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The application of probiotics, prebiotics and synbiotics to enhance the immune responses of vannamei shrimp (*Litopenaeus vannamei*) to *Vibrio harveyi* infection

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Abstract. The aim of this study was to examine the effectiveness of probiotics, prebiotics and synbiotics in enhancing the immune responses of vannameishrimp (*Litopenaeus vannamei*) to *Vibrio harveyi* infection. This research was comprised of five treatments, namely:T0(+)= Positive control, T0(-)= Negative control, T1= 1% probiotic , T2= 2% prebiotic, and T3= synbiotic (1% probiotic+2% prebiotic). The Probiotic SKT-b (dose 10^6 CFU/ml) and oligosaccharides from sweet potatos as a prebiotic were used in this study. The prebiotics and probiotics were supplemented into the shrimp diet. The study revealed that higher immune responses were observed in T3 with total heamocytes of3.36 ± 0.05 x10^6 to 9.32 ± 0.05x10^6 cells/ml, phagocytic activity of 16.48 ± 0.14 to 77.55 ± 0.22%, and phenoloxidase activity of 0.128 ± 0.03 to 0.591 ± 0.01. We concluded that the addition of synbiotics in the feed resulted in better immune responses compared to the addition of probiotics and prebiotics.
Key Words: hemocyte, phagocytic, phenoloxidase, *Ipomoea batatas,* vibriosis.

Introduction. *Litopenaeus vannamei* is an introduced shrimp in Indonesia (Briggs et al 2005). This species has been cultured intensively by local farmers. However, the farmers claim that the shrimp have been frequently infected by diseases, the most comment disease being vibriosis due to *Vibrio* spp. infection (Sharma et al 2010). This disease has been treated with antibiotics, but this technique has led to pathogenic strains that are resistant to antibiotics (Moriarty 1999). In addition, the use of antibiotics in aquaculture may contribute to an increase in the frequency of resistance in the related microflora, and next its for example bacteria on fish may also be transmitted to humans when the aquacultured products are eaten, or when other foods are eaten that have been cross-contaminated by bacteria from fish (Serrano 2005). Thus, some possible alternatives to treating this problem are the application of probiotics, prebiotics, or synbiotics.

Al-Dohail et al (2009) stated that probiotics are commonly used to substitute and restrict the use of antibiotics or chemical drugs in aquaculture, with the aim of improving growth and disease resistance. However, this method has some limitations, including competition in nutrient intake, growth, and colonization of digestive bacteria (Lisal 2005). When the probiotic bacteria do not get enough nutrients, the bacteria in the digestive tract will wash out. Thus, there needs to be another approach to overcome these limitations, such as the application of prebiotics.

Prebiotics are food ingredients that cannot be digested and that provide beneficial effects to the host by stimulating the growth and activity of one or more of the digestive bacterias in the colon (Schrezenmeir & de Vrese 2001). However, these effects do not last longer than the prebiotic supplementation period, so synbiotics are needed to overcome this limitation (Lisal 2005). Synbiotics are a balanced combination of probiotics

and prebiotics that support the survival and growth of beneficial bacteria in the digestive tract of an organism (Schrezenmeir & de Vrese 2001).

The application of probiotics and prebiotics in a diet has positive effects on growth performance, immune system, disease resistance, maintenance of intestine function, and microbial activity in the intestine tract (Merrifield et al 2010). For example, Li et al (2009) reported that the use of *Bacillus* OJ (PB) at a concentration of 108 CFU g^{-1} and 0.2% meisomalto-oligosaccharides (IMO) successfully increased the shrimp's immune systems against WSSV diseases. The probiotic *Bacillus* spp. and the prebiotic MOS have successfully increased the growth performance and survival rate of lobster *Homarus gammarus* L. (Daniels et al 2010). In addition, Hai & Fotedar (2009) reported that the addition of Bio-Mos® and β-1,3-D-glucan into feed has increased growth, survival rates and immune responses in king prawns (*Penaeus latisulcatus*). Hence, the objective of the present study was to examine the effect of probiotics, prebiotics and synbiotics in the immune response of vannamei shrimp (*L. vannamei*) against *Vibrio harveyi* infection.

Material and Method. This study was carried out from March 2011 to May 2011 in Fish Health Laboratory, Department of Aquaculture, Faculty of Fishery and Marine, Bogor Agricultural University, Indonesia.

Experimental design. A completely random experimental design was used in this study. The treatments were: T0(-) = the shrimp fed with a diet without probiotics or prebiotics and not infected with *V. harveyi* bacteria (negative control); T0(+) = the shrimp fed with a diet without probiotics and prebioticsbut infected with *V. harveyi* bacteria (postive control); T1 = the shrimp fed with a diet containing 1% probiotics (Wang 2007) and infected with *V. harveyi* bacteria; T2 = the shrimp fed with a diet containing 2% prebiotics (Mahious et al 2006) and infected with *V. harveyi* bacteria; T3 = the shrimp fed with a diet containing 1% probiotics and 2% prebiotics and infected with *V. harveyi* bacteria. Every treatment had three replicates.

Experimental shrimp. A total of 150 vannamei shrimp were obtained from Anyer STP Hatchery with an average weight of 3.5 g and an average length of 5 cm were used in this study. The experimental shrimp were reared in aquarium tanks (60 cm x 35 cm x 30 cm) at a stocking density of 10 shrimp per tank for 28 days. The shrimp were acclimatized for 7 days prior to being used for this experiment.

Probiotics and preparation of prebiotics (oligosaccharidee extraction). The SKT-b probiotic was prepared based on the method of Widanarni et al (2003). To produce 1 L of prebiotics we steamed 500 g of sweet potato (*Ipomoea batatas*) flourat 100^{o}C for 30 minutes and then dried it in a furnace at 55^{o}C for 18 hours. The dried potato was then milled and the flour was sieved. A total of 100 g of potato flour was mixed with 1 L of ethanol 70% and stirred for 15 hours at room temperature. The suspensions were filtered and the filtrates were evaporated at 40^{o}C for 30 minutes and then centrifuged at 5000 rmp for 10 minutes.

Infection test. The probiotics and prebiotics, at appropriate dosages, were mixed with 2% egg yolk and then sprayed evenly to the feed (a commercial diet that contains 40% crude protein) and dried at room temperature for 10 minutes. The experimental shrimp fed only with the commercial feed that contains 40% crude protein were fed at a ration of 10% of their body weight five times a day at 6 AM, 10 AM, 2 PM, 6 PM, and 10 PM for 14 days. However, the shrimp fed with the feeds that contained probiotics, prebiotics and synbiotics were only fed once a day at 6 PM. The shrimp fasted on day 15 and were infected with *V. harveyi* at doses of 10^6 CFU mL^{-1} (LD_{50}) on day 16. Then the experimental shrimp were reared for another 14 days and fed with a commercial diet without probiotics, prebiotics or synbiotics.

Immune response. The immune responses were examined 9 times a day on day 0, day 7, and day 14 before the injection of *V. harveyi* bacteria, and after the injection of *V.*

harveyi bacteria at 6, 12, 24, 72, 120 and 168 hours after injection. The immune response factors evaluated were total hemocyte count, phagocytic activity and phenoloxidase activity.

Data analysis. The data was subjected to one-way analysis of variance (one-way Anova) followed by the Duncan's multi-range test. The SPSS Ver.17.0 was used to performe the analysis.

Results and Discussion

Total hemocyte count. The hemocyte totals increased in week 1 and week 2 before *V. harveyi* infection. The hemocytes also increased at 6, 12, and 24 hours after *V. harveyi* injection, but decreased sharply at 72 hours after injection and then rose again at 120 and 160 hours after injection (Figure 1). The Anova test showed that the treatment had a significant effect on the total hemocytes ($p < 0.05$) where the best results were found in T3 (the shrimp fed with a diet containing 1% probiotics and 2% prebiotics), but it did not differ significantly from the other treatments.

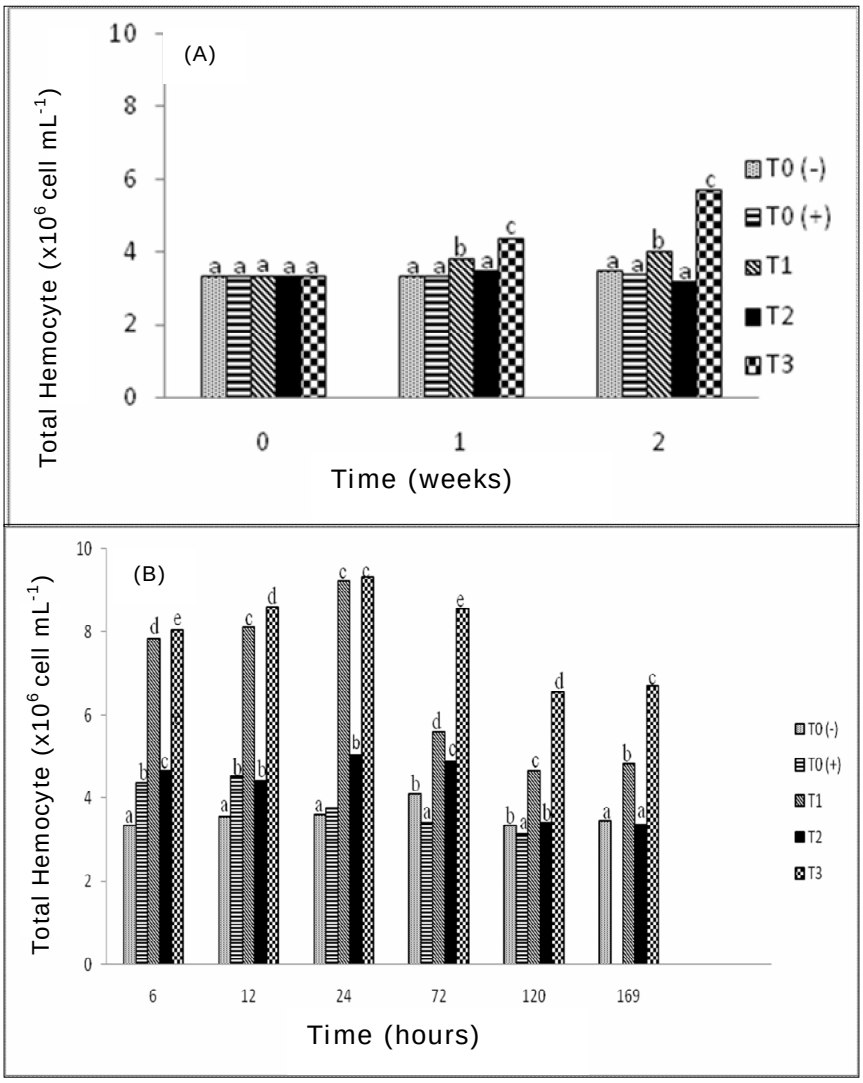

Figure 1. (A) The hemocyte total of vannamei shrimp before injection of *V. harveyi;* (B) The hemocyte total after injection of *V harveyi.* T0(-) = Negative control; T0(+) = Positive control; T1 = Probiotic; T2 = Prebiotic; T3 = Synbiotic. The bars with different letter ssuggest a significant difference ($p < 0.05$).

The hemocyte plays an important role in the immune systems of crustacea. Hemocytes are involved in the activities of phagocytosis, encapsulation, degranulation and nodular

aggregation against pathogens (Sahoo et al 2008). This study revealed that the hemocytes increased at 6, 12, and 24 hours post infection, and then decreased when higher hemocytes were found in treatment T3 (the shrimp fed with a diet containing 1% probiotics and 2% prebiotics) with the values of 8.06±0.07 (106 cells mL^{-1}), 8.6±0.08 (106 cells mL^{-1}) and 9.32±0.05 (106 cells mL^{-1}), respectively. Presumably, when the shrimp were infected with *V. harveyi* , the shrimp produced many hemocytes to fight against the pathogens entering their bodies. This is consistent with the findings of Anderson & Siwicki (1995) who found that in the case of pathogen infestation, there will be an increase in the total hemocyte count, an indication of the infection phase. The decrease in phagocitic activity was probably due to a reduction of phagocitic cells in the haemolymph. The cells were likely destroyed along with the invaded bacteria cells by the immune system of the body. Higher phagocitic activity occurred six hours after *V. harveyi* infection, indicating that the hemocytes started fighting against the *V. harveyi* pathogen before the bacteria secreted virulences.

Phagocytic activity. Phagocytic activity is the most common cellular defense reaction from shrimp. This response is an attempt to defend themselves from antigen infiltration by destroying the antigen nonspecifically via a phagocytosis process. The results showed that the phagocytic activity increased during the two weeks before *V. harveyi* injection, where the highest activity was found in P3 (69.78±0.28%). The activity increased sharply at 6 hours after injection of *V. harveyi* and then declined gradually at 12 hours to 24 hours, and then activity increased again at 120 and 168 hours after *V. harveyi* injection (Figure 2). Statistical analysis revealed that there were significant differences among the treatments, where the highest phagocytic activity was found in P3 at all monitoring times. This indicates that synbiotics gave the best results compared to probiotics and prebiotics. The Anova test showed that the treatment had a significant effect on phagocytic activity ($p < 0.05$) where the best results were found in T3 (the shrimp fed with a diet containing 1% probiotics and 2% prebiotics), indicating that the best immune responses occurred when the shrimp were fed with a diet containing 1% probiotics and 2% prebiotics.

The results showed that phagocytic activity increased from 0 to 6 hours and then declined at 120 hours and 168 hours (Figure 2). The decrease in phagocytic activity in the experimental shrimp was caused by the reduction in phagocytic cells in the hemolymph because most of the phagocytic cells were broken during the phagocytosis process fighting against the virus. This correlates with the findings of Rengpipat et al (2000) who studied phagocytic activity in tiger shrimp *Penaeus monodon.* Similar results were also reported with *L. vannamei* where the phagocytic index of treatment was higher compared to the control (Li et al 2009). An increase in phenoloxidase activity increases the ability of shrimp to recognize and fight against pathogens that enter the body, showing that the immune systems of the shrimp improve as well.

Phenoloxidase activity. The phenoloxidase is an important enzyme in the melanization process of crustaceans as a response to intruder pathogens. This study revealed that phenoloxidase activity increased during the study, illustrating that probiotic bacteria can stimulate the hemocytes of experimental shrimp (Figure 3). The Anova test showed that the treatment had a significant effect on phenoloxydase activity and the best results were found in T3 (the shrimp fed with a diet containing 1% probiotics and 2% prebiotics) followed by T1 (the shrimp fed with a diet containing 1% probiotics), but these values were significantly different from the other treatments ($p<0.05$).

Conclusions. The application of prebiotics and probiotics in the shrimp diet resulted in a positive immune response from the vannamei shrimp, where the best results were found with a combination of 2% prebiotics and 1% probiotics (synbiotics) and therefore this combination can be introduced and applied to the farmers in relation to increase farm production of the vannamei shrimp.

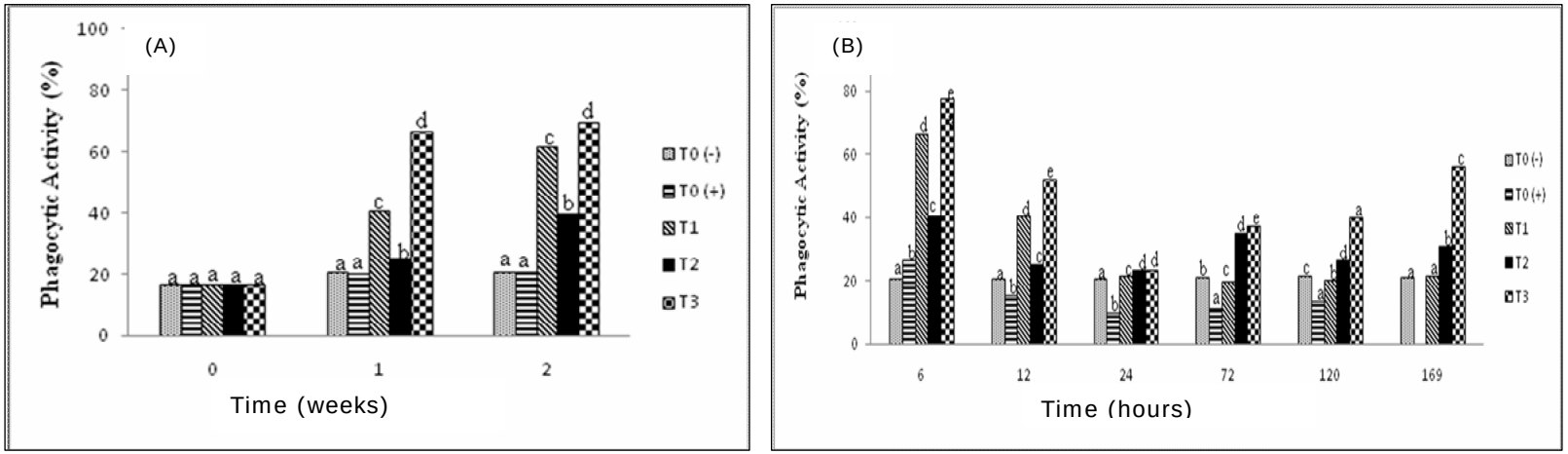

Figure 2. (A) The phagocytic activity of vannamei shrimp before injected of *V. harveyi*; (B) The phagocytic activity of vannamei shrimp after injected of *V. harveyi*. T0(-) = Negative control; T0(+) = Positive control; T1 = Probiotic; T2 = Prebiotic; T3 = Synbiotic. The bars with different letters suggest a significant difference ($p < 0.05$).

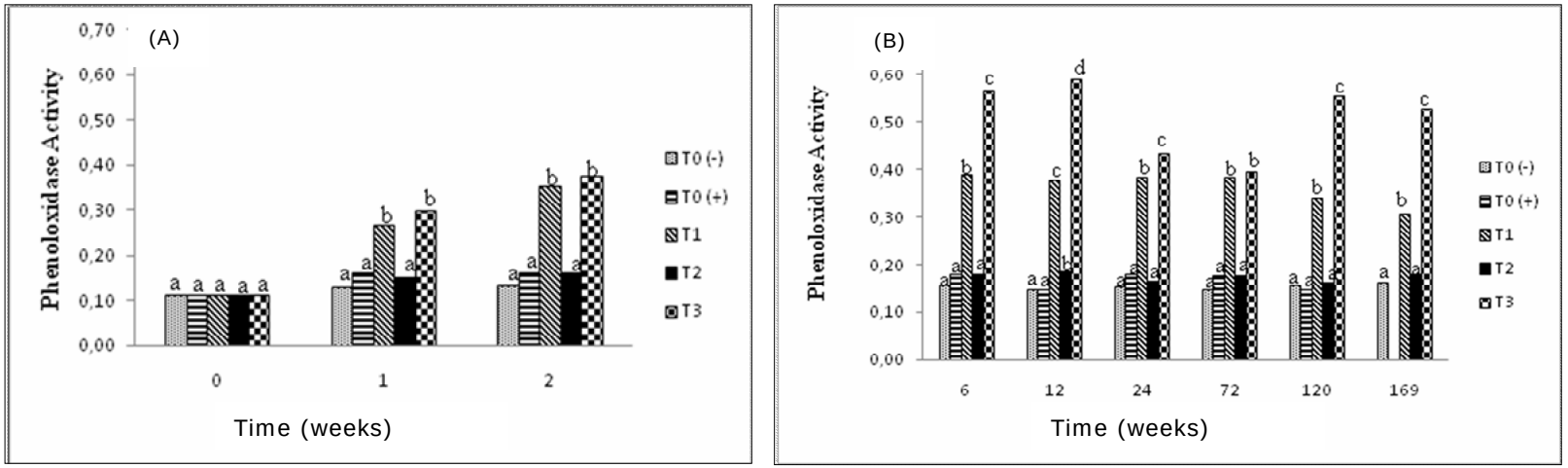

Figure 3. (A) The phenoloxidase activity of vannamei shrimp before injection of *V. harveyi;* (B) Phenoloxidase activity after injection. T0(-) = Negative control; T0(+) = Positive control; T1 = Probiotic; T2 = Prebiotic; T3 = Synbiotic. The bars with different letters suggest a significant difference ($p < 0.05$).

References

Al-Dohail M. A., Roshada H., Mohammed A., 2009 Effects of the probiotic *Lactobacillus acidophilus* on the growth performance, haematology parameters and immunoglobulin concentration in African catfish (*Clarias gariepinus*, Burchell 1822) fingerling. Aquaculture Research 40:1642-1652.

Anderson D. P., Siwicki A. K., 1995 Basic haematology and serology for fish health program. Fish Health Section, Asian Fisheries Society, Manila, Phillipines, pp. 185-202.

Briggs M., Funge-Smith S., Subasinghe R. P., Phillips M., 2005 Introductions and movement of two penaeid shrimp species in Asia and the Pacific. FAO Fisheries Technical Paper No. 476, Rome, 82 pp.

Daniels C. L., Merrifield D. L., Boothroyd D. P., Davies S. J., Factor J. R., Arnold K. E., 2010 Effect of dietary *Bacillus* spp. and mannan oligosaccharides (MOS) on European lobster (*Homarus gammarus* L.) larvae growth performance, gut morphology and gut microbiota. Aquaculture 304:49-57.

Hai N. V., Fotedar R., 2009 Comparison of the effects of the prebiotics (Bio-Mos® and β-1,3-D-Glucan) and the costomised probiotics (*Pseudomonas synxantha* and *P. aeruginosa*) on the culture of juvenile western king prawns (*Penaeus latisulcatus* Kishinouye, 1986). Aquaculture 289:310-316.

Li J., Tan B., Mai K., 2009 Dietary probiotic *Bacillus* OJ and oligosaccharides influence the intestine microbial populations, immune responses and resistance to white spot syndrome virus in shrimp *Litopenaeus vannamei*. Aquaculture 291:35-40.

Lisal J. S., 2005 [Probiotic and prebiotic concept to modulate colon microbiota]. Medical Nusantara 26:256-262 [in Indonesian].

Mahious A. S., Gatesoupe F. J., Hervi M., Metailler R., Ollevier F., 2006 Effect of dietary inulin and oligosaccharides as prebiotics for weaning turbot, *Psetta maxima* (Linnaeus, 1758). Aquaculture International 14(3):219-229.

Merrifield D. L., Arkadios-Dimitroglou A., Foey A., Davies S. J., Baker R. T. M., Bøgwald J., Castex M., Ringø E., 2010 The current status and future focus of probiotic and prebiotic applications for salmonids. Aquaculture 302:1–18.

Moriarty D. J. W., 1999 Disease control in shrimp aquaculture with probiotic bacteria. Microbial biosystems: new frontiers. Proceedings of the 8th International Symposium on Microbial Ecology. Bell C. R., Brylinsky M., Johnson-Green P. (eds), Atlantic Canada Society for Microbial Ecology, Halifax, Canada, pp. 1-7.

Rengpipat S., Rukpratanporn S., Piyatiratitivorakul S., Menasaveta P., 2000 Immunity enhancement in black tiger shrimp *Penaeus monodon* by a probiont bacterium (*Bacillus* S11). Aquaculture 191:271-288.

Schrezenmeir J., de Vrese M., 2001 Probiotics, prebiotics and synbiotic - approaching a definition. American Journal of Clinical Nutrition 73(2):361-364.

Sahoo P. K., Das A., Mohanty B. K., Pilai B. R., Mohanty J., 2008 Dietary β-1,3 glucan improve the immunity and disease resistance of freshwater prawn *Macrobrachium rosenbergii* challenged with *Aeromonas hydrophyla.* Aquaculture Research 39:1574-1578.

Serrano P. H., 2005 Responsible use of antibiotics in aquaculture. FAO Fisheries Technical Paper No. 469, Rome, 110 pp.

Sharma S. R., Shankar K. M., Sathyanarayana M. L., Sahoo A. K., Patil R., Narayanaswamy H. D., Rao S., 2010 Evaluation of immune respone and resistance to diseases in tiger shrimp, *Penaeus monodon* fed with biofilm of *Vibrio alginolyticus.* Fish and Shellfish Immunology 29:724-732.

Wang Y. B., 2007 Effect of probiotics on growth performance and digestive enzyme activity of the shrimp *Penaeus vannamei*. Aquaculture 269:259-264.

Widanarni, Suwanto A., Sukenda, Lay B. W., 2003 Potency of *Vibrio* isolates for biocontrol of vibriosis in tiger shrimp (*Penaeus monodon*) larvae. Biotropia 20:11-23.

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