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Biphasic moult cycle of the parasitic isopod *Norileca indica* (H. Milne Edwards, 1840) (Isopoda: Cymothoidea): stage-wise characterisation and haemolymph ecdysteroids titre

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ABSTRACT

Norileca indica (H. Milne Edwards, 1840) is a protandrous hermaphroditic and obligatory parasitic isopod of scombrid fishes. The posterior and anterior biphasic moult cycles in the adult phases (male, transitional, and female) of this species are characterised based on the microscopic changes reflected in the uropod exopodite and maxillule, respectively. Biphasic parturial moult as well as the moult removing the old brood pouch characteristic of ovigerous females was recorded. Assay of haemolymph ecdysteroids titre with respect to different biphasic moult cycle stages appears to be first report of its kind among aquatic and/or parasitic isopods. The ecdysteroids titre showed significant fluctuation in relation to the moult cycle stages, while the parasite was at a biphasically intermoult stage, titre was estimated to be 13.64 ± 2.09 ng ml^{-1}. By the onset of the premoult at the posterior body region, titre showed a gradual increase and reached a maximum level (328.54 ± 70.17 ng ml^{-1}; $P = 0.0060$) when the parasite was at late premoult at the posterior body part and mid premoult stage at the anterior body part. There was a subsequent sharp decline (92.8 ± 12.95 ng ml^{-1}; $P = 0.002$) soon after the ecdysis of the posterior part by the time premoult changes in the anterior part advanced as late premoult and about to moult. The level of ecdysteroids titre was 55.56 ± 14.47 ng ml^{-1} when the parasite completed anterior ecdysis and reached postmoult. Data on the monthly occurrence of biphasic moult cycles revealed that a mean average of 23.26% of the adult population undergoes premoult/ecdysis/postmoult activities throughout the year. The rate of moulting showed a decline (16.12 ± 0.60%) during the monsoon months (June-August) when compared to pre-summer/summer (February-May) (24.83 ± 2.42%) and post-monsoon/winter (September-January) (26.28 ± 1.75; $P = 0.005$).

Key words: cymothoids, exopodite, intermoult, maxillule, parasitism, postmoult, premoult, uropod ramus

INTRODUCTION

Moulting is an essential characteristic of crustaceans, affecting development, growth, and reproduction (see Hopkins, 2009; Kuballa *et al.*, 2011; Kappalli *et al.*, 2012; Nagathinkal *et al.*, 2017). Each moult cycle comprises intermoult, premoult, ecdysis, and postmoult with further subdivisions within each stage. The intermoult stage (C) is the longest period, during which normal growth takes place. The premoult stage (D) is a preparative phase for ecdysis, during which apolysis, the formation of new cuticle, takes place. In ecdysis or moulting (stage E), the individual sheds the old cuticle and absorbs water to expand newly formed flexible cuticle. The synthesis of endocuticle and the calcification of the exoskeleton take place during the postmoult (E), the recuperation period (Drach, 1939; Drach & Tchernigovtzeff,1967; Steel, 1980; Wägele, 1992; Kuballa *et al.*, 2011; Kappalli *et al.*, 2012; Mrak *et al.*, 2012; Vittori *et al.*, 2012).

Crustaceans generally follow a monophasic moult cycle by which the complete exoskeleton is removed at a time (Drach & Tchernigovtzeff,1967; George,1972). Biphasic moulting is the rule in isopods by which the old cuticle of the posterior body part exuviates prior to that of anterior part and the break between the two halves, which is an indicative sign of ecdysis, which occurs at the junction of pereonites 4 and 5 (Schöbl, 1879; Gorvett, 1947; George & Sheard, 1954; Price & Holdich, 1980; Wägele, 1992; Mrak *et al.*, 2012; Vittori *et al.*, 2012). An exception is in an Antarctic isopod, *Glyptonotus* Eights,1852, which follows the monophasic moult (George,1972). The moult-related changes in the two halves, however, are not in synchrony throughout the biphasic moult cycle (Price & Holdich, 1980). The first report of a biphasic moult among isopods was in *Porcellio scaber* Latreille, 1804 and it was subsequently confirmed in other terrestrial isopods such as *Ligia exotica* Roux, 1828, *L. oceanica* (Linnaeus, 1767), *L. italica* Fabricius, 1798, and *Oniscus asellus* Linnaeus, 1758 (Schöbl, 1879; Tait, 1917; Numanoi, 1934; Wieser, 1964; Messner, 1965; Price & Holdich, 1980; Steel, 1980, 1982; Štrus, 1990; Štrus & Compere, 1996).

Different strategies have been proposed for the characterisation of the monophasic moult cycle stages. Setagenic events in the mouthparts and pleopods have been used, for instance, for the precise characterisation of the moult stages in brachyuran crabs (Kappalli & Gopinathan, 1996; Suganthi & Gopinathan, 1999; Kappalli *et al.*, 2012; Nagathinkal *et al.*, 2017). We have used the uropod ramus to determine the monophasic moult cycle characteristics of the manca larva of the parasitic isopods *Mothocya renardi* (Bleeker, 1857) (A. Panakool and SK, unpublished data) and *Norileca indica* (H.A. Kottarathil and SK, unpublished data). The uropod ramus shows characteristic changes like the retraction of the epidermis, the appearance of the newly formed setae while the larva is under the different premoult stages. There are no reports of a stage-wise characterisation of a biphasic moult cycle based on the observation of the appendages of the respective sides of the body is concerned. During the premoult phase, terrestrial isopods reabsorb calcium from the cuticle and transport it across the anterior sternal epithelium of the first four sternites into the exuvial gap, where it remains stored until the completion of the posterior moult (Steel, 1993; Ziegler, 1996, 1997; Ziegler & Scholz, 1997; Zeigler *et al.*, 2005, 2007). Steel (1982), Štrus (1990) and Zidar *et al.* (1998) described fat bodies on the first four sternites of *O. asellus* and *L. italica* as the first sign of early premoult. The male phase of the parasitic isopods *Mothocya epimerica* Costa, 1851 and *M. renardi* undergo sequential moults until it is transformed as an ovigerous female (Trilles, 1968, 1969, 1994; Sindermann, 1990; Grabda, 1991; Leonardos & Trilles, 2003; A. Panakkool and SK, unpublished data).

The ecdysteroids, primarily regarded as the growth hormone, promote premoulting and ecdysis in crustaceans. A vast body of literature pertaining to the ecdysteroids titre in relation to the crustacean monophasic moult cycle is available (Chan *et al.*, 1988; Snyder & Chang 1991; Lee *et al.*,1998; Suganthi & Gopinathan, 1999; Hopkins 2009; Kappalli *et al.*, 2012; Shyamal *et al.*, 2014; Subramoniam, 2016; Nagathinkal *et al.*, 2017). Except the data in a free-living terrestrial isopod *Armadillidium vulgare* (Latreille, 1804) (Suzuki *et al.*, 1996), no information on the ecdysteroids titer is available in any other free living or parasitic isopods, even though this group follows the characteristic biphasic moult cycle. A comprehensive study on moult cycle stages of parasitic isopods parallel with ecdysteroids titre in a life-cycle-stage-dependent manner is highly warranted as these protandrous hermaphrodites massively infect and severely damage commercially important fishes worldwide.

We present the stage-wise characterisation of the biphasic moult cycle of a parasitic isopod, *N. indica*, during the adult stages. This cymothoid isopod profusely infects the gills and branchial chambers of the scombrid fish *Rastrelliger kanagurta* (Cuvier, 1816), distributed along the southwestern coast of India (Kottarathil & Kappalli, 2019). Previous attempts helped us choose the maxillule as a possible appendage to characterise the anterior biphasic moult stages in *M. renardi* and *N. indica* (A. Panakkool and SK, unpublished data; H.A. Kottarathil and SK, unpublished data). The posterior and anterior biphasic moult cycle stages in *N. indica* were characterised based on morphological as well as microscopic level changes well reflected in their uropod exopodite and maxillule respectively. The level of ecdysteroids titre parallel with biphasic moult cycle stages was also quantitatively determined. The assessment on the monthly incidence of biphasic moult cycles helped us reveal the seasonality in its occurrence in the adult population of *N. indica*.

MATERIALS AND METHODS

Collection of N. indica

Live specimens of *N. indica* were collected from the branchial cavity of fresh or live host fish, *R. kanagurta* obtained from the Ayyikkara (11°51′N, 75°22′E) and Madakkara (12°21′N, Long.75°12′E) fish landing centers, North Malabar Coast, India. In order to assess the moult cycle frequency at population level, the collection of the parasite was made three to four times a week during July 2017 to June 2019. Different adult life cycle phases (male, transitional, and females) of *N. indica* were identified from the collected samples based on morphological characters (Bruce, 1986; Kottarathil, 2016). The samples were maintained live without the host for 8–10 hr irrespective of stage.

Identification of the biphasic moult stages

Identification of the biphasic moult cycle stages in *N. indica* was performed by microscopic observation of the characteristic moult-related sequential changes in the appendages such as the exopodite (uropod ramus) and maxillule (Kappalli *et al.*, 2012; Kottarathil, 2016; Nagathinkal *et al.*, 2017). The uropod ramus was cut off from the parasite using a sharp blade and the exopodite separated under a dissection microscope (MS 24; Magnus, New Delhi, India). The microscopically visible maxillule was also obtained using fine forceps (Kottarathil, 2016) from the same individual. Both appendages were mounted in filtered seawater on a clean glass slide and immediately observed under a microscope (Axiolab A1; Zeiss, Oberkochen, Germany). The posterior moult cycle stages (intermoult, premoult (early, mid- and late), ecdysis, and postmoult) were identified by the observation of the changes, if any, in the epidermis of the exopodite and differences in the ecdysial gap visible under the microscope. The appearance of the newly formed maxillule within the existing maxillule and difference in the ecdysial gap were used to identify the moult cycle stages in the anterior body part of parasite. Both appendages showing different moult stages were photographed (Axiocam 305; Zeiss) and processed with the ZEN software (Zeiss). The ventral side of the transitional as well as the ovigerous females under the biphasic (posterior and anterior) premoult stages was observed with help of a Leica stereozoom microscope (S8APO; Leica, Wetzlar, Germany) and documented with an attached camera (MC170hd; Leica).

Ecdysteroids titre assay

Haemolymph from the adult (male/transitional and female) stages of *N. indica*, in relation to the biphasic moult cycle stages, was collected using the Hamilton TN 250 µl syringe (Hamilton, Reno, NV, USA) and subjected to ecdysteroids titre assay using 20 hydroxy ecdysone EIA kit (A05120; Cayman Chemicals, Ann Arbor, MI, USA). The methodology used followed the manufacturer's instructions.

Incidence of biphasic moulting in infective stages

All the adult phased individual parasites recovered during the study period (July 2017-June 2019) were subjected to the identification of the biphasic moult stages (intermoult/premoult/ecdysis/postmoult) and the monthly data on the incidence of moulting activities were prepared. The monthly data were grouped as pre-summer/summer (February-March/April-May), monsoon (June-August), post-monsoon/winter (September-October/November-January) to assess seasonality, if any, in the moulting events. The moulting frequency of the individuals in the population was calculated from samples obtained in the same month of different years (July 2017-June 2018 and July 2018-June 2019).

Statistical analysis

We used one-way ANOVA and paired t-test using the software Graphpad prism 8 (version 8.1.0; GraphPad Software, San Diego, CA, USA) to assess the level of significance of the generated data from the ecdysteroid-titre assay in relation to the biphasic moult cycle stages as well as the monthly moulting. The values were presented as mean ± SE. Tukey test was done for the P values corrections for the number of comparisons using the software SPSS (Version 24.0; IBM, Armonk, NY, USA).

RESULTS

Biphasic moult cycles in adult stages

A biphasic moult cycle was frequently observed in the adult phases. The onset of ecdysis was obvious with the appearance of a fissure between pereonites 4 and 5. The pre-ecdysial events, in the posterior half of the body were evidenced from the microscopic changes in the exopodite of the uropod ramus (Fig. 1). While the posterior half exuviated, the anterior half was under the late-premoult stage as reflected in the maxillule (Fig. 2). There was a marked difference in body texture and colour between the posterior and the anterior halves undergoing respectively postmoult and premoult stages; the soft, translucent and pale-coloured postmoult posterior half also appeared relatively wide compared to the premoult-stage anterior half (Fig. 3). At the premoult stage of the anterior half, the eye turned white in contrast to the black eye during the postmoult/intermoult stages. Ecdysis of the anterior half ensued while the posterior half attained intermoult stage.

Changes in the exopodite of the uropod ramus during the moult cycle of the posterior body part

The changes in the onset of the biphasic moult cycle in the posterior half of the body was well reflected in the exopodite of the uropod ramus. The cuticle and the epidermis were closely apexed at the intermoult stage (Fig. 1A) and the epithelial layer of the hypodermis appeared as a thin lining (Fig. 1A). The early premoult changes were indicated with the retraction of epidermis from the old cuticle along with the visibility of the epithelial layer (Fig. 1B). The mid-premoult stage the ecdysial gap between the old cuticle and epidermis was clearly visible under the microscope (Fig. 1C), and in the late premoult stages, the width of the ecdysial gap (between the old cuticle and the retracted epidermis) was increased. The newly formed cuticle layer was also visible as the premoult stage advanced towards its late stage (Fig. 1D). The matrix of the exopodite appeared translucent in the immediately moulted posterior half. The prominent visibility of the hypodermal epithelial lining was also observed in the exopodite of the uropod ramus throughout the premoult and the postmoult periods (Figs. 1D, E).

Changes in the maxillule during the moult cycle of the anterior body part

At intermoult, the maxillule matrix was opaque (Fig. 2A). By the onset of premoult, the retraction of hypodermis from the old cuticle was visible at the apical region of the maxillule, the same phenomenon was also observed at the apical recurved spines and the matrix become translucent. At early premoult stage, separation of the epidermis from the old cuticle was completed and four newly formed apical recurved spines were visible (Fig. 2B) and at the mid-premoult stage a narrow ecdysial gap between the tip of the new apical spine and the base of the old apical spine also appeared (Fig. 2C). The ecdysial gap between the tip of the new apical spine and the base of old apical spine widened at the late premoult stage (Fig. 2D). By this time, the posterior half of the body completed ecdysis and attained the intermoult stage. Moulting the anterior half (cephalon and first four pereonites) along with their respective appendages (cephalic appendages and pereopods) ensued apparently within 48 hr after the posterior half ecdysis. After ecdysis, the matrix of maxillule was more or less translucent compared to that at intermoult/premoult stage (Fig. 2E).

Biphasic parturial moult cycle in the breeding female

The first biphasic parturial moult started precisely right from the transitional phase by which the female phase with complete brood pouch (with four pair of oostegites) was formed (Fig. 4B, 4C). The female began its breeding cycle with the oviposition of the eggs into the brood pouch in which eggs were maintained until the larval development and their subsequent release (Fig. 4D). Subsequently, the brood pouch was partially (fourth pair of old oostegites) removed with the moulting of the posterior body part. The complete removal of the brood pouch (first three pairs of the old oostegites) was accomplished through the moulting of the anterior body part (Fig. 4E, 4F). This biphasic moulting event was then followed by the biphasic parturial moult by which the brood pouch was reformed to receive the new clutch of eggs. The ovigerous females follows this pattern of the biphasic moult cycle apparently multiple times to renew their brood pouch throughout their breeding life.

Ecdysteroids titre in relation to the biphasic moult cycle stages

The estimated level of haemolymph ecdysteroids titre was 13.64 ± 2.09 ng ml^{-1} when both the anterior and posterior body parts were at the intermoult stage. The level registered a marked increase (45.31 ± 4.66 ng ml^{-1}) as the posterior half of the parasite entered premoult. The ecdysteroids titre (51.43 ± 2.14 ng ml^{-1}) was found to be comparable ($P = 0.2$) with the previous value when the parasite showed the sign of premoult initiation at its anterior body. There was nevertheless a drastic increase in the level of ecdysteroids titre (328.54 ± 70.17ng ml^{-1}; $P = 0.006$) as the anterior and posterior body parts attained mid-premoult and late-premoult stages, respectively. This was followed by an abrupt decline in the level (92.89 ± 12.95 ng ml^{-1}) when the posterior body completed ecdysis and attained postmoult, with the titre difference (328.54 ± 70.17 and 92.89 ± 12.95) was statistically significant ($P = 0.0025$). The titers measured when the anterior and posterior body parts at the postmoult and intermoult stages was 55.56 ± 14.47ng ml^{-1}, respectively (Fig. 5). Overall statistical analysis also supports the moult-stage wise difference in the haemolymph ecdysteroids titre (ANOVA $F = 14.52$, $P < 0.0001$). The level of significance shown by the Tukey test is indicated in Figure 5.

Monthly incidence of biphasic moult cycles in the adult stage

A total of 1,998 adult individuals of *N. indica* including 74 males, 18 transitional, and 1,906 females collected from July 2017 to June 2019 were observed to assess moulting activities. A large proportion (77%) of the population of *N. indica* remained at intermoult stage throughout the study period. The incidence of premoulting/ecdysis/postmoulting was also noticed throughout the year,

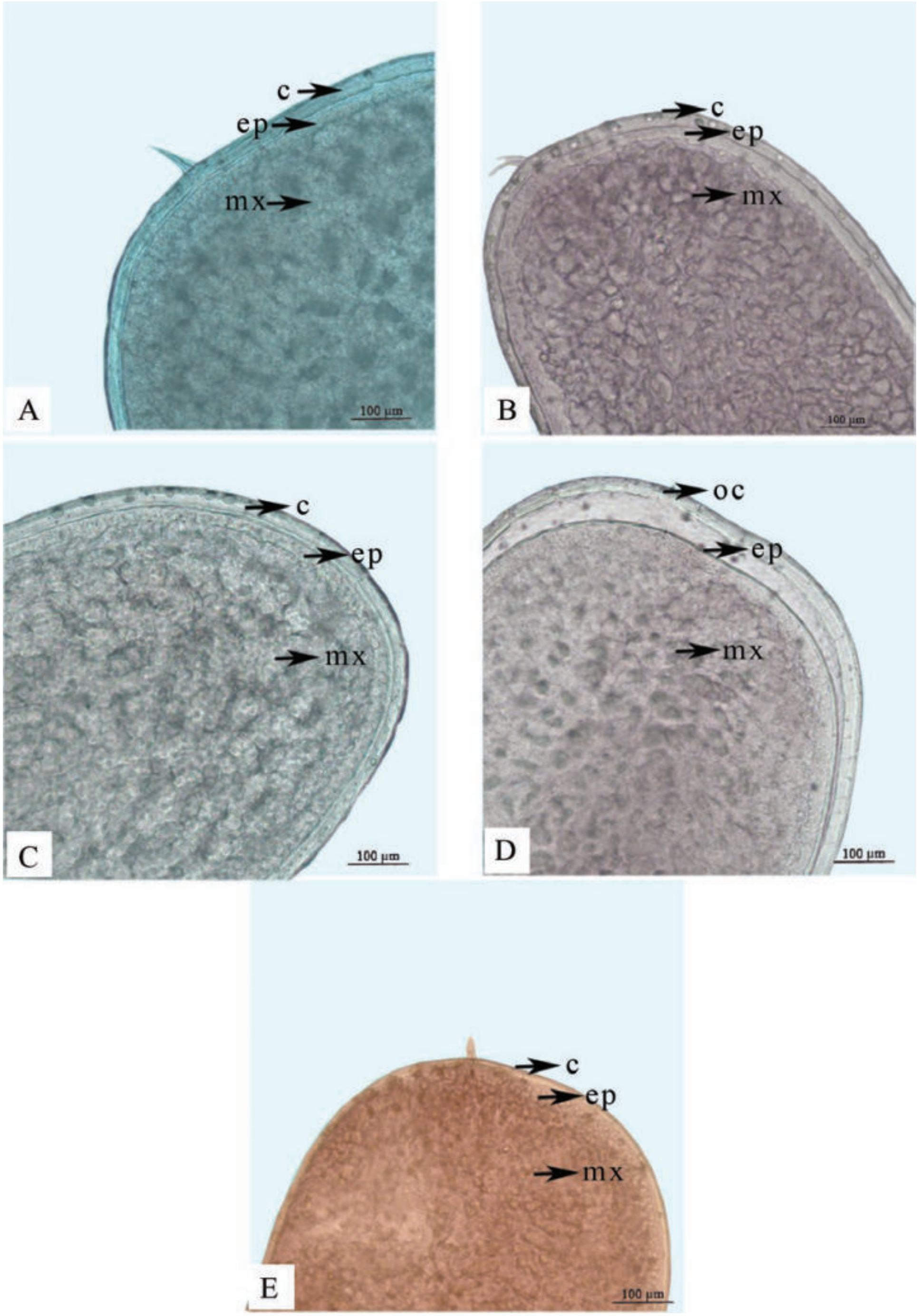


Figure 1. Exopodite of the uropod ramus from the infective stages of *Norileca indica* showing moult-related changes (200×): intermoult (**A**), early premoult (**B**), mid-premoult (**C**). late premoult (**D**), postmoult (**E**). c, cuticle; ep, epidermis; mx, matrix; nc, new cuticle; oc, old cuticle. Scale bars = 100µm

with a range of 16–31% (Fig. 6); maximum and minimum rate of incidence was noticed in the months of May (31%) and June (16%), respectively. There was a significant difference ($F = 6.98$, $P = 0.014$) in the moulting rate when we grouped the months according to season (pre-summer/summer, monsoon, post-monsoon/winter), (Fig. 7); during the monsoon months (June-August), the rate showed a decline (16.12 ± 0.60%) compared to that of pre-summer/summer (February-May) (24.83 ± 2.42%) and post-monsoon/winter (September-January) (26.28 ± 1.75; $P = 0.005$). The significance shown by the Tukey test is shown in Figure 7.

DISCUSSION

The present study demonstrates that the adult phase of *N. indica* adopts a biphasic moult cycle, which so far appears to be the rule among isopods. Unlike adults, however, the marsupial staged larva of this species follows a monophasic moult (Kottarathil, 2016), which is characteristic of other malacostracan crustaceans (Drach & Tchernigovtzeff, 1967) (with the exception of *Glyptonotus*) by which the entire exuvium sheds at the same time. In the morphological sign of a biphasic ecdysis in *N. indica* appears with a break between the base of the pereonite 4 and the apex of pereonite 5, with the posterior half preceding the anterior half in moulting.

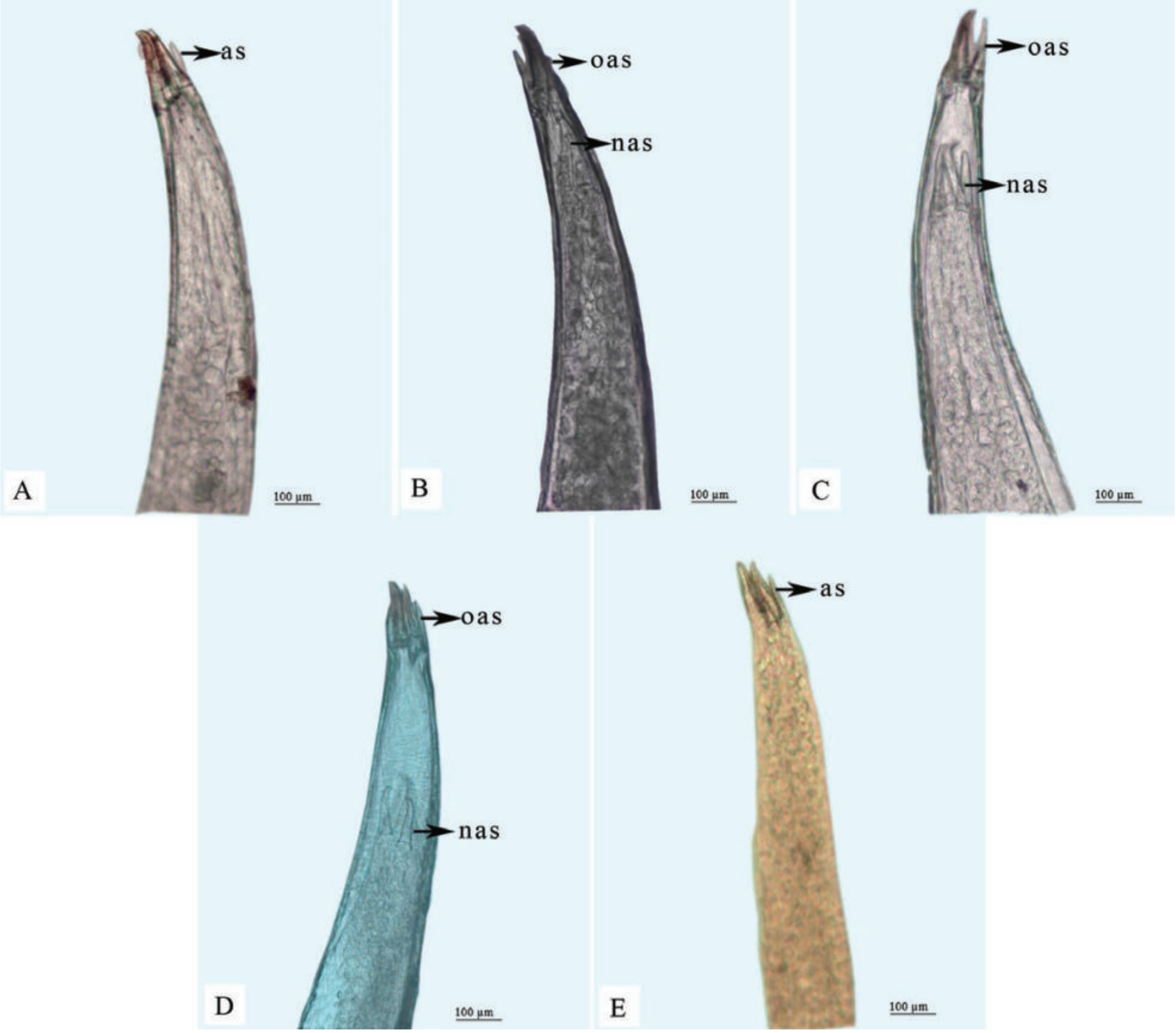


Figure 2. Maxillule from the infective stages of *Norileca indica* showing moult-related changes (200×): intermoult (**A**); early, mid- and late premoult stages (**B–D**), postmoult (**E**). as, apical spine; ep, epidermis; nas, new apical spine; nc, new cuticle; oas, old apical spine; oc, old cuticle. Scale bars: = 100µm

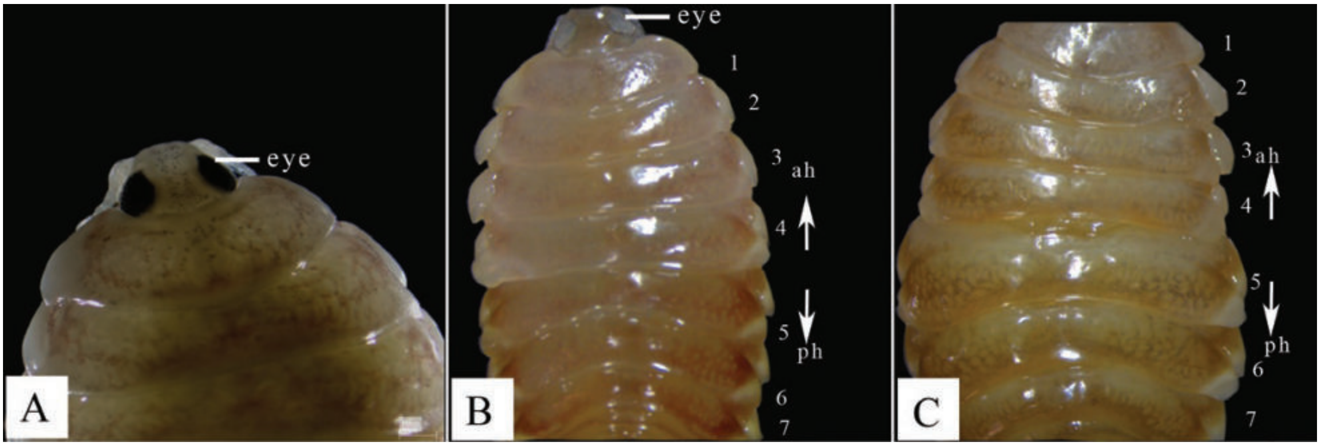


Figure 3. Morphological changes related to biphasic moult in *Norileca indica*. Black eye indicates the intermoult stage (**A**), white eye indicates the mid-premoult stage at the anterior body half (**B**). Size difference between the two body halves indicates the late-premoult stage at the anterior body half (ah) and postmoult stage at the posterior body half (ph) (**C**).

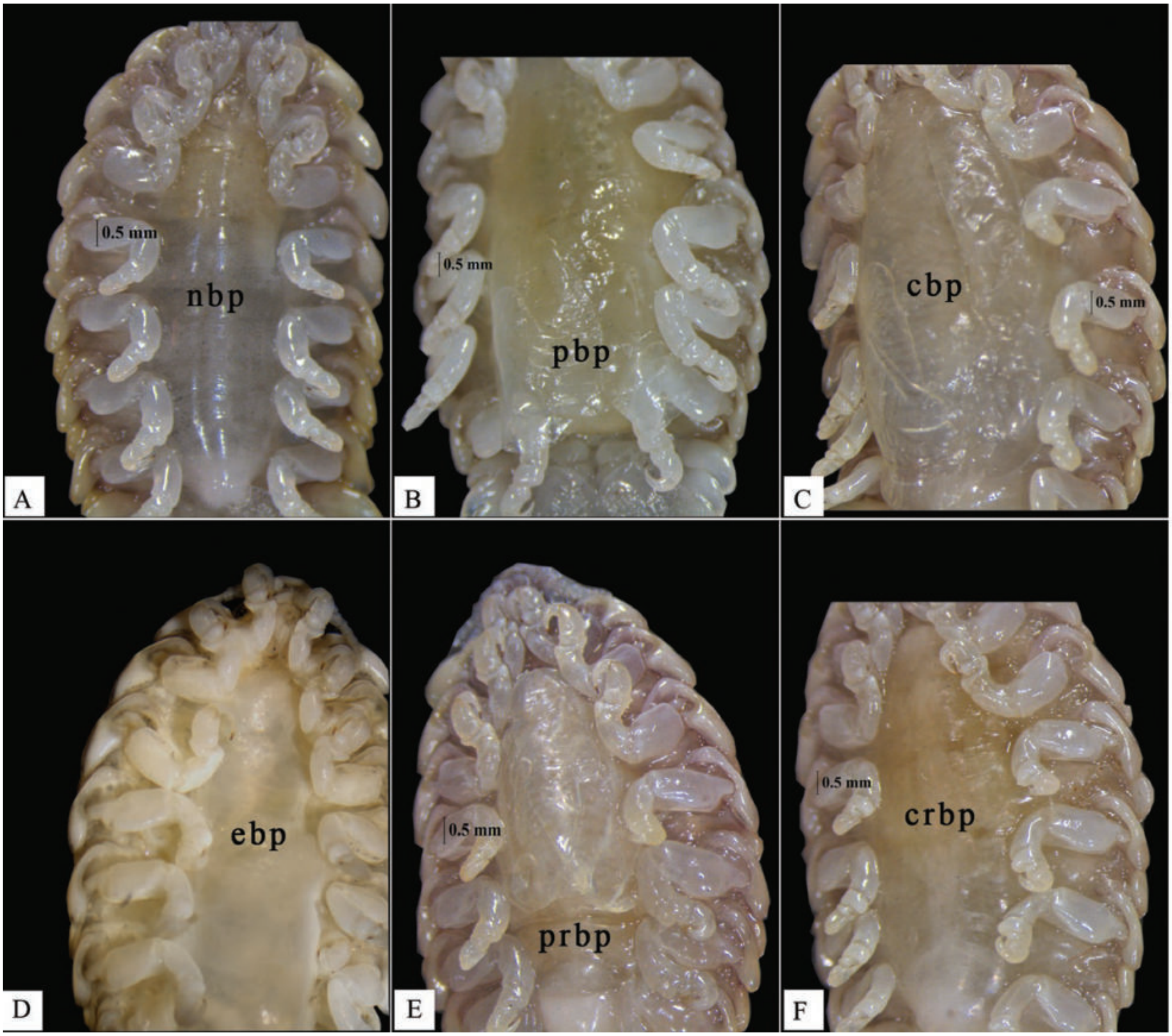
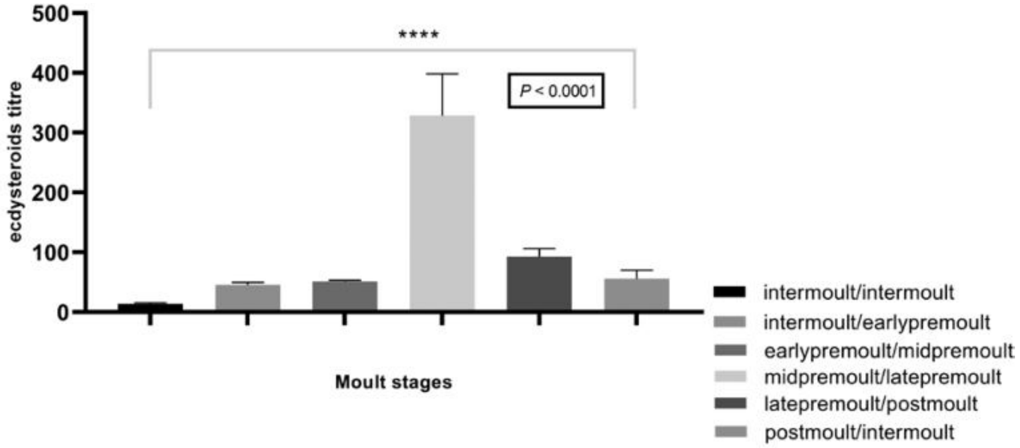


Figure 4. Ventral side of the transitional and female phases of *Norileca indica* showing the changes in the brood pouch in connection with the biphasic moulting: transitional stage without brood pouch and about to undergo biphasic parturial moult (**A**); female with partially formed brood pouch (after posterior parturial moult) (**B**); female with fully formed brood pouch (after anterior parturial moult) (**C**); female with empty brood pouch and about to undergo biphasic moult (**D**); female with partially removed brood pouch (after posterior moult) (**E**); female without brood pouch (after anterior moult (**F**). cbp, complete brood pouch; crbp, completely removed brood pouch; ebp, empty brood pouch; nbp, no brood pouch; pbp, partially formed brood pouch; prbp, partially removed brood pouch.

Such pattern appears to be comparable to that reported in terrestrial isopods such as *L. exotica*, *L. oceanica*, *L. italica*, *P. scaber*, and *O. asellus* (Tait, 1917; Numanoi, 1934; Wieser, 1964; Messner,1965; Price & Holdich, 1980; Steel, 1980, 1982; Štrus, 1990; Štrus & Compere, 1996) and in a cymothoid, *M. renardi* (Panakkool & Kappalli, unpublished data) *Sphaeroma walkeri* (Stebbing, 1905), deviating from this typical pattern, moults the anterior half prior to the posterior half (Vallabhan, 1979).

Except the report on *M. renardi* (Kottarathil, 2016; A Panakkool and SK, unpublished data), the biphasic moult in parasitic isopods has only been mentioned without any descriptions (Trilles, 1969, 1994; Grabda, 1991; Leonardos & Trilles, 2003, 2004). Despite moult-related microscopic changes in all the appendages in the male, transitional, and female of *N. indica*, the maxillule and the exopodite of the uropod ramus are the most suitable appendages reflecting all characteristic changes related to the biphasic moult stages. In adult brachyuran crabs and in the larva of parasitic isopods, the endopodite of the maxilliped 3 and the exopodite of the uropod ramus, respectively, have been found as the most suitable appendages for categorising the monophasic moult stages (Kappalli *et al.*, 2012; Kottarathil, 2016; Nagathinkal *et al.*, 2017). The subepithelial accumulation of fat has been described for moult staging in *L. italica* by Štrus (1990. The sternal fat bodies as well as calcium deposits have been reported as the indicator of premoult changes in *O. asellus*, *P. scaber*, and *Armadillo officinalis* Duméril, 1816 (Steel, 1982; Ziegler, 1996, 1997; Ziegler & Scholz, 1997; Zidar *et al.*, 1998; Montesanto & Cividini, 2018). According to Price & Holdich (1980), *O. asellus* ingests the exuviae after each moult to use the calcium to mineralise the new cuticle. This behaviour was not found in *N. indica*, apparently because following each ecdysis, the parasite could derive the calcium directly from the seawater.



Tukey analysis

Parameter	Moult stages					
	Im/Im	Im/ePm	ePm/mPm	mPm/lPm	lPm/Pom	Pom/Im
Ecdysteroids level	^{a}13.64 ± 2.09	^{a}45.31 ± 4.66	^{a}50.92 ± 2.14	^{b}328.54 ± 70.17	^{a}92.89 ± 12.95	^{a}55.56 ± 14.47

$^{a\text{-}f}$ Mean ± S.E with different superscript letters within the raw differ significantly ($P < 0.05$)

Figure 5. Ecdysteroids level (ng ml^{-1}) in relation to moult cycle (ANOVA test among the moult stages): intermoult/intermoult ($N = 5$); intermoult/early premoult ($N = 5$); early-premoult/mid- premoult ($N = 5$); mid-premoult/late-premoult ($N = 6$); late premoult/postmoult ($N = 8$); postmoult/intermoult ($N = 6$); *, level of significance ($P < 0.05$). Im, intermoult; ePm, early premoult; mPm, mid premoult; lPm, late premoult; Pom, postmoult.

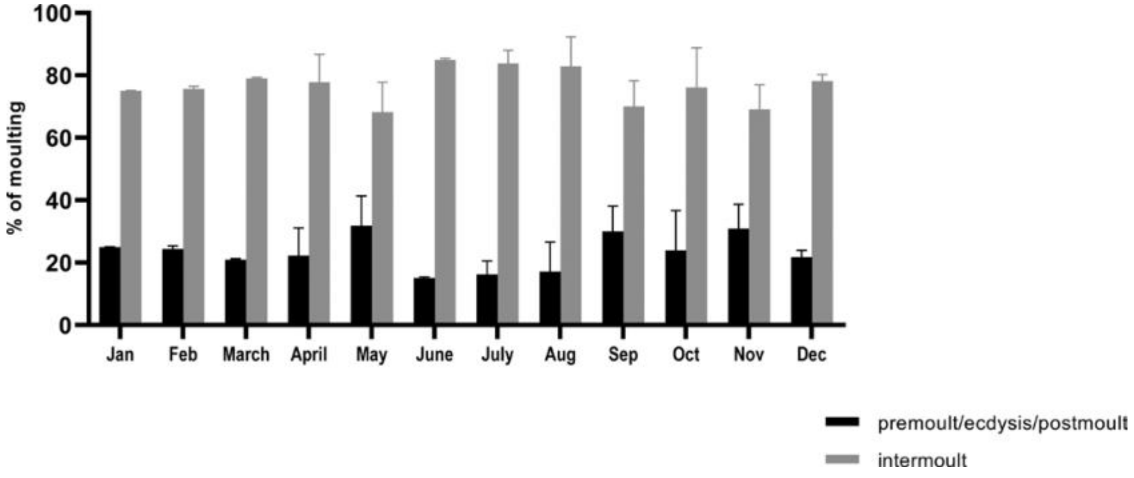
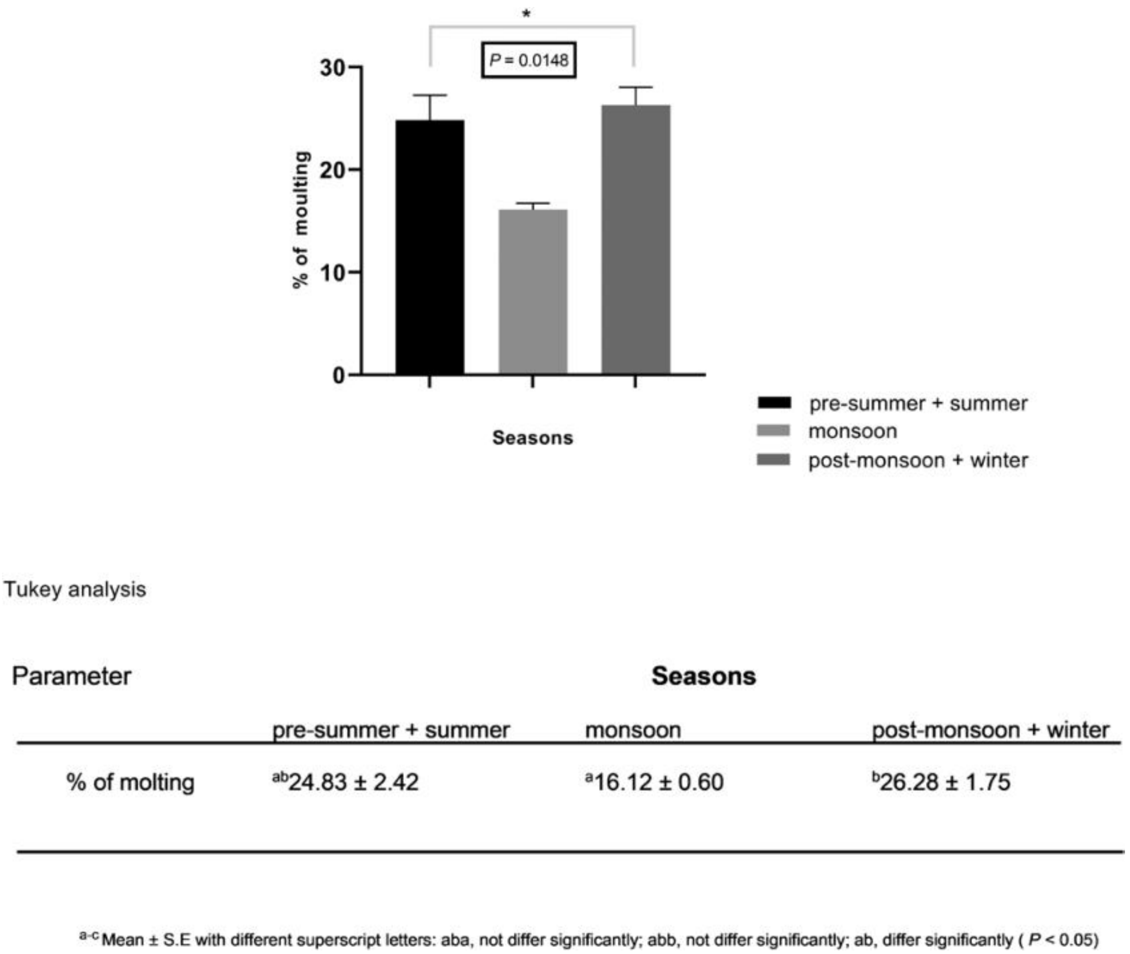


Figure 6. Moulting (per-cent month^{-1}) in *Norileca indica*

The biphasic premoult events reflected in the maxillule of the male, transitional, and female phases of *N. indica* are quite comparable. The premoult starts with the detachment of the old cuticle followed by the appearance of a new apical spine and the widening of the ecdysial gap between the old and new cuticle. All of events culminate in exuviation. According to Mrak *et al.* (2012), moulting in *P. scaber* involves apolysis of the old cuticle from the underlying epithelium, which secretes a new cuticle starting with the epicuticle followed by an extracellular compartment (ecdysial space) containing different materials. Based on the appearance and shape of calcium deposits, six to nine premoult substages have been reported in terrestrial isopods (*P. scaber* and *O. asellus*) (Steel, 1982; Wieser, 1964; Messner, 1965; Zidar *et al.*, 1998). In *P. scaber*, the premoult duration in the male is 5.7 ± 0.9 d and 5.5 ± 1.4 d in females (Zidar *et al.*, 1998), whereas in *Titanethes albus* (C. Koch, 1841), a trichoniscid isopod, it is reported to be 7 weeks (Vittori *et al.*, 2012). Additional observations are warranted to determine the duration of the moult cycle in *N. indica*.

The duration between the anterior and posterior moults is apparently 24–48 h in *N. indica*. In *Sphaeroma quadridentatum* (Say, 1818), the time period between the moulting of the two halves is approximately 13 h (Borowsky, 1996). The duration in *L. oceanica* is relatively long, as it takes an average of 3.5 d and shedding of the exoskeleton of the posterior half needs 10–12 min (Tait, 1917; Carlisle, 1956; George, 1972). The interval of the posterior and anterior moults in *O. asellus* and *L. exotica* is approximately 1.8 d and 24 h, respectively (Price & Holdich, 1980). Alhough their exoreceptors and defensive tegumental glands are functional, the



Tukey analysis

Parameter	Seasons		
	pre-summer + summer	monsoon	post-monsoon + winter
% of molting	^{ab}24.83 ± 2.42	^{a}16.12 ± 0.60	^{b}26.28 ± 1.75

$^{a\text{-}c}$Mean ± S.E with different superscript letters: aba, not differ significantly; abb, not differ significantly; ab, differ significantly ($P < 0.05$)

Figure 7. Seasonal moulting activities in *Norileca indica*. ANOVA test among the seasons (pre- summer/summer ($N = 4$); monsoon ($N = 3$); post monsoon/winter ($N = 5$); *, level of significance ($P < 0.05$).

biphasic moult limits the movement of isopods, a defence from predators (Price & Holdich, 1980). Biphasic moulting seems to be highly adaptive in parasitic isopods. The appendages used to attach to the host immediately after the postmoult are soft and pliable, but the biphasic moult ensures clinging to the host by the clawed appendages that are functional in one half of the body. The mouthparts and pereopods of first four pereonites of the anterior half are apparently used to firmly attach the host during the moulting of the posterior part. When the posterior part moults, followed by recuperation, the function of pereopods 5 to 7 is restored, enabling the parasite to cling the host by the time the anterior half undergoes moult (Kottarathil, 2016).

The question as to why isopods follows biphasic moulting is yet to be adequately addressed. According to George (1972), biphasic moulting is related to the lack of a carapace in isopods. Copulation takes place during the female's biphasic moult to the brooding stage moult and thereby facilitates breeding by rejuvenating the brood pouch (Steel, 1980; Wilson, 1991). While the females with fully developed oocytes in ovaries moults the posterior half of the body and the exoskeleton becomes soft, the male mounts and inseminates the female. After the insemination, the female then moults the anterior half of the body and deploy the oostegites that form the brood pouch (Wilson, 1991).

Our assay on ecdysteroids titre in haemolymph of *N. indica* appears to be first report of its kind among parasitic and marine isopods. The measured level of ecdysteroids at the intermoult stage is 13.64 ± 2.09 ng ml^{-1},which is comparable to that (10–20 ng ml^{-1}) reported in the terrestrial *A. vulgare*. The level showed a significant increase by the onset of premoult (45.31 ± 4.66 ng ml^{-1}) and attained its maximum level (328.54 ± 70.17 ng ml^{-1}) at late premoult before decreasing in postmoult (92.89 ± 12.95 ng ml^{-1} after posterior-part ecdysis and 55.56 ± 14.47 ng ml^{-1} after anterior-part ecdysis) similar to reports in *A. vulgare* (Suzuki *et al.*, 1996) as well as in other crustacean groups (Chan *et al.*, 1988, Snyder & Chang 1991, Lee *et al.*, 1998). The maximum reported titre value in *A. vulgare* is 150–175 ng ml^{-1} at midpremoult, with the level decreasing (20–30 ng ml^{-1}) at the end of the premoult stage (Suzuki *et al.*, 1996). Although the general rule of ecdysteroids titre is that ecdysteroids increases in premoult and decreases in intermoult and postmoult, there is a significant difference in the level of ecdysteroids titre even among the same crustacean group. The titre is relatively low even in the premoult stage (12–34 ng ml^{-1}) of the brachyuran crab *Uca triangularis* (A. Milne-Edwards, 1873) (Kappalli *et al.*, 2012; Nagathinkal *et al.*, 2017). During the premoult stage, however, the titre value is as high as 1,886.5 ± 186.2 ng ml^{-1} and 233.89 ± 14.38 ng ml^{-1}, respectively, in the crabs *Cancer magister* Dana, 1852 and *Metopograspus messor* (Forskål, 1775) (Thomton *et al.*, 2006; Kappalli & Gopinathan, 2007).

Frequent moulting has been reported in terrestrial isopods (Price & Holdich, 1980). Under laboratory conditions, males and females of *P. scaber* moult every 33 ± 3.6 and 33 ± 7.5 d, respectively (Zidar *et al.*, 1998). The breeding females of *P. siculoccidentalis* undergo one to four successive parturial moults and the time interval between successive moults ranges from 20 to 40 d (Montesanto *et al.*, 2012). Premoulting is highly pronounced during the warmer days (Steel, 1980) in *O. asellus* and *A. vulgare*

(Latreille 1804), and the average premoult period reported at 15.7 ºC–17.4 ºC is 47 d (Carlisle, 1956) in *L. oceanica*. Our study reveals that in *N. indica* approximately 23% of the population engages in moulting activities throughout the year. No statistically significant difference was noticed as far as the overall months are concerned. Moulting, however, has discrete seasonality The rate of moulting high during pre-summer/summer months (February-May) and postmonsoon/winter months (September-January), but there was a drastic and significant decline ($P = 0.0307$) during the monsoon months (June-August), indicating that moulting is apparently temperature- and salinity-dependent.

Biphasic moult is therefore the rule in *N. indica* during its obligatory parasitic life on a host fish. Breeding females furthermore engage in a parturial moult for the formation/reformation of the brood pouch followed by the another moult to remove the old one apparently throughout their reproductive life. The results of the present study indicate the necessity of further and adequate attention from an interdisciplinary perspective, using physiological, biochemical, endocrinological, and molecular tools to unravel the intricacies of the unique mechanism of biphasic moult in cymothoids. Despite extensive research investigating the physiological process underlying crustacean moulting, there is no clear understanding of the cascade of events that regulate this event. Classical molecular approaches have focused on genes specifically related to moulting but have failed to comprehensively cover this complex process. New and powerful technologies like next generation sequencing (NGS) offer a holistic approach to derive the information on the regulation of biphasic moult cycle. *Norileca indica* is as an ideal model for such studies, which would open a rich area for future research.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Crustacean Biology* online.

S1 Table. Monthly moulting activity in the adult phases of *Norileca indica.*

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