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The Side-Effects of Polyvinylpyrrolidone-Coated Nanosilver Particles on Controlling Total *Vibrio* during *Artemia parthenogenetica* Hatching and Naupliar Development

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Abstracts

Artemia parthenogenetica is an emerging species used in marine hatcheries in Vietnam. Disinfection during their cyst incubation is important, however, disinfectants might have side-effects. This study examined the effects of polyvinylpyrrolidone-coated nanosilver particles (AgNPs) on *A. parthenogenetica* cyst hatching and nauplii development. Cysts were exposed to low (0.1, 0.4, and 0.7 mg L^{-1}) and high (1, 4, and 7 mg L^{-1}) levels of AgNPs for 48h. The total *Vibrio* in the incubated water and nauplii significantly decreased when AgNPs were applied, however, the hatching success significantly reduced when the AgNPs level exceeded 1 mg L^{-1}. Nauplii Instar I and Instar II were exposed to 10, 50, 100, 200, 500, 600, 700, 750, 800, 900, and 1000 mg L^{-1} to determine their LD50. The LD_{50}-36h and LD_{50}-48h of AgNPs on Instar I were 136.5 and 53.21 mg L^{-1}, respectively, while those on Instar II were 22.15 and 12.1 mg L^{-1}, respectively. Thus, the safe level of AgNPs, which could be used to control *Vibrio* during *Artemia* cyst incubation in fish/shrimp hatcheries, is 0.7-1 mg L^{-1}.

Keywords

Artemia, development, nanosilver particles, toxicity, Vibrio

Introduction

Artemia spp., commonly known as brine shrimp, are the most common form of live feed in fish and shellfish hatcheries due to their pathogen-free cysts and ability to enrich the nutrient content of nauplii. *Artemia* cysts hatch to become Instar I nauplii, which not only have a size suitable for the jaws of newly hatched marine and freshwater larvae but also have a high nutrient value of amino acids and fatty acids (Stappen *et al.*, 2024). When the Instar I molt into Instar II nauplii, their nutritional value is further increased by the enrichment process since the Instar II nauplii start to filter non-selectively (Campbell *et al.*, 1993; Dixon *et al.*, 1995; Sorgeloos *et al.*, 2001). The successful enhancement of highly unsaturated fatty

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acids together with polar lipids in enriched Instar II nauplii has been obtained using various additives (Navarro *et al.*, 1999; Garcia *et al.*, 2008). Besides the biometric and nutrient values, the bacterial quality of *Artemia* nauplii is an important factor in the culture success of fish and shrimp larvae.

The hatching and enrichment processes might be contaminated with bacteria (López-Torres & Lizárraga-Partida 2001). The hydration of cysts releases a high organic loading condition, mainly glycerol, which induces a proportional growth of opportunistic bacteria such as *Vibrio* spp. despite a short incubation period (Stappen *et al.*, 2024). Most *Vibrio* loads have been shown to cause disease and mortality outbreaks in *Artemia* larval rearing (Lightner & Lewis 1975; Baticados *et al.*, 1990; Lavilla-Pitogo *et al.*, 1990; Gomez-Gil *et al.*, 2004), hence, a disinfection step must be applied during the hatching process. The most commonly used disinfectants are calcium hypochlorite, sodium hypochlorite (bleach), iodine, formaldehyde, and lime powder (Tunsutapanich, 1979; Stappen *et al.*, 2024). However, a hypochlorite treatment may not kill all germs present in the alveolar and cortical layers of the outer shell (Stappen *et al.*, 2024). Meanwhile, probiotics only suppress *Vibrio* in *Artemia* (Sahandi *et al.*, 2022). Complete sterilization can be achieved through cyst decapsulation, which would be troublesome for some *Artemia* producers.

Silver nanoparticles (AgNPs) have been tested for disease control in aquaculture due to their mechanical and toxic properties against bacteria (Bahabadi *et al.*, 2016; Dananjaya *et al.*, 2016). The small size of AgNPs facilitates better interaction with cell membranes and better penetration into cells (Rai *et al.*, 2009). AgNPs can also release Ag^+ ions that are toxic to most bacteria (Lara *et al.*, 2011). To increase their bioavailability, AgNPs are coated with polyvinylpyrrolidone (PVP) to stabilize the particles and reduce their toxicity (Hou *et al.*, 2017). PVP-coated nanosilver particles have a slower dissolution rate in seawater than other coated AgNPs (*i.e.* citrate-AgNPs) (Angel *et al.*, 2013).

However, there is still a lack of information about their safety for organisms and the environment (Gambardella *et al.*, 2015), including their use with *Artemia*. The acute toxicity of AgNPs on *Artemia salina* was shown to increase with increasing nanoparticle concentrations (Arulvasu *et al.*, 2014; An *et al.*, 2019). Rahmani *et al.* (2016) and Lacave *et al.* (2017) showed trophic transfer in fish fed with *A. salina* previously treated with AgNPs. Besides *A. salina*, *Artemia parthenogenetica* is an emerging species used in Asian marine hatcheries. However, the impacts of PVP coated AgNPs on early *A. parthenogenetica* stages are not known. Hence, our aims were to evaluate the effects of PVP coated AgNPs on *Vibrio* control efficacy during *A. parthenogenetica* cyst incubation and to determine the embryo hatching success and how the nauplii development.

Materials and Methods

Nanosilver particles

The PVP coated AgNPs were purchased from Sil-Life^{TM} (Taiwan) as a brown aqueous solution. The properties of the AgNPs were provided in Do *et al.* (2023). The AgNPs were diluted in distilled water and the tested concentrations were verified with linear calibration by measuring the optical determination at 410nm (Tran *et al.*, 2015) using a UV-Vis Specord 200 spectrophotometer (Analytik Jena, Germany). The concentrations of the Ag nanoparticle solutions were determined through analysis of the absorption spectrum. An absorption peak observed at 410 nm is indicative of the surface plasmon resonance characteristic of Ag nanoparticles approximately 40 nm in diameter. Furthermore, the extinction coefficient of the Ag nanoparticle solution has been calculated to be 336×10^8 M^{-1} cm^{-1}. Consequently, the concentration of Ag in the solution could be estimated (Paramelle *et al.*, 2014).

Experiment 1: Effect of low and high AgNPs levels on the hatching ratio of Artemia cysts

The tested low concentrations of AgNPs were 0, 0.1, 0.4, and 0.7 mg L^{-1}. The tested high concentrations of AgNPs were 0, 1, 4, and 7 mg L^{-1}. Each treatment had three replicates. *Artemia* cysts (1.6g) were incubated in a 1-L Imhoff cone for each replicate. The temperature and salinity

of the UV-filtered natural seawater were maintained at 28°C and 29PSU, respectively. The seawater was aerated to provide oxygen and stir the eggs evenly to avoid settling at the bottom. The water parameters (pH, DO, NH_3, NO_{2-}) were measured every 8h. Dissolved oxygen (DO) was measured with a DO200 probe sensor (YSI, USA) while pH, NH_3, and NO_{2-} were measured with an A3 Tomota test kit (Otanics, Vietnam). The hatching ratio and deformities of *Artemia* nauplii were monitored at 24, 36, and 48h post-incubation.

Hatching ratio and deformity determination

The method of determining the hatching ratio was according to the National Standards: Aquaculture feeds – Brine shrimp (*Artemia*) cysts – Technical requirements and test methods (TCVN 11754:2016). After 24, 36, and 48h of incubation, six 250µL-samples were randomly collected from each Imhoff cone and transferred into a 24 well tray. A drop of 3% Lugol solution was added to each sample for fixation. The total number of nauplii and number of deformed nauplii of each sample were counted. The average total number of each cone (Ni) and the average number of malformed nauplii (Fi) were calculated. Then, five drops of Javel water solution (NaOCl) were added into each well to separate unhatched eggshells and dissolve empty eggshells. The number of unhatched embryos (orange color) in each sample was counted. The average number of unhatched embryos (Ei) of each cone was calculated.

The hatching ratio (Hi) of each incubation cone was determined according to the formula:

$$Hi = (Ni+Ui)/(Ni+Ui+Ei) \times 100$$

in which Ni is the average nauplii number of the cone, Ui is the average number of eggs in the umbrella stage of the cone, and Ei is the average number of unhatched embryos in the cone.

The hatching ratio of each treatment (H%) was the average value of the hatching ratio of the three incubation cones.

The deformity ratio (Ti) of each incubation cone was determined according to the formula:

$$Ti = Fi/(Ni+Ui+Ei) \times 100$$

in which Fi is the average number of malformed nauplii of the cone.

The deformity ratio of each treatment (T%) was the average value of the deformity ratio of the three incubation cones.

Total Vibrio density in seawater and Artemia determination

Samples of the *Artemia* cysts and water before incubation, and of *Artemia* nauplii and water after 24h of incubation were collected to determine the bacterial infection. *Artemia* cysts were weighed out to 0.1g and crushed, and then 0.9mL of distilled water was added. *Artemia* nauplii were weighed out to 0.3g and ground. The nauplii and water samples were diluted 100 times before inoculation. Fifty microliters (50µL) of each prepared sample was inoculated on TCBS agar. The petri dishes were incubated at 28°C and the number of *Vibrio* colonies was counted after 24h.

Experiment 2: LC50 of AgNPs on Artemia Instar I & II nauplii

The twelve AgNPs treatments were 0 (Control), 10, 50, 100, 200, 500, 600, 700, 750, 800, 900, and 1000 mg L^{-1}. Each treatment was repeated five times. Ten *Artemia* Instar I nauplii were pipetted into each 2mL well. Similarly, 10 *Artemia* Instar II nauplii were pipetted into each well. The salinity and temperature in each well were maintained at 29PSU and 28°C, respectively. The mortality rates of the *Artemia* I & II nauplii were monitored after 24h and over 7 days.

Mortality K (%) was determined according to the formula:

$$K(\%) = C/F \times 100$$

in which K(%) is the mortality ratio, C is the number of dead *Artemia* nauplii, and F is the initial *Artemia* number.

The mortality ratio (%) of each treatment was the average value of the mortality ratio of the five replicates.

Statistical analysis

The hatching, survival, and abnormal ratio values were log transformed and normalized. Transformed data among treatments at a

particular sampling event were compared using one-way analysis of variance (ANOVA) with pair-wise Tukey's post hoc tests ($\alpha = 0.05$) using MiniTab v.16. The LD50 of AgNPs on *Artemia* Instar I and II nauplii were obtained by fitting the two-parameter log-logistic function and then plotted using GraphPad Prism v9 for Windows (Dinh *et al.*, 2022).

Results

Microbial and water quality conditions

The total *Vibrio* densities in both the water and nauplii were significantly decreased when AgNPs were applied. The total *Vibrio* count of the water after 24h in the AgNPs ranged from 0.2-0 × 10^3 CFU mL^{-1} while that in the control was 16.8 × 10^3 CFU mL^{-1}. The total *Vibrio* count of the *Artemia* nauplii after 24h could not be detected in the AgNP treatments while that in the control was 2 × 10^3 CFU mL^{-1}.

The environmental conditions of experiment 1 were within the suitable ranges for hatching *Artemia* cysts. There were no differences in all the environmental conditions between the control and the AgNPs treatments. The temperature ranged from 29-31.3℃. Salinity was constant at 29PSU. The DO was 6.3-6.5 mg L^{-1}. pH was 7.6-7.7. Nitrite was not detectable. NH_3 was at a low level of 0-0.03 mg L^{-1}.

Similarly, the environmental conditions of experiment 2 were within the optimal ranges for hatching and no differences were found between the control and the AgNPs treatments. Temperature ranged from 30.2-30.5℃. Salinity was 29 PSU. The DO was 6.3-6.4 mg L^{-1}. pH was 7.6-7.8. Nitrite was 0-0.3 mg L^{-1}. NH_3 was 0-0.3 mg L^{-1}.

Hatching success at low levels of AgNPs

The hatching success was not significantly different among the control, AgNP0.1, and AgNP0.4 throughout the experimental period (P >0.05; **Figure 1**). The AgNP0.7 treatment showed significantly higher hatching ratios than the control and AgNP0.1 at 24h (P <0.05). The hatching success of AgNP0.7 was significantly higher than those of AgNP0.1 and AgNP0.4 at 36 and 48h (P <0.05). However, the different margins (< 3%) were not substantial in the shrimp hatchery's practices. We did not spot any abnormal Instar I nauplii in any of the treatments over 48h.

Hatching success at high levels of AgNPs

Within the first 24h there were no signs of abnormal Instar I nauplii in any of the treatments. We found no abnormal Instar I nauplii in the control and AgNP1 after 48h. In contrast, the abnormal Instar I ratio increased in AgNP4 and AgNP7 over time. Between these treatments, the

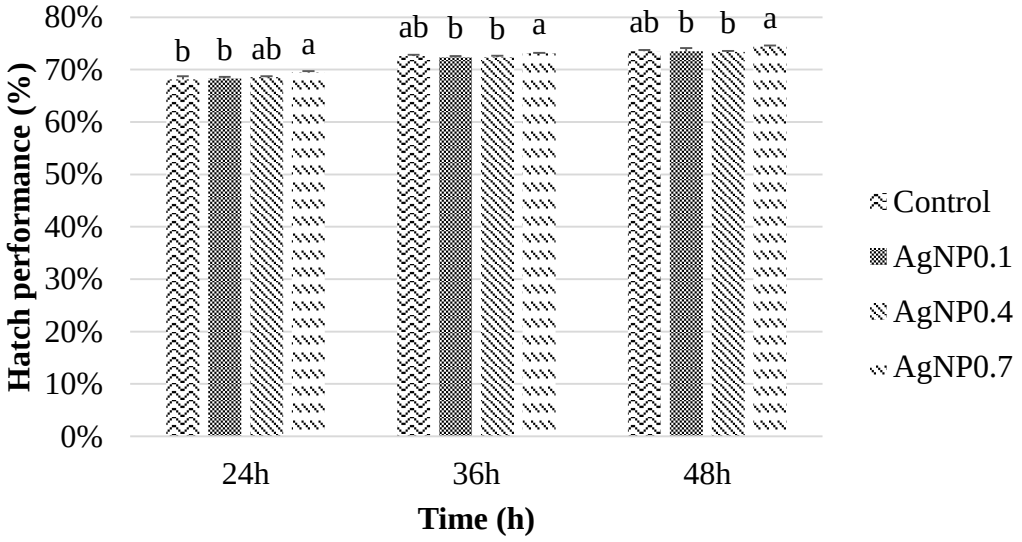

Figure 1. Hatching success of *Artemia parthenogenetica* cysts in the treatments of 0, 0.1, 0.4, and 0.7 mg L^{-1} AgNPs over time (The same letters above the bars indicate no significant differences among treatments at each sampling point, P >0.05)

abnormal Instar I ratios in AgNP7 were higher than in AgNP4 at both 36h and 48h (P <0.05, **Figure 2**).

Both hatching ratios in the controls of the low and high AgNP doses were ~70%, and therefore, within the normal range. The hatching success of the AgNP1.0 treatment was the highest at 24h while that of AgNP7.0 was the lowest (P <0.05). There was no difference in the hatching ratio between the control and AgNP4.0 at 24h (P >0.05). There was a decreasing trend of the hatching ratio with the increase of AgNP concentrations at any sample event (P <0.05). No difference in the hatching ratio between the control and the AgNP0.1 treatment was found at 36h and 48h (P >0.05).

LD50 of AgNPs on Instar I & II nauplii

Mortality of Instar I nauplii increased with the increase of the AgNP concentration at 24h, 36h, and 48h (**Figure 3**). After 72h of exposure, no surviving Instar I nauplii were observed in all the AgNP treated groups while the survival of the control was 8 ± 3.74% (Mean ± S.E.M).

The dose-response curves at 36h and 48h followed by the typical sigmoidal pattern. The LD50 values of AgNP at 36h and 48h were 136.5 and 53.21 mg L^{-1}, respectively (R-square = 0.9 and 0.95, respectively). We could not determine the LD50 at 24h since the mortality had not reached 100%. Although we could not determine the exact LD50 at 72h afterward since the mortality reached 100% after 48h, it was estimated to be lower than 10 mg L^{-1} (**Figure 3**).

Similar to Instar I, the mortality of Instar II nauplii increased with the increase of the AgNP concentration at 24h, 36h, and 48h (**Figure 4**). All Instar II nauplii that were exposed to AgNP died after 72h. The dose-response curves at 24h, 36h, and 48h followed the typical sigmoidal pattern. The LD50 values of AgNP at 24h, 36h, and 48h were 242.5, 22.15, and 12.1 mg L^{-1}, respectively (R-square = 0.95, 0.94, and 0.85, respectively).

Discussion

The toxicity of AgNPs on the cyst hatching success of *Artemia parthenogenetica* seems to be dependent on the permeability of Ag^{+} ions through its shell. The PVP-AgNPs toxicity in this study was lower than in the study of Arulvasu *et al.* (2014) in which 0.2-1.3 mg L^{-1} of citrate-AgNPs significantly reduced the hatching ratio to 21-56%. The reason for this difference might be because we used non-decapsulated cysts while Arulvasu *et al.* (2014) used sodium hypochlorite decapsulated cysts. The chorion layer is known to be resistant to different hazard factors to protect the embryo (Stappen *et al.*, 2024). Also, citrate-coated AgNPs are known to be more toxic to zebrafish than PVP coated AgNPs (Liu *et al.*, 2019). Similar to our result, Rekulapally *et al.* (2019) found significant drops in the hatching ratios to 32.2% and 29.3% with 10 and 100 mg L^{-1} PVP-AgNPs, respectively. The toxicity of PVP-AgNPs was even reduced when *Artemia* cysts were dried and hatched on tryptic soy agar

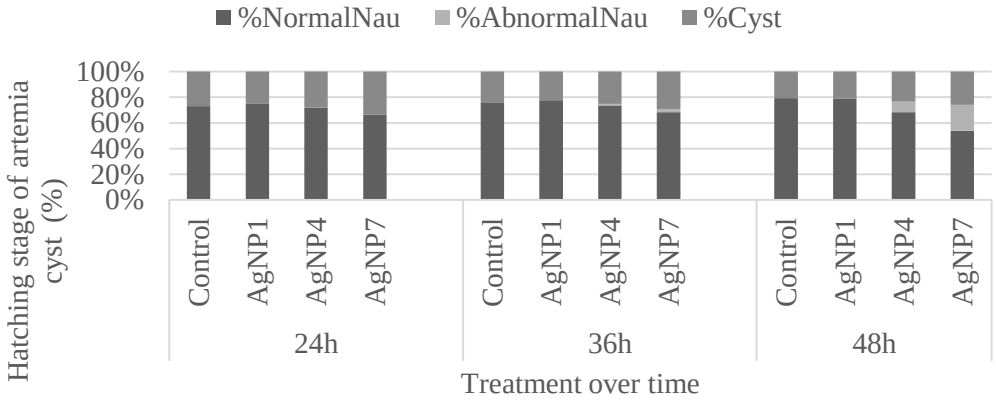

Figure 2. Stage of hatching *Artemia parthenogenetica* cysts in treatments of AgNP at 0 (control), 1, 4, and 7 mg L^{-1} over time

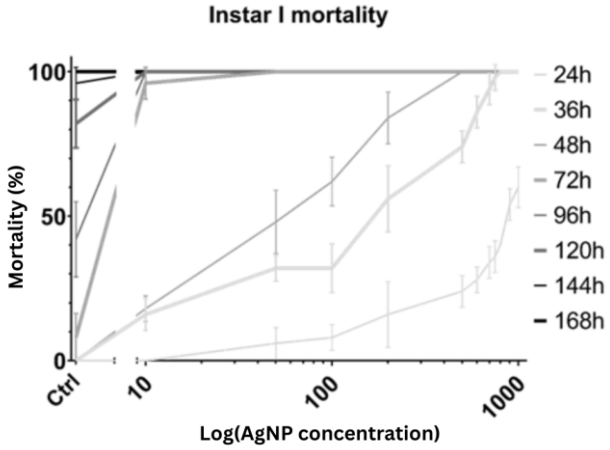

Figure 3. AgNP lethal doses on *Artemia parthenogenetica* Instar I nauplii (AgNP concentrations are presented in the logarithmic scale)

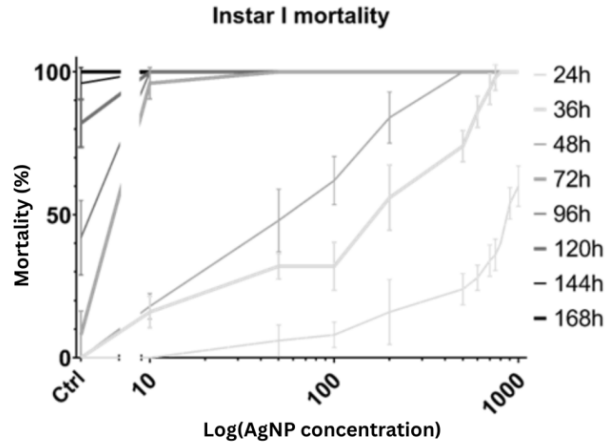

Figure 4. AgNP lethal doses on *Artemia parthenogenetica* Instar II nauplii (AgNP concentrations are presented in the logarithmic scale)

(Do *et al.*, 2023) due to fewer dissolved Ag^+ ions. The toxicity of AgNPs was suggested due to the cuticle damage through the binding of ionic silver (Pinheiro *et al.*, 2024).

The acute toxicity of AgNPs on both *Artemia* Instar I and Instar II nauplii was significant, although the toxicity levels seem to vary among studies. The LD50 values in this study appear to be higher than those found by some previous studies on *A. salina*. All nauplii Instar I of *A. salina* died at 50 and 100 mg L^{-1} AgNPs after 24h of exposure (Pinheiro *et al.*, 2024). Kumar *et al.* (2012) reported the LC_{50}-24 h value of 10^{-3} mg L^{-1} AgNPs on *A. salina*. Falugi *et al.* (2013) obtained an LC_{50}-48h value of 7.3×10^{-3} mg L^{-1} AgNPs on *A. salina*. Becaro *et al.* (2015) reported the EC_{50}-48h immobilization value of 5.5×10^{-2} mg L^{-1} AgNPs on *A. salina*. However, our result appears to be similar to An *et al.* (2019) in which the EC_{50}-72h of AgNPs for *A. salina* nauplii was 10.7 mg L^{-1}. Within 24h, the impact of < 10 mg L^{-1} AgNPs on *A. salina* was also minimal (An *et al.*, 2019) as found in this study.

Instar II nauplii seem to be more susceptible to AgNps than Instar I nauplii. *A. salina* Instar II were more sensitive to AgNPs than Instar I nauplii in the 48h exposure (Lacave *et al.*, 2017). This result also aligns with previous studies on other chemicals. Sogerloss *et al.* (1978) demonstrated that Instar II were more sensitive to copper than Instar I nauplii. Nguyen Tac An *et al.* (1996) also found that Instar II were more susceptible to $CuSO_4$, $K_2Cr_2O_7$, and chemicals used in the petroleum industry than Instar I nauplii. The obvious difference in the lethal doses between Instar I and Instar II nauplii might

be because Instar II nauplii start to feed so their digestive tract comes in contact with the tested chemicals (Sogerloss *et al.*, 1978). Indeed, the aggregation of nanoparticles in the nauplii's gut increased with the AgNP concentrations and those filled with particles showed substantial mortality within 24h (Aruvasul *et al.*, 2014; An *et al.*, 2019). Nanoparticle intake induces gut necrosis of epithelial cells and severe edema, indicated by swelling of the cells with enlarged cytoplasms (Kachenton *et al.*, 2019). Consequently, nanoparticle accumulation leads to oxidative stress with the generation of reactive oxygen species or lysosomal damage (Ates *et al.*, 2020; Do *et al.*, 2023).

Conclusions

This study demonstrated the potential application of AgNPs during *Artemia* cyst incubation in spite of their toxicity at different hatching stages. *A. parthenogenetica* cysts that were exposed to low levels of AgNPs (up to 0.7-1 mg L^{-1}) showed similar hatching success rates to the control while total *Vibrio* was eradicated in the water and nauplii. The higher concentrations of AgNPs caused the side-effects of reducing the hatching success and increasing the abnormal nauplii ratio. The toxicity of AgNPs increased with the developing stages of the *Artemia* cysts, and Instar I and Instar II nauplii. The LD_{50}-36 h and LD_{50}-48h of AgNP on Instar I were 136.5 and 53.21 mg L^{-1}, respectively, while those on Instar II nauplii were 22.15 and 12.1 mg L^{-1}, respectively. Future studies will verify the application of AgNPs at a mass scale production of *Artemia* nauplii.

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