to mesohaline zone and therefore, females from this river could reach the breeding area earlier to breeding stocks from southern rivers. Higher preponderance of spent females in this river was also reported (Harikrishnan and Kurup, 1997) which also point out to prolonged occurrence of larvae in mesohaline zone extending up to premonsoon period.

In polyhaline and oligohaline zones, *M. rosenbergii* larvae could be detected only irregularly which in former, appeared to have drifted along current from mesohaline zone. Such drifting larvae would succumb to antagonistic ecological conditions in polyhaline zone. In oligohaline zone, positive detections were made in February, August, October and November. The positive detections in oligohaline zone (southern part of salinity barrier) in February was unanticipated as the barrier remained closed from mid December to mid March preventing the movement of egg bearing females. The closure coincided with very low river discharges from south and increased tidal inundations from north (Kurup and Harikrishnan, 2000). In the present study, maximum salinity recorded south of salinity barrier during December to March was 10 ppt. Raman (1967) has reported that prior to breeding season, mature and egg bearing females were encountered near river mouths as a second wave of breeding migration. *M. rosenbergii* larvae detected during closure of barrier would have been released by such late migrant females.

This study is the first molecular approach towards the detection of commercially important *M. rosenbergii* larvae among plankton samples. We attempted to acquire an account of this species across its natural abode, Vembanad Lake using periodic sampling and molecular analysis. However, a more extensive study incorporating more sampling stations representing the confluent rivers of this lake could help to generate a more detailed account the occurrence and ecology of *M. rosenbergii*.

5 Conclusion

We have successfully detected and quantified *M. rosenbergii* larvae from plankton samples using specific primers for COI gene. This approach could be implemented for detecting and quantifying *M. rosenbergii* larvae over its entire accounted stations. In addition, the ecological factors complementing its survival could be correlated along with their occurrence for future research in stock development.

Data sharing

Data sharing is not applicable to this article as no new data were created or analysed in this study.

Conflict of interest

The authors declare responsibility for the entire contents of this paper and have no conflicts of interest.

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References

Anger K. The Biology of Decapod Crustacean Larvae, Crustaceana 14. AA Balkema Publications, Lisse, Netherlands, 2001, pp. 1–420.

Anger K. 2006. Contributions of larval biology to crustacean research: a review. *Invert Reprod Develop* 49: 175–205.

Bauer RT, Delahoussaye J. 2008. Life history migrations of the amphidromous river shrimp *Macrobrachium ohione* from a continental large river system. *J Crust Biol* 28: 622–632.

Bauer RT. Amphidromy and migrations of freshwater shrimps. II. Delivery of hatching larvae to the sea, return juvenile upstream migration, and human impacts, in: A. Asakura (Ed.), New Frontiers in Crustacean Biology: Proceedings of the TCS Summer Meeting, Tokyo, 20–24 September 2009. Crustaceana Monographs 15, 2011, pp. 145–156.

Bilodeau AL, Lankford WS, Kim TJ, Felder DL, Neigel JE. 1999. An ultrasensitive method for detection of single crab larvae (*Sesarma reticulatum*) by PCR amplification of a highly repetitive DNA sequence. *Mol Ecol* 8: 683.

Bucklin A, Guarnieri M, Hill RS, Bentley AM, Kaartvedt S. 1999. Taxonomic and systematic assessment of planktonic copepods using mitochondrial COI sequence variation and competitive, species-specific PCR. *Hydrobiology* 401: 239–254.

Chow S, Suzuki N, Imai H, Yoshimura T. 2006. Molecular species identification of spiny lobster phyllosoma larvae of the genus *Panilirus* from the Northern Pacific. *Mar Biotechnol* 8: 260–267.

DeLong EF, Wickman GS, Pace NR. 1989. Phylogenetic strains: ribosomal RNA-based for the identification of single cells. *Science* 243: 1360–1363.

Dixon DR, Jollivet DA, Dixon LR, Nott JA, Holland PW. 1995. The molecular identification of early life-history stages of hydrothermal vent organisms. *Geo Soc Lond Spec Pub* 87: 343–350.

Dos Santos A, González-Gordillo JI. 2004. Illustrated keys for the identification of the Pleocyemata (Crustacea: Decapoda) zoeal stages, from the coastal region of south-western Europe. *J Mar Biol Assoc UK* 84: 205–227.

Dos Santos A, Santos AM, Conway DV, Bartilotti C, Lourenço P, Queiroga H. 2008. Diel vertical migration of decapod larvae in the Portuguese coastal upwelling ecosystem: implications for offshore transport. *Mar Ecol Prog Ser* 359: 171–183.

Fell JW. 1995. rDNA targeted oligonucleotide primers for the identification of pathogenic yeasts in a polymerase chain reaction. *J Ind Microbiol* 14: 475–477.

Goffredi SK, Jones WJ, Scholin CA, Marin R, Vrijenhoek RC. 2005. Molecular detection of marine invertebrate larvae. *Mar Biotechnol* 8: 149–160.

Goodwin HL, Hanson JA. Aquaculture of the Freshwater Prawn *Macrobrachium* species. The Oceanic Institute, Waimanalo, HI, 1975.

Guerao G, Cuesta JA. 2014. Caridea. *Atlas Crust Larv* 48: 250–255.

Gurney R. Larvae of Decapod Crustacea. Ray Society, London, 1942.

Harikrishnan M. Population characteristics, fishery and post larval distribution of *Macrobrachium rosenbergii* (de Man) and *M. idella* (Hilgendorf) of Vembannad lake. PhD thesis, Cochin University of Science and Technology, 1997, p. 276.

Harikrishnan M, Kurup BM. Temporal and spatial availability of ovigerous females of *Macrobrachium rosenbergii* (de Man) in

Vembanad lake, in Proceedings of the Eighth Kerala Science Congress, 1996, pp. 441–443.
Harikrishnan M, Kurup BM. 2001. Fishery of *Macrobrachium rosenbergii* (de Man) in the Vembanad lake and confluent rivers. *Ind J Fish* 48: 189–198.
Harikrishnan M, Kurup BM. On the sustainability issues of the fishery of the giant freshwater prawn *Macrobrachium rosenbergii* (de Man) in the Vembanad lake, South India, in: B.M. Kurup, K. Ravindran (Eds.), Sustain Fish. Cochin University of Science and Technology, 2006, pp 502–510.
Harikrishnan M, Madhusoodana Kurup BM. 1997. On the Intensity of Exploitation of Berried Females of *Macrobrachium rosenbergii* (de Man) in the Vembanad Lake. *Fish Technol* 34: 13–20.
Harikrishnan M, Unnikrishnan U, Maju MS, Reena Greeshma AR, Kurup BM. 2010. Size at sexual maturity, egg number and reproductive output of the snapping shrimp *Alpheus euphrosyne euphrosyne* De Man, 1897. *Invert Reprod Dev* 54: 195–202.
Hartmann G. 1958. Apuntessobre la biología de camarón de río, *Cryphiops caementarius* (Molina) Palaemonidae, Decapoda. *Pesca y Casa* 8: 15–28.
Hebert PD, Ratnasingham S, De Waard JR. 2003. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proc R Soc Lond Ser B Biol Sci* 270: 96–99.
Ibrahim KH. 1962. Observations on the fishery and biology of the freshwater prawn *Macrobrachium malcolmsonii* Milne Edwards of River Godavari. *Ind J Fish* 9: 433–467.
Jacob B, Revichandran C, Kumar NK. 2013. Salt intrusion study in Cochin estuary-using empirical models. *Ind J Geo-Mar Sci* 42: 304–313.
Jensen PC, Purcell MK, Morado JF, Eckert GL. 2012. Development of a real-time PCR assay for detection of planktonic red king crab (*Paralithodes camtschaticus* (Tilesius 1815)) larvae. *J Shellfish Res* 31: 917–924.
Jose D, Harikrishnan M. 2016. Non-homologous COI barcode regions: a serious concern in decapod molecular taxonomy. *Mitochondrial DNA Part A* 28: 482–492.
Jose D, Harikrishnan M. 2019. Evolutionary history of genus *Macrobrachium* inferred from mitochondrial markers: a molecular clock approach. *Mitochondrial DNA Part A* 30: 92–100.
Joseph J, Kurup PG. 1989. Volume transport and estuarine features at Cochin inlet. *Mahasagar* 22: 165–172.
Kobayashi G. 2020. Small-scale population genetic structure of the sand bubbler crab Scopimera ryukyuensis in the Ryukyu Islands, Japan. *Mol Biol Rep* 47: 2619–2626.
Kurup BM, Harikrishnan M. 2000. Reviving the *Macrobrachium rosenbergii* (de Man) Fishery in Vembanad lake, India, Vol. 23. Naga, The ICLARM Quarterly, April–June, 2000, pp. 4–9.
Kurup BM, Sebastian MJ, Sankaran TM, Rabindranath P. 1992. Fishery and biology of *Macrobrachium* spp. of the Vembanad lake, in: E.G. Silas (Ed.), Freshwater prawns, Kerala Agricultural University, Kochi 1992, pp. 76–89.
Lakshmanan PT, Shynamma CS, Balchand AN, Kurup PG, Nambisan PN. 1982. Distribution and seasonal variation of temperature and salinity in Cochin backwaters. *Ind J Mar Sci* 11: 170–172.
Ling SW. 1969. The general biology and development of *Macrobrachium rosenbergii* (de Man). *FAO Fish Rep* 57: 589–606.
McManus GB, Katz LA. 2009. Molecular and morphological methods for identifying plankton: what makes a successful marriage? *J Plankton Res* 31: 1119–1129.
Medeiros-Bergen DE, Olson RR, Conroy JA, Kocher TD. 1995. Distribution of holothurian larvae determined with species-specific genetic probes. *Limnol Oceanogr* 40: 1225–1235.
Menon MK. 1938. The early larval stages of two species of Palaemon. *Proc Ind Acad Sci Sec B* 8: 288–294.
Menon NN, Balchand AN, Menon NR. 2000. Hydrobiology of Cochin backwater system-a review. *Hydrobiologia* 430: 149–183.
Morgan SG. 1995. Life and death in the plankton: larval mortality and adaptation. *Ecol Mar Invert Larvae* 1: 279–322.
Ngoc-Ho NN. 1976. The larval development of the prawns *Macrobrachium equidens* and *Macrobrachium* sp. (Decapoda: Palaemonidae), reared in the laboratory. *J Zool* 178: 15–55.
Odero J, Gomes B, Fillinger U, Weetman D. 2018. Detection and quantification of Anopheles gambiae sensu lato mosquito larvae in experimental aquatic habitats using environmental DNA (eDNA). *Wellcome Open Res*. 3: 26.
Olson RR, Runstadler JA, Kocher TD. 1991. Whose larvae? *Nature* 351: 357–358.
Palumbi SR, Benzie J. 1991. Large mitochondrial DNA differences between morphologically similar penaeid shrimp. *Mol Mar Biol Biotechnol* 1: 27–34.
Pan M, McBeath AJ, Hay SJ, Pierce GJ, Cunningham CO. 2008. Real-time PCR assay for detection and relative quantification of *Liocarcinus depurator* larvae from plankton samples. *Mar Biol* 153: 859–870.
Pechenik JA. 1999. On the advantages and disadvantages of larval stages in benthic marine invertebrate life cycles. *Mar Ecol Prog Ser* 177: 269–297.
Pillai NN. 1990. *Macrobrachium striatus*: a new species from the southwest coast of India. *J Mar Biol Assoc Ind* 32: 248–253.
Pillai NN. Studies on the life history of some carideans of the southwest coast of India. PhD thesis, Cochin University of Science and Technology, 1991, 278 p.
Pillai NN, Mohamed KH. 1973. Larval history of *Macrobrachium idella* (Hilgendorf) reared in the laboratory. *J Mar Biol Assoc Ind* 15: 359–385.
Pineda J, Hare JA, Sponaugle SU. 2007. Larval transport and dispersal in the coastal ocean and consequences for population connectivity. *Oceanography* 20: 22–39.
Qasim SZ. 2003. Indian estuaries. Allied publishers.
Queiroga H, Blanton J. 2005. Interactions between behaviour and physical forcing in the control of horizontal transport of decapod crustacean larvae. *Adv Mar Biol* 47: 107–214.
Raman K. 1964. On the location of nursery ground of the giant prawn *Macrobrachium rosenbergii* (De Man). *Curr Sci* 33: 27–28.
Raman K. 1967. Observations on the fishery of giant freshwater prawn *Macrobrachium rosenbergii* (de Man), in: Proc Symp Ser Crust Part II. *Mar Biol Assoc Ind* B2-33B: 253–279.
Rao RM. 1967. Studies on the biology of *Macrobrachium rosenbergii* (de Man) in the Hooghly estuary with notes on its fishery. *Proc Nat Inst Sci Ind* 358: 1–27.
Roy MKD, Nandi NC, Khan RA. 2009. Invertebrate diversity. Faunal Diversity of Vembanad Lake. A Ramsar site in Kerala, India. *Wetland Ecosyst Ser* 10: 69–128.
Rumrill SS. 1990. Natural mortality of marine invertebrate larvae. *Ophelia* 32: 163–198.
Schwamborn R, Ekau W, Silva AP, Silva TA, Saint-Paul U. 1999. The contribution of estuarine decapod larvae to marine zooplankton communities in North-East Brazil. *Archiv Fish Mar Res* 47: 167–182.
Sureshkumar S. Systematics, bionomics and seed production of *Macrobrachium* spp. of the Vembanad lake. PhD thesis, Cochin University of Science and Technology, 1998, 231.
Thasneem TA, Nandan BS, Geetha PN. 2018. Water quality status of Cochin estuary, India. *Ind J Geo Mar Sci* 47: 978–989.

Thomas MM. 1976. New records of four Alpheid shrimps from the Indian waters. *J Mar Biol Assoc Ind* 18: 666–669.

Uno Y, Kwon CS. 1969. The larval development of *Macrobrachium nipponense* (de Man) reared in the laboratory. *Bull Soc Franco-Japnaised Oceanograph* 7: 278–294.

Vadopalas B, Bouma JV, Jackels CR, Friedman CS. 2006. Application of real-time PCR for simultaneous identification and quantification of larval abalone. *J Exp Mar Biol Ecol* 334: 219–228.

Wang J, Zhen Y, Mi T, Yu Z, Wang G. 2015. Development of a real-time PCR assay (SYBR Green I) for rapid identification and quantification of scyphomedusae *Aurelia* sp. 1 planulae. *Chin J Oceanol Limnol* 33: 974–987.

Wight NA, Vadopalas B, Friedman CS. 2008. Identification and quantification of burrowing shrimp larvae (*Neoptrypaea californiensis* and *Upogebia puggetensis*) from plankton samples using quantitative polymerase chain reactions (QPCR). *J Shellfish Res* 27: 462.

Williamson DI. 1982. Larval morphology and diversity, in: L.G. Abele (Ed.), The Biology of Crustacea 2; Embryol Morphol Genet 2: 43–110.

Zhang T, Fang HH. 2006. Applications of real-time polymerase chain reaction for quantification of microorganisms in environmental samples. *Appl Microbiol Biotechnol* 70: 281–289.

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