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Research Article

Prevalence, site of infection, and differentiating oocytes of the cymothoid isopod, *Norileca indica* (Milne Edwards, 1840) from its infected short mackerel, *Rastrelliger brachysoma*, in the Upper Gulf of Thailand

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Abstract

Norileca indica, a cymothoid isopod crustacean, is an obligate parasite infecting marine fishes in tropical areas. This study attempted to investigate the prevalence, site of infection and distinct stages of oogenesis of this parasitic isopod obtained from short mackerel (*Rastrelliger bachysoma*). All *N. indica* samples were collected and processed through histological techniques. *N. indica* showed higher prevalence during the dry season than the rainy season and its parasitic infections were more prevalent in the female *R. brachysoma* (60% prevalence) compared to males. Two distinct sites of *N. indica* attachment on the host (infection sites) including gill and integument were clearly observed. Interestingly, this isopod preferred to attach more on the integument surface than gill, especially in the female *R. brachysoma* (55% prevalence). Using histological observation, *N. indica* exhibited asynchronous development of oocyte in the ovary. The oocytes were enclosed by a thin layer of connective and muscular tissues. Three stages of oogenesis can be identified in the ovary: oogonium, primary oocyte, and secondary oocyte. Oogonium located inside the cyst near the germinal epithelium. A large nucleus of oogonium was surrounded by a weak basophilic cytoplasm. Unlike oogonium, multiple nuclei (2 - 3 nuclei) surrounded by basophilic cytoplasm was a key characteristic of the primary oocyte. The secondary oocyte contained spherical yolk granules with multiple layers of follicular cells, which might function to support yolk uptake into the ooplasm. In addition, early embryonic development of *N. indica* was also observed in this study. Taken together, this finding highlights parasitic prevalence and infection sites of *N. indica* in short mackerel, as well as a better resolution of its oogenesis, early development and reproductive cycle.

Keywords: microanatomy, cymothoid crustacean, short mackerel, parasitic isopod

Introduction

Rastrelliger brachysoma, a short mackerel, is a fish with high economic value in Thailand. Senarat et al. (2015) reported that *R. brachysoma* collecting from the Upper Gulf of Thailand exhibited deteriorated health with the occurrence of the hepatic histopathology. This severe health problem of short mackerel is possibly due to contamination of pollutants from untreated water released from communities, aquaculture and industries around Chaopaya, Tha-Chin, Bangprakong and Maeklong Rivers, and it has been clearly demonstrated that contamination in the marine environment has a negative impact on marine organisms and ecosystem status (Hungspreugs & Yuangthong, 1983; Cheevaporn & Menasveta, 2003; Wattayakorn, 2012). In addition, the deterioration of the mackerel health is likely related to the presence of a common parasite infecting *R. brachysoma*, which was identified to be *Norileca indica* (Milne Edwards, 1840). *N. indica* is a cymothoid isopod crustacean (Isopoda, Cymothoidae), mostly infecting fishes in tropical and temperate regions of Indo-Pacific Ocean (Rameshkumar et al., 2015). It has been shown that *N. indica* specifically infected the gill structure in bigeye scad (*Selar crumenophthalmus*) collecting from Thailand (Nagasawa & Petchsupa, 2009). Although taxonomy, molecular biology and ecology of the parasitic *N. indica* has been clearly defined (Cruz-Lacierda & Nagasawa, 2017; van der Wal et al., 2017), its reproductive cycle in tropical fishes visualized by histological techniques is still unknown.

In this study, our attempt was to record the site of infection and prevalence of *N. indica* in the *R. brachysoma*. In addition, the description of differentiating oocytes found in the parasitic isopod *N. indica* ovary was conducted using an accurate histological method. This study gives a better picture of how *N. indica* oocytes developed as well as structure of the isopod embryos, potentially increasing our understanding on infection mechanism and the reproductive biology of *N. indica*. It could also provide an insight on how to solve health problem from the isopod infection of *R. brachysoma* as well as the parasite–fish health relationships in this ecosystem.

Materials and methods

Animal collection and sampling site

The sexually maturated, *Rastrelliger brachysoma* (total length: male, 16.50 ± 0.98 cm; female, 16.70 ± 1.12 cm), as a fish host of *Norileca indica*, was obtained (40 samples/sexes/season) using bamboo strake trap. Fish collection was done during rainy season (October to November 2013) and dry season (December 2013 to February 2014) at a station (13°16'18.4" N, 100°02'13.4" E) locating in the Upper Gulf of Thailand, Samut Songkhram province, Thailand.

Assessment of infection sites and parasitic prevalence

All fish were euthanized by rapid cooling shock (Wilson et al., 2009). The external and internal anatomies were assessed and inspected via stereomicroscopy and visual observation to identify the existence of the parasitic isopods. If parasitic isopod *Norileca indica* is present, total length and prevalence percentage throughout infection sites were measured.

Histological observation

Norileca indica samples were fixed in 4% paraformaldehyde at 4°C overnight and were processed according to histological techniques (Presnell & Schreibman, 1997; Suvarna et al.,

2013). Paraffin sections were cut at 4 µm thickness and stained with haematoxylin–eosin (H&E) (Presnell & Schreibman, 1997; Suvarna et al., 2013). The ovarian structure and oogenesis of *N. indica* were examined under the light microscopy and photographed with a Leica DM750 light microscope (Boston Industries, Inc; USA).

Results and discussion

Prevalence and site of infection of the parasitic isopod *Norileca indica*

A total of twenty-six *Norileca indica* with the mean total length of 33.10 ± 0.98 (SD) mm were collected from their infected *Rastrelliger bachysoma*. Results of parasitic prevalence and site of infection for *N. indica* are summarized in Table 1. In all seasons, the prevalence of *N. indica* was higher in the females compared to the males. A highest prevalence of this isopod was recorded in the dry season of female *R. brachysoma* (60% prevalence), compared to the rainy season. Two distinct infection areas of *N. indica* included gill and integument surface, similar to the previous observations (Rivichandran et al., 2009; Rivichandran et al., 2010). A highest presence of *N. indica* was obviously found in the integument surface, especially in female *R. brachysoma* (55% prevalence in the dry season) and (40% prevalence in rainy season). The mechanism of why this isopod preferred to infect the female fish than the male one and how it preferred to populate more on the fish integument than the fish gill are still unknown and this should be addressed in the future works harboring integrative knowledge of histology, cellular and molecular biology.

Table 1. Prevalence (%) and site of infection in *Norileca indica*

Seasons / Fish	Rainy season		Dry season	
	Males	**Females**	**Males**	**Females**
Prevalence of infected isopod	15%(3/20)	50%(10/20)	5%(1/20)	60%(12/20)
Site of infection / organs				
Gill	5% (1/20)	10% (2/20)	0	5% (1/20)
Integument	10%(2/20)	40% (8/20)	5%(1/20)	55%(11/20)

Differentiating oocytes in the parasitic isopod *Norileca indica*

The developing oocyte and oocyte maturation of *Norileca indica* was present in all samples, indicating asynchronous development of oocyte in the ovary (Figures 1A-1B). It was suggested that *N. indica* might favor a multiple spawning seasons or a long spawning period. A correct evaluation of the reproductive and spawning seasons of this isopod is hence required for further studies. Histologically, the outermost layers of the ovary contained a thin layer of connective and muscular tissue (Figure 1B), called as “ovarian capsule”. The ovary carried both developing oocytes and nurse cells (Figures 1B-1C). Based on considerable differences in cell size, histological staining properties and histological features, differentiation of oocyte was classified into three major stages: oogonium stage, primary oocyte stage and secondary oocyte stage (Figures 1D-1F). This pattern of oocyte differentiation was similar to previous observations in *Bopyrina abbreviata* (Romero-Rodriguez et al., 2017) and other crustaceans (Krol et al., 1992; Van Herp & Soyes, 1997; Smija & Devi, 2015). However, some studies showed that the five distinguished stages of ovarian maturation were identified in penaeid shrimps (Tan-Fermin, 1991; Medina et al., 1996). This difference in staging system might be

due to different duration of oocyte development in a species-specific manner, different classification system and standard criteria and guidelines of the morpho-histological features.

Oogonium (Og)

Microscopically, it was revealed that Og located in the oogonial cyst near the germinal epithelium (Figure 1D). Og was oval in shape (10-15 µm in diameter). The oval nucleus (about 5 µm in diameter) was stained faint blue with the H&E method. Heterochromatin condensation was slightly observed within the nucleus, which was surrounded by a slightly eosinophilic cytoplasm (Figure 1D). This Og feature was similar to *Eoleptestherita ticinensis*, reported by Scanabissi & Tommasini (1990). The pre-follicular cell could not be observed at a light microscopic level. Electron microscopic level is needed to further investigate in this event.

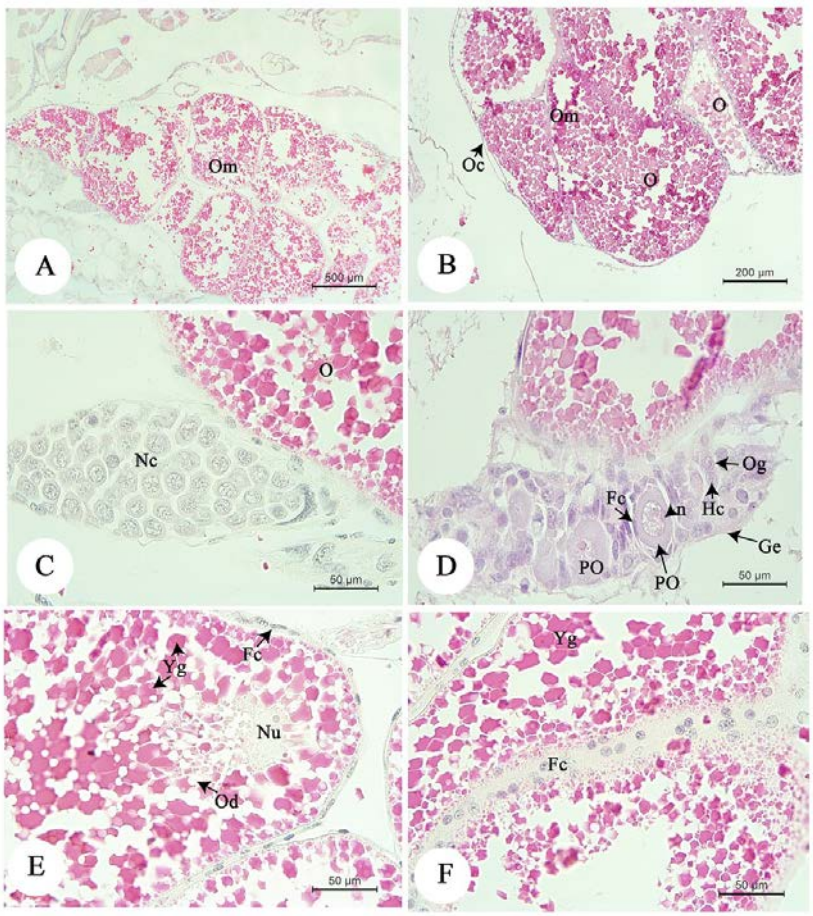

Figure 1. Light photomicrograph of the ovarian structure (A-C) and differentiating oocytes (D-F) in *Norileca indica*. Note: Fc = follicular cell, Ge = germinal epithelium, Hc = heterochromatin condensation, n = nucleolus, Nc = nurse cell, Nu = nucleus, O = oocytes, Oc = ovarian capsule, Od = oil droplet, Og = oogonium, Om = ovarian maturation, PO = primary oocyte, Yg = yolk granules.

Primary oocyte stage (PO)

The PO, undergoing 1st meiosis, had a spherical shape. PO is bigger than the oogonium (about 20-30 µm in diameter), A large nucleus with about 20 µm in diameter carried the small dense cords of heterochromatin. Multiple nucleoli (2-3 nucleoli) were found close to the nuclear membrane (Figure 1D). The ooplasm became a strong basophilic staining. The follicular cells were present and enclosed PO (Figure 1D). Similarly, this character of PO was also reported in pink shrimp *Penaeus paulensis* (Pérez Farfante, 1967) (previously known as *Farfantepenaeus paulensis*) (Dumont et al., 2007).

Secondary oocyte stage (SO)

SO was a developing oocyte stage and found the most in the ovary. The SO is bigger than Og and PO (ranging from 300-400 µm in diameters) because a lot of the yolk granules were accumulated in the ooplasm. Spherical yolk granules were detected as the acidophilic stained granules (H&E method) (Figure 1E). These unique SO characteristics are similar to what found in other crustaceans including giant river prawn *Macrobrachium rosenbergii* and Chinese mitten crab *Eriocheir sinensis* (Dhainaut & De Leersnyder, 1976; Erribabu & Shyamasundari, 1978; Meeratana & Sobhon, 2007). According to Erribabu and Shyamasundari (1978), they speculated that yolk granule was produced/ transported from vitellogenin in the haemolymph under hormonally regulated synthesis. The decrease of encentric nucleo-cytoplasmic ratio was obviously seen (Figure 1E). The oil droplets were seen and widely distributed (Figure 1E). Ravid et al. (1999) also reported that the lipid storage was produced from the hepatopancreas before it was transported to the ovary during vitellogenesis in crustaceans. The function of lipid played a key role as the energy source (triacylglycerols and phospholipids) for hormone synthesis (Lubzens et al., 1997; Ravid et al., 1999). A single layer of the supporting follicular cell was also well developed (Figure 1E), which might function to support yolk uptake into the ooplasm (Souty, 1980). This SO pattern is well agreed with Souty (1980) investigating another marine isopod, *Idotea balthica basteri*.

In the last stage, the oocyte had the maximum diameter around 500 µm due to the abundance of yolk granule in the ooplasm. No nucleus was detected under the germinal vesicle breakdown (GVBD) (Figure 1F).

Moreover, the embryonic development was also observed (Figure 2A). The fertilized egg was filled as yolk plate (Figures 2B-2C), whereas its surface was well developed by multilayers of blastomeres showing the mesodermal plug (Figures 2C-2D), similar to what found in isopod, *Irona* sp. (Nair, 1956) (Figure 2). Embryonic development in *N. indica* is little known and further studies are needed.

Conclusion

To investigate the pattern of isopod infection which might affect the heath of marine fishes, here we firstly demonstrated the prevalence and infection site of cymothoid isopod *Norileca indica* (parasite), a common ectoparasite of fishes in temperate and tropical water of Indo-Pacific region, in short mackerel *Rastrelliger bachysoma* (host) collected from the Upper Gulf of Thailand. Parasitic infection took place in dry season more than rainy season, and the parasite had prevalence in infecting females more than the male mackerel. Additionally, three distinct stages of oocyte development toward oocyte maturation were identified: oogonium, primary oocyte, and secondary oocyte. Further investigation the effect of *N. indica* on immune response in *R. brachysoma* is required to understand isopod infection. This study could provide a foundation to investigate the advanced reproductive biology of isopod, which could be applied to resolve the problem of *R. brachysoma* health.

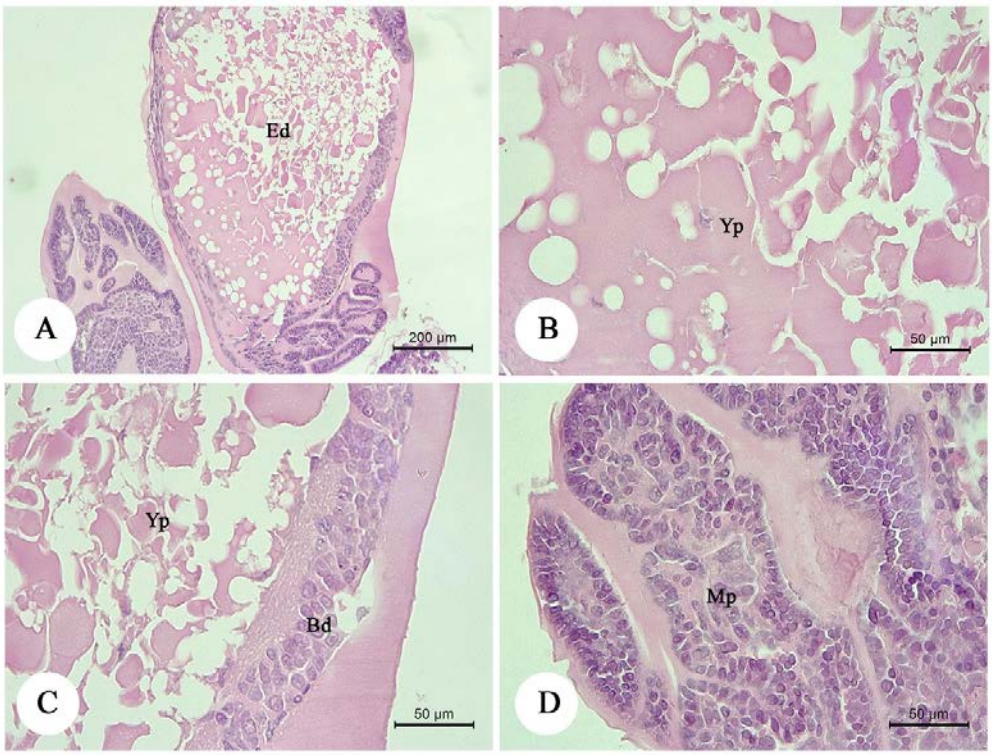

Figure 2. Light photomicrograph of the embryonic development (Ed) in *Norileca indica*. Note: Bd = blastodisc, Mp = mesodermal plug, Yp = yolk plate.

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