Biochemical Analyses of Trichogramma dendrolimi (Hymenoptera: Trichogrammatidae) in Vitro and in Vivo Rearing for 10 Generations

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Biochemical analyses of *Trichogramma dendrolimi* (Hymenoptera: Trichogrammatidae) in vitro and in vivo rearing for 10 generations

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Abstract

Trichogramma dendrolimi Matsumura (Hymenoptera: Trichogrammatidae) is an economically important egg parasitoid with a wide host range and is mass produced for biological control programs. With the development of in vitro rearing techniques, an artificial medium has been developed that is believed to hold potential for the large-scale production of the parasitoid. We sought to evaluate the effectiveness of an artificial rearing medium by including biochemical analyses and not just by comparing the biological parameters of reared wasps. In particular, this study was conducted to determine differences in proportions of nutrients between the artificial medium and a natural host used for mass rearing *T. dendrolimi* by biochemical analyses of carcasses of the latter. Biochemical parameters including protein concentration, lipid content, trehalose content, and trehalase activity in *T. dendrolimi* continuously reared in an optimized artificial medium versus on *Antheraea pernyi* Guérin-Méneville (Lepidoptera: Saturniidae) eggs were compared. The adults produced in vitro showed lower protein concentrations and higher lipid and trehalose contents and trehalase activity compared with those reared in vivo. Different—and even contrary—evolution of biochemical parameters was found when comparing adults reared in the artificial medium with those reared in natural host eggs over 10 generations. The results suggested that the artificial medium had a non-optimal composition for the in vitro rearing of *T. dendrolimi,* and that it could be improved by altering the composition of the medium, especially its protein concentration and lipid content. Biochemical analysis may be a useful method for optimizing artificial rearing media for other entomophagous insect species.

Key Words: artificial medium; protein; lipid; trehalose; trehalase activity; carcass analysis

Resumen

Trichogramma dendrolimi Matsumura (Hymenoptera: Trichogrammatidae) es un parasitoide de huevos económicamente importante con amplia gama de hospederos, que está siendo producido en masa para los programas de control biológico. Con el desarrollo de técnicas de cría in vitro, se ha desarrollado un medio artificial que se cree que posee un potencial para la producción a gran escala del parasitoide. Hemos tratado de evaluar la eficacia de un medio artificial de cría mediante la inclusión de los análisis bioquímicos y no sólo mediante la comparación de los parámetros biológicos de la avispas criadas. En particular, se realizó este estudio para determinar las diferencias en las proporciones de nutrientes entre el medio artificial y un hospedero natural utilizado para la cría masiva de *T. dendrolimi* por los análisis bioquímicos de los cadáveres de estos últimos. Los parámetros bioquímicos, incluyendo la concentración de proteína, contenido de lípidos, el contenido y actividad de trehalosa de *T. dendrolimi* criado continuamente en un medio artificial optimizado fueron comparados con los huevos de *Antheraea pernyi* (Guérin-Méneville) (Lepidoptera: Saturniidae). Los adultos producidos in vitro mostraron concentraciones más bajas de proteínas y lípidos, un contenido y actividad más alto de trehalosa en comparación con aquellos criados in vivo. Diferente—e incluso contrarias—se encontraron la dinámica de los parámetros bioquímicos al comparar los adultos criados en el medio artificial con los criados de huevos hospederos naturales de más de 10 generaciones. Los resultados sugirieron que el medio artificial tiene una composición no óptima para la cría in vitro de *T. dendrolimi*, y que podría ser mejorada mediante la alteración de la composición del medio, especialmente su concentración de proteína y el contenido de lípidos. El análisis bioquímico también puede ser un método útil para la optimización de los medios de cría artificial para otras especies de insectos entomófagos.

Palabras Clave: medio artificial; proteína; lípidos; trehalosa; actividad trehalasa; análisis de la canal

For decades, researchers have been committed to the development of in vitro rearing techniques for mass producing *Trichogramma* species to overcome the high costs and inadequate supplies of factitious hosts. However, in vitro rearing systems for *Trichogramma* species have not been implemented on a wide scale, because stable quality of in vitro–produced *Trichogramma* has proven difficult to obtain (Cônsoli & Grenier 2010). Clearly, further studies are needed to improve the performance of parasitoids reared in artificial media if the aim is to use them in mass rearing systems.

Most of the studies focused on the parasitoid quality by assessing biological traits of in vitro–reared insects compared with those reared in factitious and/or natural hosts and examining just a single generation (Grenier et al. 1995; Nurindah et al. 1997), whereas few investigators were able to evaluate parasitoid fitness after rearing in an artificial medium continuously (Gao et al. 1982; Nordlund et al. 1997). Few investigated the quality of insects produced in artificial media by comparing biochemical parameters between adults reared in vivo and those reared in vitro (Grenier et al. 1995; Cônsoli & Grenier 2010). Bio-

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chemical analyses of hosts could be used for establishing the quantity of each component of the artificial medium (Grenier 2012). For example, Xie et al. (1982) analyzed the amino acid contents of host eggs to determine their various requirements and define the medium composition for *Trichogramma*.

Besides the host, the biochemical analysis could be done with the parasitoid itself. The carcass analyses may provide information about the nutritional needs of the parasitoid and serve as a necessary reference for deficiency and imbalance corrections in the medium composition by comparison with insects grown on control hosts (Grenier & De Clercq 2003; Dindo et al. 2006; Grenier 2012). The composition of key components in living organisms, such as proteins, lipids, carbohydrates, enzymes, and hormones, might be a good criterion for estimating the quality of artificially reared natural enemies (Grenier & De Clercq 2003).

Trichogramma dendrolimi Matsumura (Hymenoptera: Trichogrammatidae) is an economically important egg parasitoid, which has a wide host range and is mass produced for biological control programs in China. The parasitoid could be reared in various lepidopteran insect eggs such as *Corcyra cephalonica* (Stainton) (Pyralidae)*, Ephestia kuehniella* Zeller (Pyralidae), *Antheraea pernyi* Guérin-Méneville (Saturniidae), or *Samia cynthia ricini* Boisduval (Saturniidae). In recent years, we attempted to develop an optimal artificial medium for in vitro rearing of *T. dendrolimi*, whose offspring should have similar quality as those reared in vivo. A basic artificial medium was developed by Li et al. (1986) containing a large proportion (40%) of pupal hemolymph of *A. pernyi*, egg yolk, fresh milk, and Neisenheimer's salt solution. More recently, this basic artificial medium was adjusted by supplementing it with trehalose in sterile water to partially replace the pupal hemolymph, which was believed to hold potential for the mass production of *T. dendrolimi* and reduce the associated production cost (Lü et al. 2013). Then Lü et al. (2014) revealed that trehalose is an essential ingredient and developed an optimized artificial medium by orthogonal array design. The aim of the present study was to determine the quality of this medium and *T. dendrolimi* produced for 10 generations by comparing the biochemical parameters of the parasitoids reared in artificial medium and those reared in *A. pernyi* eggs as the control.

Materials and Methods

EXPERIMENTAL INSECTS

Trichogramma dendrolimi was originally obtained from the Institute of Plant and Environment Protection, Beijing Academy of Agricultural and Forestry Sciences, Beijing, China. In the laboratory, stock cultures of *T. dendrolimi* were reared in eggs of *A. pernyi* as a factitious host. Adults were fed on a honey solution (30% in water). Climatic conditions were 27 ± 1 °C, 75 ± 5% relative humidity (RH), and a 16:8 h L:D photoperiod (Lü et al. 2013).

ARTIFICIAL MEDIUM PREPARATION

The artificial medium used in this study is the optimized artificial medium (Lü et al. 2014), consisting of 3 mL pupal hemolymph of *A. pernyi*, 2.5 mL egg yolk, 1 mL of 10% malted milk solution, 1 mL Neisenheimer's salt solution, 0.1 g trehalose (Sigma, St. Louis, Missouri, USA), and 1.5 mL sterile water.

Pupal hemolymph was collected from live *A. pernyi* pupae that were immersed in a water bath at 60 °C for 10 min to avoid melanization of the hemolymph. After surface sterilization of the pupae with 75% ethanol, the hemolymph was collected by pressing the pupae under sterile conditions. Neisenheimer's salt solution was prepared with 7.5 g NaCl, 0.1 g KCl, 0.2 g $CaCl_2$, 0.2 g $NaHCO_3$, and 1,000 mL distilled water. The solution was used after sterilization.

PREPARATION OF ARTIFICIAL EGG CARDS AND *A. PERNYI* EGG CARDS

For artificial egg cards, 20 semispherical domes (2–3 mm in diameter × 3 mm high) were produced by pressing a heated glass rod onto one half of a sheet (8 × 7 cm) of polyethylene and polypropylene copolymer film (30 µm thick) through a plastic semispherical mold. After the sheets were sterilized by UV irradiation, 4 µL medium was transferred to each dome using a pipette. The half of the sheet containing the domes (convex side) to be exposed to oviposition by the parasitoid was folded over the other half and sealed using a plastic sealer, so that the concave side and the bottom piece of film provided sufficient space to allow aeration for parasitoid development. Afterwards, the external surface of the egg cards was treated with 10% (wt/v) polyvinyl alcohol to stimulate oviposition (Lü et al. 2014). For *A. pernyi* egg cards, 20 eggs were attached to cardboard strips (2 × 2 cm) with 10% (wt/v) polyvinyl alcohol.

EXPOSURE OF EGG CARDS TO *T. DENDROLIMI* WASPS AND SAMPLE COLLECTION

For the 1st generation, artificial egg cards and *A. pernyi* egg cards were placed in plastic trays (30 × 20 × 5 cm) for exposure to *T. dendrolimi* adults for 24 h. Parasitoids of both sexes were released in the trays using a ratio of parasitoids to artificial eggs of 6:1. Sex ratios were approximately 8:1 (female: male) in all trays. Trays were tightly covered by paper to avoid escape of the parasitoids and placed in climatic incubators set at 27 ± 1 °C, 75 ± 5% RH, and a 16:8 h L:D photoperiod. After 24 h of exposure, the egg cards were taken out and the wasps were removed. Before the *T. dendrolimi* adult emergence began, each egg card was inserted into a glass tube (3 cm in diameter × 9 cm high), which was covered by a cotton cloth with a rubber band. When adults emerged, a new egg card was inserted into the glass tube for the next generation of in vitro and in vivo rearing. Adults of each generation from both cultures were collected on ice and maintained at −80 °C for biochemical assessments.

PREPARATION OF *T. DENDROLIMI* EXTRACT AND BIOCHEMICAL ANALYSES

For each sample, 0.0100 ± 0.0002 g of adults were accurately weighed and homogenized in either 1,000 µL pre-cooled phosphate buffered saline (PBS, 0.02 mol/L, pH 5.8) or in 1,000 µL ultrapure water on ice. The homogenate was centrifuged twice at 4 °C and 7,378 *g* for 10 min. Supernatants (2 mL) in PBS were maintained at −80 °C to analyze protein concentration, trehalose content, and trehalase activity.

Protein Concentration Determination

Protein concentrations were determined by the Bradford method (Bradford 1976) using bovine serum albumin (BSA) as a standard. Extracts (20 µL) were homogenized in 130 µL PBS. The homogenate was mixed with 150 µL Coomassie Brilliant Blue G-250 and measured at 595 nm after incubation for 8 min. Each treatment was performed in triplicate.

Lipid Content Determination

To determine the lipid content, 0.02 g (DM) *T. dendrolimi* adults were accurately weighed and added to a 2 mL mixture of chloroform

and methanol (chloroform: methanol = 2:1). The homogenate was centrifuged twice at 498 *g* for 20 min. The pellet was baked for 72 h at 60 °C to a constant weight (LDM). The lipid content was calculated as follows:

$$\text{Lipid content} = [(DM - LDM)/DM]$$

Trehalose Content Determination

Trehalose extracts were subjected to the reaction with anthrone (Sigma, St. Louis, Missouri, USA) and colorimetric measurement at 630 nm, according to the anthrone method (Brin 1966; Ferreira et al. 1997). Trehalose served as external standard for quantification. Each treatment was performed in triplicate.

Trehalase Activity Measurements

Trehalase activity was determined by the 3,5-dinitrosalicylic acid colorimetric method (DNS method) (Miller 1959; Wang 2004) with absorbance measured at 540 nm. One unit (U) of enzyme activity was defined as the amount of enzyme capable of releasing 1 mg of reducing sugar per minute. Each treatment was performed in triplicate.

STATISTICAL ANALYSES

Statistical contrasts were analyzed between the artificial medium (in vitro) and *A. pernyi* eggs (in vivo). All data were analyzed with SPSS 17.0 software (SPSS Inc. Chicago, Illinois, USA). Paired-sample *t*-tests were done to analyze differences between treated and control groups. In all experiments, differences among means were considered significant at $P \leq 0.05$.

Results

PROTEIN CONCENTRATION

In vitro and in vivo rearing of *T. dendrolimi* had an effect on the protein concentration in different generations (Fig. 1). Protein concentrations in adults of generations F1– F4 and F7– F10 reared in *A. pernyi* eggs (range: 81.17 ± 0.77 to 99.06 ± 0.70 mg/g) were significantly higher compared with those reared in artificial medium (range: 59.22 ± 0.11 to 87.21 ± 0.71 mg/g), whereas no differences existed in F5 and F6 (F1: $t = 44.47$, df = 2, $P = 0.001$; F2: $t = 61.11$, df = 2, $P = 0.001$; F3: $t = 31.87$, df = 2, $P = 0.001$; F4: $t = 10.05$, df = 2, $P = 0.010$; F5: $t = 0.17$, df = 2, $P = 0.880$; F6: $t = 3.33$, df = 2, $P = 0.079$; F7: $t = 28.33$, df = 2, $P = 0.001$; F8: $t = 24.47$, df = 2, $P = 0.002$; F9: $t = 43.76$, df = 2, $P = 0.001$; F10: $t = 13.12$, df = 2, $P = 0.006$).

LIPID CONTENT

Fig. 2 shows that the lipid content in the in vitro–reared adults (range: 0.74 ± 0.01 to 0.83 ± 0.14 g/g) was significantly higher than in the in vivo–reared adults (range: 0.62 ± 0.71 to 0.72 ± 0.13 g/g) except for generations F6 and F7 (F1: $t = 4.78$, df = 2, $P = 0.041$; F2: $t = 12.33$, df = 2, $P = 0.007$; F3: $t = 5.69$, df = 2, $P = 0.029$; F4: $t = 228.63$, df = 2, $P = 0.001$; F5: $t = 14.19$, df = 2, $P = 0.005$; F6: $t = 2.21$, df = 2, $P = 0.158$; F7: $t = 0.71$, df = 2, $P = 0.550$; F8: $t = 14.19$, df = 2, $P = 0.005$; F9: $t = 8.87$, df = 2, $P = 0.012$; F10: $t = 8.04$, df = 2, $P = 0.015$).

TREHALOSE CONTENT

Data concerning the content of trehalose are shown in Fig. 3. The parasitoid adults reared in vitro showed significantly higher values of trehalose than those reared in vivo. However, only slight differences were observed in F4 and F10 (F1: $t = 29.15$, df = 2, $P = 0.001$; F2: $t = 17.33$, df = 2, $P = 0.003$; F3: $t = 9.81$, df = 2, $P = 0.010$; F4: $t = 2.01$, df = 2, $P = 0.182$; F5: $t = 11.68$, df = 2, $P = 0.007$; F6: $t = 26.05$, df = 2, $P = 0.001$; F7: $t = 16.13$, df = 2, $P = 0.004$; F8: $t = 30.93$, df = 2, $P = 0.001$; F9: $t = 5.55$, df = 2, $P = 0.031$; F10: $t = 3.85$, df = 2, $P = 0.160$). Trehalose fluctuated with generation. For in vitro and in vivo rearing, the highest content was 4.5 times and 7 times higher, respectively, than the lowest content.

TREHALASE ACTIVITY

Trehalase activity in *T. dendrolimi* adults grown in vitro and in vivo were basically the same over 10 generations. Trehalase activity in generations F3, F4, F7, and F10 were significantly higher when wasps were reared in vitro compared with *A. pernyi* eggs; no differences occurred between in vitro and in vivo rearing of other generations (F1: $t = 0.52$, df = 2, $P = 0.658$; F2: $t = 0.09$, df = 2, $P = 0.934$; F3: $t = 4.30$, df = 2, $P = 0.050$; F4: $t = 10.30$, df = 2, $P = 0.009$; F5: $t = 2.66$, df = 2, $P = 0.117$; F6: $t = 3.06$, df = 2, $P = 0.092$; F7: $t = 14.44$, df = 2, $P = 0.005$; F8: $t = 4.04$, df = 2, $P = 0.056$; F9: $t = 3.31$, df = 2, $P = 0.080$; F10: $t = 10.04$, df = 2, $P = 0.010$) (Fig. 4).

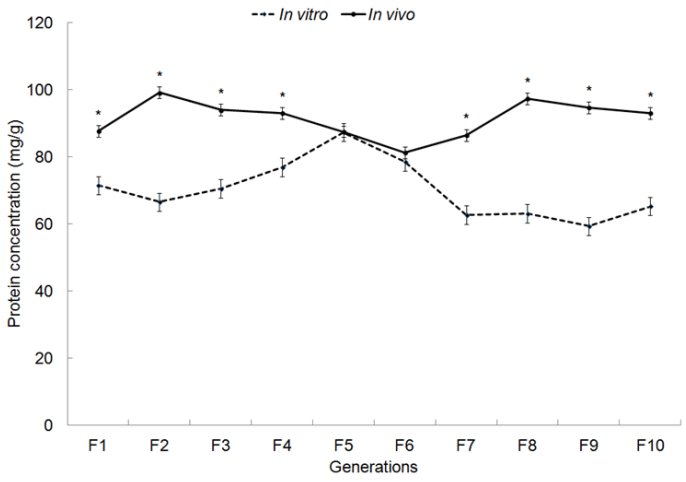

Fig. 1. Protein concentration in *Trichogramma dendrolimi* reared in vitro and in vivo for 10 generations. Means (± SE) were calculated from 3 replicates. Data with an asterisk differ significantly according to paired-sample *t*-tests at $P = 0.05$.

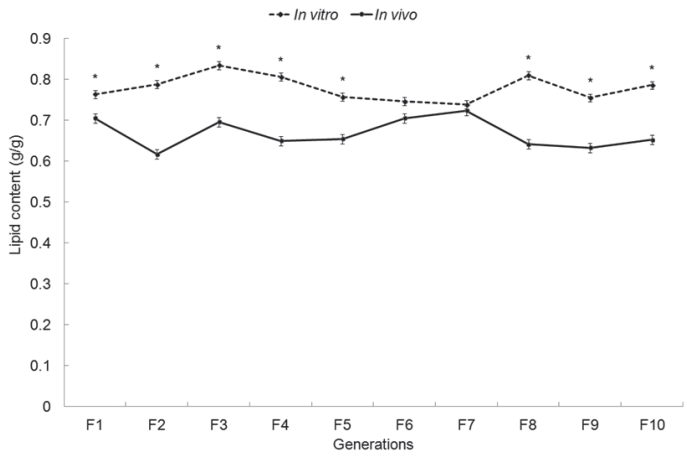

Fig. 2. Lipid content in *Trichogramma dendrolimi* reared in vitro and in vivo for 10 generations. Means (± SE) were calculated from 3 replicates. Data with an asterisk differ significantly according to paired-sample *t*-tests at $P = 0.05$.

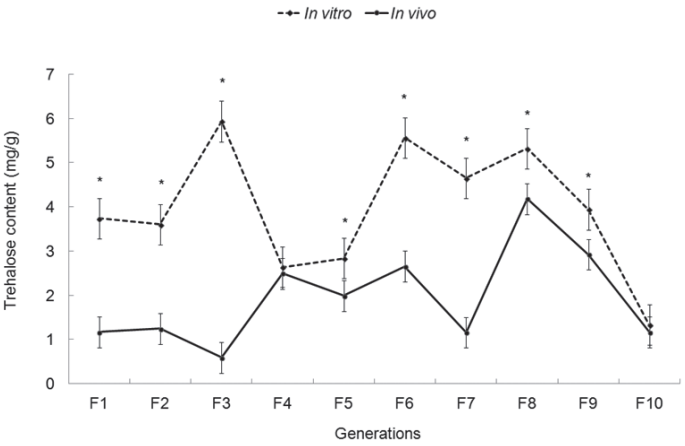

Fig. 3. Trehalose content in *Trichogramma dendrolimi* reared in vitro and in vivo for 10 generations. Means (± SE) were calculated from 3 replicates. Data with an asterisk differ significantly according to paired-sample *t*-tests at $P = 0.05$.

Discussion

It is important to consider not only the composition of food defining a medium (Grenier & Bonnot 1988) but also the composition of *Trichogramma* itself developed in natural host eggs (Barrett & Schmidt 1991). Media containing high amounts of nitrogen permit the development of *Trichogramma* to the adult stage more or less successfully. Protein, lipid, and carbohydrate contents and enzyme activities in *T. dedrolimi* adults successively reared in vitro and in vivo for 10 generations were analyzed in the present study. Our aim was to compare an artificial medium and a natural host for mass rearing *T. dendrolimi*.

Proteins are key constituents required by parasitoids. Grenier et al. (1995) found that total amino acid content in *T. dendrolimi* grown in vitro was increased and reached nearly the level of that in parasitoids grown in vivo when proteins were added to the medium. Free amino acid levels in *T. dendrolimi* grown in an artificial medium with hemolymph revealed an excess of some amino acids, notably serine (Grenier 1994). Pupal hemolymph of *A. pernyi*, egg yolk, and milk solution used in this study may provide adequate protein requirements for *T. dendrolimi*. In many insects, hemolymph functions as a storage reservoir and is characterized by a high level of free amino acids, often occurring in excess compared with protein synthesis (Chen 1985).

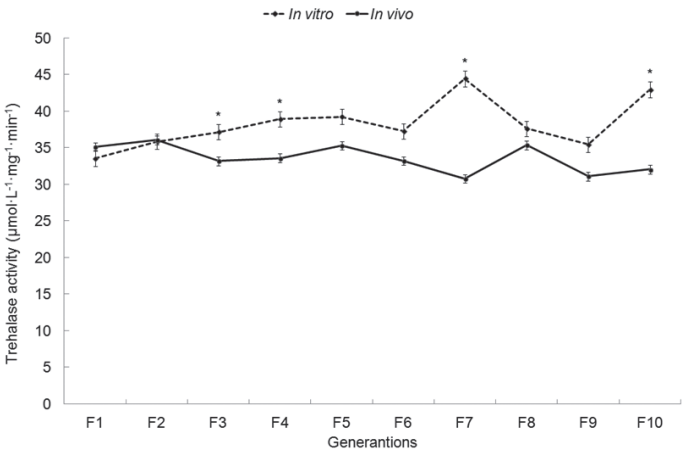

Fig. 4. Trehalase activity in *Trichogramma dendrolimi* reared in vitro and in vivo for 10 generations. Means (± SE) were calculated from 3 replicates. Data with an asterisk differ significantly according to paired-sample *t*-tests at $P = 0.05$.

Protein concentration of in vitro–reared adults was similar to the level observed among in vivo–reared adults of generations F5 and F6. The result showed that the in vitro–reared parasitoid may accumulate protein during the first few generations when grown in new media or hosts. Although the protein content in generations F5 and F6 reached the in vivo level, parasitoids continuously reared in artificial medium contained less protein compared with those reared in the natural host.

Lipid and trehalose (carbohydrates) are energy sources and serve as cold-protective substances, which are very important for overwintering and diapause (Lee 1991). *Exeristes roborator* (F.) (Hymenoptera: Ichneumonidae) adults reared in vitro contained half the total lipids of those reared in the host insect (Thompson & Johnson 1978), whereas the parasitoid *Phryxe caudata* (Rondani) (Diptera: Tachinidae) contained similar fatty acid concentrations when reared in vitro and in vivo (Grenier & De Clercq 2003). There were no differences between the nutritional components of parasitoids reared in the in vivo or in vitro diets at generations F6 and F7. This result indicated that there may have been autoregulation when *T. dendrolimi* was reared in different hosts. Overall, our results indicated that lipids and fatty acids had higher concentrations in the artificial medium than in *A. pernyi* eggs. Egg yolk frequently incorporated into artificial media for egg parasitoids provides well-emulsified concentrations of fatty acids, cholesterol, and lecithin (Grenier 2012).

Trehalose has the potential to partially replace insect hemolymph and was proven to be the most important ingredient affecting the whole developmental process of *T. dendrolimi* (Lü et al. 2013, 2014). In the tested medium, trehalose may act as a protein stabilizer and a component of a feedback mechanism regulating feeding behavior and nutrient intake (Thompson 2003), and may serve as stress protectant (Wingler 2002; Elbein et al. 2003) to influence many physiological processes. Variation of the trehalose content in the 2 media indicated that metabolism of *T. dendrolimi* is not the same in different hosts and among different generations. The parasitoid may need higher energy to adapt to artificial medium in the first few generations. Metabolism seems more active when the parasitoid is reared in *A. pernyi* eggs.

Trehalase is a specific enzyme in glucose metabolism of insects. It is involved in the chitin synthesis pathway and mainly hydrolyzes trehalose into two molecules of glucose to provide energy. Although trehalose content in artificial medium was significantly higher than in the natural host, the trehalase activity in *T. dendrolimi* adults grown in vitro and in vivo was similar. The results showed that the effect of enzyme activity on the parasitoid was not obvious when wasps were reared in vitro and in vivo and may further explain the adaptability of *T. dendrolimi* to the artificial medium.

To establish the exact composition of the food, it is necessary to take into account intermediate metabolism; the existence of catabolism and inter-conversion metabolism makes it difficult to identify the exact form (monomers or polymers) of each component to incorporate into the food (Grenier 2012). Changes in biochemical parameters in this study showed different trends between in vitro and in vivo rearing during 10 generations. Different biochemical conversions may have been caused by imbalances in the main nutrient classes (proteins, lipids, and carbohydrates) in the artificial medium. Furthermore, it is known that parasitoids reared in different hosts express different metabolism patterns. Wu et al. (1978) found commonalities between the biochemical composition of different hosts, which may explain why *Trichogramma* could grow in hundreds of host species. At the same time, the detected differences in biochemical composition among these species' eggs suggested that the parasitoid can tolerate some variation in nutrient composition. Of the synthetic insect media, only those with well-adjusted proportions of the essential nutrients could support normal growth in many insects (Dadd 1985). Therefore, it is

necessary to consider the proportion of each ingredient and the physicochemical properties of the artificial medium rather than the precise content of each component.

In conclusion, based on the biochemical parameters examined, our study indicates that the artificial medium for *T. dendrolimi* in vitro rearing could be improved by altering its composition, especially in protein concentration and lipid content. Biological parameters should be assessed in the future to develop an optimal artificial medium for *T. dendrolimi*.

Acknowledgments

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