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Evaluation of frozen Umbrella-stage Artemia as first animal live food for Litopenaeus vannamei (Boone) larvae

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Abstract

An alternative larval shrimp feeding regime, in which umbrella-stage *Artemia* were constituting the first zooplankton source was evaluated in the culture of *Litopenaeus vannamei*. In a preliminary experiment, umbrella-stage *Artemia* were fed to larvae from zoea 2 (Z2) to mysis 2 (M2) stages to identify the larval stage at which raptorial feeding starts and to determine daily feeding rates. The following experiment evaluated the performance of two feeding regimen that differed during the late zoea/early mysis stages: a control treatment with frozen *Artemia* nauplii (FAN), and a treatment with frozen umbrella-stage *Artemia* (FUA). The ingestion rate of umbrella-stage *Artemia* increased from nine umbrella per larvae day^{-1} at Z2 stage to 21 umbrella per larvae day^{-1} at M2. A steep increase in ingestion and dry weight from Z3 to M2 suggests a shift to a raptorial feeding mode at the M1 stage. Treatment FUA exhibited a significantly higher larval stage index ($P < 0.05$) during the period that zoea larvae metamorphosed to the mysis stage, and a higher final biomass, compared with treatment FAN. Based on these results and on practical considerations, a feeding regime starting with umbrella-stage *Artemia* from Z2 substage can be recommended for *L. vannamei* larvae rearing.

Keywords: Umbrella-stage Artemia, *Litopenaeus vannamei*, Larvae, live food

Introduction

Feeding regimen and feeding practices are major factors affecting the success of a commercial shrimp hatchery (Yúfera, Rodríguez & Lubián 1984; D'Abramo, Perez, Sangha & Puello-Cruz 2006). The development of shrimp larvae is characterized by a number of stages and sub-stages that differ in behaviour, morphology and nutritional requirements (Samocha, Uziel & Browdy 1989; Lavens & Sorgeloos 1996). In nature, the larval shrimp diet generally consists of diverse phytoplankton, zooplankton and bioflocs of various sizes and with different biochemical composition, therefore more likely to meet the overall nutritional requirements of the larvae. In contrast, feeding regimen used in commercial shrimp hatcheries rely on a limited number of live food items or on a combination of live and artificial food. The larval rearing diet used in commercial hatcheries consists of a few species of microalgae (Loya-Javellana 1989; Gallardo, Alfonso, Gaxiola, Soto & Rosas 1995) and newly-hatched *Artemia* nauplii (New 1976; Sorgeloos 1980; Kumlu & Jones 1995). *Artemia* nauplii constitute the principal animal protein source used in commercial shrimp larviculture, because of their nutritional value and size, easy acceptance by the larvae and convenient storage as cysts.

Determining the suitable particle size, feed quantity and feeding schedule are of great importance and therefore greatly affect larval development (Kurmaly, Jones, Yule & East 1989; Samocha *et al.*

1989; Jones, Yule & Holland 1997). Zoeal stages feed mainly on phytoplankton (3–30 μm diameter) by filter-feeding, although from stage Z2, depending on the penaeid species, they can capture animal prey of up to 100 μm raptorially (Jones, Kumlu, Le Vay & Fletcher 1997). Feeding on animal organism as early as Z2 has been documented by several authors, using rotifers (Emmerson 1984; Samocha *et al.* 1989; Naessens, Cobo, Van Hauwaert, Van Horenbeeck & Sorgeloos 1995), nematodes (Wilkenfeld 1984; Biedenbach, Smith, Thomsen & Lawrence 1989; Focken, Schlechtriem, von Wuthenau, García-Ortega, Puello-Cruz & Becker 2006), copepods (D'Abramo *et al.* 2006) and *Artemia* nauplii in different forms such as live freshly-hatched nauplii (Kuban, Lawrence & Wilkenfeld 1985; Samocha *et al.* 1989; Lavens & Sorgeloos 1996) and frozen nauplii (Wilkenfeld, Fox & Lawrence 1981). Some commercial hatcheries start feeding Instar 1 *Artemia* nauplii as early as possible, because a number of studies have documented the benefit of an introduction of an animal protein source in the zoea stages (Wilkenfeld 1984; Samocha *et al.* 1989; Naessens *et al.* 1995). However, shrimp larvae at that stage are poor hunters and the use of live *Artemia* nauplii results in an inefficient food uptake. Therefore, heat-killed, blended or frozen *Artemia* Instar 1 nauplii can be fed to late zoea stages and mysis to ease capture. Also, the use of inactive *Artemia* displays the advantage to overcome problems associated with ongrowing of uneaten *Artemia* in the larval rearing tanks (Sorgeloos, Coutteau, Dhert, Merchie & Lavens 1998). On the other hand, the used of heat-killed and blended *Artemia* nauplii may affect the nutritional quality and may deteriorate rearing water quality. García-Ortega, van Hoornyck, Segner, Coutteau and Verreth (1995) studied the effect of heat treatment on the nutritional quality of decapsulated *Artemia* cysts, observing that already at 60°C around 30% of the protein was denaturalized and the enzymatic activity decreased by more than 50%. The continued use of frozen *Artemia* may deteriorate water quality as well. Soares, Peixoto, Wasielesky and D'Incao F. (2006) furthermore observed that feeding frozen *Artemia* nauplii (FAN) resulted in significantly lower weight in *Farfantepenaeus paulensis* (Peréz-Farfante) postlarvae.

Umbrella-stage *Artemia* can be harvested during the hatching process of *Artemia* cysts after only 12 h. The cyst shell or chorion breaks upon a pre-nauplius larva, which is still surrounded by its hatching membrane, and protrudes from the cyst shell hanging underneath the empty shell, thus the name umbrella-stage. The 'breaking' process of the cyst ends when the hatching membrane breaks and the newly-hatched Instar 1 nauplius emerges (Fig. 1). Umbrella-stage *Artemia* display some advantages over *Artemia* nauplii such as higher energy content, smaller size and are easy to be captured (Lavens & Sorgeloos 1996). Umbrella-stage *Artemia* is sometimes used to feed shrimp, mud crab and fish larvae (Nghia 2004; Nhu, Dierckens, Nguyen, Tran & Sorgeloos 2009; Wouters, Cobo, Dhont & Wille 2009).

In the present study, we studied the use of a feeding regime for *Litopenaeus vannamei* larvae, introducing umbrella-stage *Artemia*. To set-up a feeding regime with umbrella-stage *Artemia*, it was necessary to determine at what stage they could be offered to the shrimp larvae and document ingestion rates. Subsequently, the effect of their use on the larval performance was evaluated by comparison with a feeding regime that is typically used in commercial hatcheries.

Figure 1 Umbrella stage hanging underneath the empty cyst shell and instar I nauplius stage of *Artemia*. (Lavens & Sorgeloos 1996).

Materials and methods

Experimental animals

Nauplii 5 (N5) of *L. vannamei* were obtained from EGIDIOSA hatchery (San Pablo, Santa Elena Province) and transported to the research facilities of the ESPOL-CENAIM. Nauplii were disinfected with 100 mg L^{-1} Argentine® (ARGENT, Redmond, WA, USA) for 1 min and acclimatized to the experimental conditions.

Two experiments were performed: firstly, an ingestion experiment to determine the consumption rates of *L. vannamei* larvae feeding on umbrella-stage *Artemia*. Secondly, an experiment to evaluate the larval performance with two alternative feeding schedules.

Ingestion experiment

Ingestion of umbrella-stage *Artemia* was assessed at different larval stages from Z2 until M2. Z2 larvae were stocked in 1 L plastic Imhoff settling cones at a density of 100 larvae L^{-1}. The settling cones were held in a water bath to maintain temperature at 32 ± 1°C. Salinity was 35 ± 1 g L^{-1} and pH averaged 8.22 ± 0.21. Gentle aeration was provided through a 1 mL glass pipette from the bottom of each cone to assure homogeneous distribution of the prey and larvae and to maintain dissolved oxygen near saturation levels.

During the experiment, larvae were fed the microalga, *Chaetoceros gracilis* (Pantocsek), at 150 000 cells mL^{-1}. Great Salt Lake (UT-USA) *Artemia* cysts (INVE Aquaculture SA, Dendermonde, Belgium) were incubated at 28°C in 35 g L^{-1} seawater provided with continuous light (2000 lux) and strong aeration (Lavens & Sorgeloos 1996). After 12 h incubation, umbrella-stage *Artemia* were collected on a 125 μm sieve and washed several times with tap water in order to remove empty shells and membranes. They were kept in a freezer at −20°C. Umbrella-stage *Artemia* were fed at 1 umbrella mL^{-1} day^{-1} for the zoea sub-stages and 5 umbrella mL^{-1} day^{-1} for the mysis sub-stages**.** Four replicates per larval stage were used.

Daily ingestion was monitored every three hours. The umbrella count was computed by three replicate counts of 5 mL samples fixed with lugol. The ingestion rate, *I*, was calculated using the equation described by Paffenhoffer (1971):

$$I = V(C_t - C_o)/nt$$

where, $(C_t\text{-}C_o)$ is the decrease in concentration of umbrella within the experimental period, t (24 h). V is the water volume and n is the number of shrimp larvae (the mean of the initial and final number of larvae in the 24 h period).

Larval counts were recorded every 24 h to calculate the survival rates at each sub-stage. Total length of the larvae (TL, mm) was measured from the eyestalk base until the end of the last abdominal segment using a profile projector. This measurement was carried out on samples of 30 larvae per replicate. For determination of dry weight (DW, mg), triplicate samples of 50 larvae per replicate were taken randomly, washed briefly with distilled water and placed into pre-weighed aluminium foil cups. The samples were dried in an oven at 60°C for 24 h and then weighed. The larval stage index (LSI) was determined by daily microscopic observations following the procedure described by Kanazawa, Teshima and Sakamoto (1985).

Larval rearing experiment

Experimental design

This experiment evaluated the performance of two feeding regimen (Table 1) that differed during the late zoea/early mysis stages: a control treatment in which FAN were fed and a treatment in which FUA. Each dietary treatment was randomly assigned to 10 replicate tanks.

Rearing conditions, live food and feeding regime

N5 were stocked at 100 N5 L^{-1} and maintained in 50 L cylindro-conical fibreglass tanks filled with sand-filtered and UV-treated seawater (salinity 34 g L^{-1}). Temperature was maintained at 32 ± 1°C. Dissolved oxygen concentration was kept above 4 mg L^{-1} in each tank.

During the sub-stages N5 and zoea 1 (Z1), the microalga *C. gracilis* was supplied. Starting from the transition of Z2–Z3, *Tetraselmis* sp. gradually replaced *C. gracilis* (Table 1). The feeding regimen of the treatments began to differ when 90% of the larvae had moulted to Z2. *Artemia* were incubated, harvested and enriched according to standard procedures as described in Lavens and Sorgeloos (1996). *Artemia* cysts (INVE Aquaculture SA, Dendermonde, Belgium) were incubated at 28°C

Table 1 Feeding regimes applied in the *Litopenaeus vannamei* (Boone) larval rearing experiment

	Microalgae		Artificial Diets		*Artemia*		Dietary	Treatments
Larval stage	*Chaetoceros gracilis* (10^3 cells mL^{-1})	*Tetraselmis* sp. (10^3 cells mL^{-1})	Liquid diet (g million $larvae^{-1}$)	Dry diet (g million $larvae^{-1}$)	Live nauplii larvae	Enriched metanauplii $larvae^{-1}$	Frozen nauplii (FAN)	Frozen umbrella (FUA)
N5	100							
ZI	120		10					
Z2	160		17	10			8	10
Z3	80	80	20	11			10	15
MI		60	28	14			12	20
M2		60	35	20			14	25
M3		60	45	23	19			
PL1		60		34	24			
PL2		60		44		17		
PL3		60		64		20		
PL4		60		76		25		
PL5		60		88		30		
PL6		60		100		35		
PL7		60		112		40		
PLS		60		124		45		
PL9		60		136		50		
PL10		60		150		55		
PL11		60		162		60		
PL12		60		175		65		

in 35 g L^{-1} seawater provided with continuous light (2000 lux) and strong aeration. After 12 h incubation, umbrella-stage *Artemia* were collected as described earlier. They were kept in a freezer at −20°C. For Instar I production, *Artemia* cysts (INVE Aquaculture SA, Dendermonde, Belgium) were decapsulated, incubated as described above and harvested after 24 h. Instar I *Artemia* nauplii were used as live newly-hatched nauplii, frozen nauplii (frozen at −20°C) and enriched metanauplii. *Artemia* nauplii, *Artemia* umbrella and enriched metanauplii *Artemia* were disinfected with 50 mg L^{-1} formaldehyde for 30 min before being given to the larvae. Artificial feeds EPIFEED-LHF® (Epicore, Eastampton, NJ, USA), FRIPPAK® (Inve Aquaculture SA, Dendermonde, Belgium), LARVA Z PLUS® (Zeigler, Gardners, PA, USA) were given from Z2 until PL12 (Table 1). Live and artificial feeds were administered alternately and provided manually in twelve daily rations. Survival of *L. vannamei* larvae was counted at every stage of development in order to adjust the feeding regime. As a prophylactic treatment, the probiotic *Vibrio algynolyticus* (ILI strain) was added daily to the culture water at a concentration of 1×10^{10} colony forming units (CFU) mL^{-1} in order to obtain a final concentration of 1×10^5 CFU mL^{-1}.

Evaluation criteria

Survival was determined by taking three 125 mL samples from each culture tank and counting all live larvae. All sampled larvae were then returned to their respective culture tank. Survival was determined at PL1 and at the termination of the experiment when the larvae reached postlarvae 12 (PL12). Total length, dry weight and larval stage index were determined as described above.

A salinity stress test was applied at PL12 on three replicate groups of 100 postlarvae from each dietary treatment. For the salinity stress test, PL12 were transferred abruptly from 35 g L^{-1} seawater to 1 L plastic beakers containing tap water (<3 g L^{-1}) for a period of 30 min. Thereafter, postlarvae were transferred to their original beaker for another 30 min. Percentage survival was assessed considering postlarvae that did not present movement of pleopods and did not react when prodding with a pipette (Palacios, Perez-Rostro, Ramirez, Ibarra & Racotta 1999; Martins, Cavalli, Martino, Rezende & Wasielesky 2006).

Statistical analysis

Results are expressed as means ± standard error of mean (SEM). Normal probability plots and the

Bartlett test for homogeneity of variances were used to verify the assumptions for further analysis. A one way ANOVA was used to detect differences between the treatments. An arcsin transformation of percentage data was applied before the data were analyzed. Tukey's HSD multiple comparison test was used to identify differences between treatments. All references to statistical significance were at the 5% level or lower. The statistical analyses were performed using STATISTICA 4.1 (Statsoft®, Tulsa, Oklahoma, USA).

Results

Ingestion experiment

Survival, dry weight, length and larval stage index are presented in Table 2. For length, dry weight and LSI increasing values were observed demonstrating a normal larval stage development. Figure 2 shows the consumption rates of umbrella by *L. vannamei* larvae from Z2 to M2. Larvae consumed a significant amount of umbrella (a mean of 6.33 umbrella mL^{-1} day^{-1}.) from the initial concentration (1 umbrella mL^{-1}) in the early stage Z2. Consumption did not increase at Z3. However, umbrella consumption doubled at mysis 1 (M1) and further increased at M2 at a concentration of 5 umbrella mL^{-1} day^{-1}.

Larval rearing experiment

Table 3 presents survival, dry weight and length results at PL1 stage of larvae reared under the different dietary treatments. Survival rates of larvae were generally high (88–91%) and did not differ between treatments ($P > 0.05$). Dry weight and length seemed to be higher in treatment FUA as compared with control FAN, but the difference was not statistically different ($P > 0.05$).

At culture day three and four, a significant ($P < 0.05$) higher LSI was observed for larvae of treatments FUA as compared with control treatment FAN (Fig. 3). From culture day five onwards, the LSI was equal in all treatments. The share of larvae that metamorphosed to PL1 ranged from 90% to 100% in all treatments.

Survival at PL 12 stage ranged from 52% to 64%. No significant differences were found between dietary treatments for survival and length ($P > 0.05$ Table 4). Dry weight exhibited the same trend as in PL1 with higher values for larvae fed FUA, although not significantly different from FAN ($P > 0.05$). On the other hand, significantly higher biomass ($P < 0.05$) was obtained in treatments FUA compared with FAN. PL12 exposed to a salinity stress presented the same percentage survival for all the dietary treatments ($P > 0.05$).

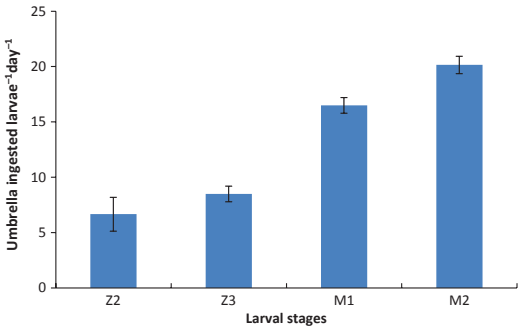

Figure 2 Ingestion rates of umbrella-stage *Artemia* by *Litopenaeus vannamei* larvae from Z2 to M2.

Table 3 Survival, dry weight and length at PL1 of *Litopenaeus vannamei* larvae receiving different dietary treatments: FAN (frozen *Artemia* nauplii) and FUA (frozen umbrella-stage *Artemia*)

Dietary treatments	Survival (%)	Dry weight (mg per larvae)	Length (mm)
FAN	91 ± 2[a]	0.180 ± 0.013[a]	3.70 ± 0.026[a]
FUA	89 ± 4[a]	0.197 ± 0.012[a]	3.80 ± 0.076[a]

Means ± standard error are presented. Within columns, superscript letters indicate significant diffferences ($P < 0.05$).

Table 2 Survival, dry weight, length and larval stage index of *Litopenaeus vannamei* larval stages during the ingestion experiment

Larval stages	Survival (%)	Dry weight (mg)	Length (mm)	larval stage Index (LSI)
Z2	94 ± 2	0.019 ± 0.001	1.45 ± 0.01	2.97 ± 0.001
Z3	96 ± 3	0.029 ± 0.002	2.29 ± 0.01	4.01 ± 0.005
MI	89 ± 1	0.065 ± 0.003	2.84 ± 0.07	4.94 ± 0.001
M2	87 ± 3	0.085 ± 0.003	3.47 ± 0.11	6.00 ± 0.001

Means ± standard error are presented.

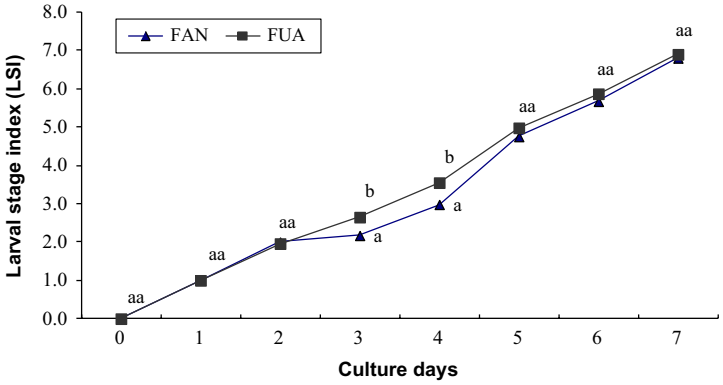

Figure 3 Larval stage index (LSI) of *Litopenaeus vannamei* larvae receiving different dietary treatments: FAN (frozen *Artemia* nauplii) and FUA (frozen umbrella-stage *Artemia*).

Table 4 Survival, dry weight, length, biomass and salinity stress survival at PL12 of *Litopenaeus vannamei* larvae receiving different dietary treatments: FAN (frozen *Artemia* nauplii) and FUA (frozen umbrella-stage *Artemia*)

Dietary treatments	Survival (%)	Dry weight (mg per larvae)	Length (mm)	Biomass (g)	Osmotic stress survival (%)
FAN	56 ± 2[a]	0.440 ± 0.021[a]	6.0 ± 0.116[a]	8.46 ± 0.42[a]	61 ± 3.7[a]
FUA	58 ± 3[a]	0.471 ± 0.022[a]	6.05 ± 0.094[a]	9.70 ± 0.50[b]	59 ± 3.5[a]

Means ± standard error are presented. Within columns, superscript letters indicate significant diffferences ($P < 0.05$).

Discussion

Freshly-hatched *Artemia* nauplii are the most commonly used animal food source in penaeid larval culture systems. It has been demonstrated by Wilkenfeld *et al.* (1981) Emmerson (1984) and Hirata, Anastasios and Yamasaki (1985) that *Artemia* nauplii consumption starts from Z3 sub-stage by, *Marsupenaeus japonicus* (Bate), *Fenneropenaeus indicus* (Milne Edwards) and *L. setiferus* (Burkenroad) respectively. Samocha *et al.* (1989) discerned no significant difference in survival when *Artemia* nauplii were offered to *L. setiferus* larvae from Z2, Z3 or M1, although trends towards increasing larval dry weights were evident as the stage of *Artemia* nauplii introduction was advanced from M1 to Z2. These findings are in accordance with Kuban *et al.*(1985), who supplemented larval diets for four penaeid species (*F. aztecus* (Ives), *L. setiferus, L. stylirostris* (Stimpson) and *L. vannamei*) with *Artemia* nauplii beginning at Z2 versus M1 improved their growth rates in terms of dry weights but did not improve their survival or metamorphosis rate. Introducing *Artemia* nauplii already from Z2 as compared with the classical feeding regime with *Artemia* supplementation as of M1 stage, better growth rates in terms of dry weights were obtained but neither survival nor metamorphosis rate had changed. Introducing *Artemia* nauplii already from Z2 is a common practice in commercial hatcheries using different processing methods: frozen, blended or heat-killed. However, to avoid the use of processing methods that could degrade the quality of the *Artemia* offered to the larvae, we introduced the possibility to use umbrella-stage *Artemia* in the feeding regime of *L. vannamei* larvae.

Umbrella were ingested at stage Z2 as the prey size of umbrella-stage *Artemia* (348.9 ± 23.2 μm) is considerably smaller than that of *Artemia* nauplii (455 ± 25.4 μm). Nevertheless, a higher experimental variability in ingestion rate at Z2, suggests that not all Z2 larvae were equally efficient at catching and ingesting umbrella-stage *Artemia*. The highest consumption rate of umbrella-stage *Artemia* by *L. vannamei* larvae determined in this study (20 umbrella per larvae day^{-1} at M2) was considerably lower than ingestion rates of newly-hatched *Artemia* nauplii ranging from 30 to over 100 nauplii per larvae day^{-1} as reported by Cook and Murphy (1969), Yúfera *et al.* (1984), Hirata *et al.* (1985) for *F. aztecus*, *Melicartus kerathurus* (Forskål) and *M. japonicus* (Bate) respectively. On the other hand, Chun and Shing (1986) reported an ingestion rate of 10 nauplii per larvae day^{-1} for mysis

3 (M3) of *Metapenaeus ensis* (de Haan). The results in our study showed a rather sharp increase in ingestion of umbrella-stage *Artemia* at metamorphosis from zoea to mysis stages. This can be explained by the feeding mode employed by penaeid larvae, shifting from filter to raptorial feeding, at the same time changing from herbivorous feeding at zoea stages to carnivorous during mysis stages (Kurmaly *et al.* 1989; Jones, Yule, *et al.* 1997; Wouters & Van Horenbeeck 2003). Emmerson (1984) indicated that the changeover point occurs around M3 for *F. indicus*, while Chun and Shing (1986) reported that such transition in feeding mode takes place at a later stage for *M. ensis*. Our observations suggest that *L. vannamei* shifts to a raptorial feeding mode at the M1 stage.

In our study, a significantly ($P < 0.05$) higher biomass was obtained for larvae fed umbrella (FUA) compared with those fed FAN. Moreover, larvae fed umbrella-stage *Artemia* exhibited a significantly ($P > 0.05$) higher larval stage index at culture day three and four compared with those fed *Artemia* nauplii. It is observed that feeding umbrellas early at Z2 markedly accelerates the larval development to the mysis stage.

Hence, in this study, umbrella has demonstrated to be valuable life food, potentially improving culture performance as compared with *Artemia* nauplii. Results from this study demonstrate that umbrella-stage *Artemia* could replace live, frozen, heat-killed or blended *Artemia* nauplii typically used in many commercial shrimp hatcheries. In conclusion, umbrella-stage *Artemia* are being proposed for application in commercial feeding regimen of *L. vannamei* larvae, starting from the early Z2 sub-stage and continued until sub-stage M2. Although further studies are needed to establish the optimal concentration of FUA as an initial live food for *L. vannamei* larvae, based on the present results, it is recommended to start feeding 10 umbrella per larvae day^{-1} at Z2 and further increase to 25 umbrella per larvae day^{-1} at sub-stage M2.

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