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Original article (Orijinal araştırma)

Biochemical changes in hemolymph of spinning and non-spinning silkworm larvae, *Bombyx mori* (L., 1758) (Lepidoptera: Bombycidae), reared on fresh mulberry leaves: possible reasons for non-spinning syndrome

Taze dut yapraklarında yetiştirilen koza ören ve koza öremeyen ipekböceği larvalarının *Bombyx mori* (L., 1758) (Lepidoptera: Bombycidae) hemolenfinde biyokimyasal değişimler: koza örememe sendromunun olası nedenleri

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Abstract

Non-spinning syndrome in *Bombyx mori* (L., 1758) (Lepidoptera: Bombycidae) is a serious issue for the sericulture industry. Determination of urea metabolism as an important parameter at the onset of spinning has shown the need for examining the role of urea metabolism in the non-spinning syndrome. The aim of this study was to investigate role of urea metabolism in the non-spinning syndrome by evaluating urease activity and L-arginine concentrations in the silkworm hemolymph and mulberry leaves. Additionally, urea concentrations were determined in hemolymph samples. Urease activities in hemolymph samples were almost twice as high in spinning larvae (SL) than in non-spinning larvae, 25 ± 5.8 vs 10.9 ± 2.4 units/l ($P < 0.05$). Urea concentrations in the SL hemolymph decreased significantly from day 5 (137 ± 13 mg/l) to day 7 (97 ± 17 mg/l) of the fifth instar ($P < 0.01$), it remained almost constant in NSL hemolymph (149 ± 19 to 167 ± 4 mg/l). Additionally, L-arginine concentrations in hemolymph samples obtained from NSL of 4.55 ± 0.48 mM were significantly higher than in SL at 2.72 ± 0.45 mM ($P < 0.01$). Changes in urease activity and L-arginine concentrations in hemolymph were similarly observed in mulberry leaves. These results suggested that changes in urea metabolism may cause or contribute to non-spinning syndrome in silkworms.

Keywords: L-arginine, non-spinning syndrome, silkworm, urea, urease

Öz

Bombyx mori (L., 1758) (Lepidoptera: Bombycidae)'de görülen koza örememe sendromu, ipekböcekçiliği sektöründe ciddi bir sorun olarak görülmektedir. Üre metabolizmasının koza örme başlangıcında önemli olması, bu metabolizmanın koza örememe sendromunda olası rolünün incelenmesi gereğini doğurmuştur. Bu çalışmanın amacı, ipekböceği hemolenfinde ve dut yapraklarında üreaz aktivitesi ve L-arginin konsantrasyonlarını saptayarak, koza örememe sendromunda üre metabolizmasının rolünü araştırmaktır. Bu parametreler ek olarak hemolenf örneklerinde üre konsantrasyonları da ölçülmüştür. Koza ören grubun hemolenfinde üreaz aktivitesi (25 ± 5,8 units/l), koza örmeyen gruba (10,9 ± 2,4 units/l) göre iki kat daha yüksek bulunmuştur ($P < 0.05$). Koza ören larvaların hemolenfinde üre konsantrasyonu 5. dönemin 5. gününden (137 ± 13 mg/l) 7. gününe önemli seviyede azalırken (97 ± 17 mg/l; $P < 0.01$), koza öremeyen larvalarda hemen hemen sabit kalmıştır (149 ± 19 mg/l'den 166 ± 4 mg/l'ye). Koza öremeyen larvaların hemolenf örneklerinde L-arginin konsantrasyonu (4.55 ± 0.48 mM), koza ören larva grubundan anlamlı ölçüde yüksek bulunmuştur (2.72 ± 0.45 mM; $P < 0.01$). Hemolenfteki üreaz aktivitesi ve L-arginin konsantrasyonlarında gözlemlenen değişiklikler benzer şekilde dut yapraklarında da saptanmıştır. Bu sonuçlar, üre metabolizmasındaki olası bir baskılanmanın ipekböceklerinde görülen koza örememe sendromuna katkıda bulunabileceği ve/veya bunun sonucu olabileceğini düşündürmektedir.

Anahtar sözcükler: L-arginin, koza örememe sendromu, ipekböceği, üre, üreaz

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Introduction

Silkworms, *Bombyx mori* (L., 1758) (Lepidoptera: Bombycidae), are most common monophagous insect in human domestication and the sericulture industry is an important economic resource in the world (Savithri et al., 2013). Sericulture has experienced serious problems with non-spinning syndrome in *B. mori* in sericulture practicing countries affecting silkworm rearing and silk production. Although cocoon and raw silk production have continued to be historically, traditionally and culturally practiced in Turkey (Sahan, 2011), recently, both cocoon and silkworm egg production have faced this problem in different regions of Turkey like other countries (Monconduit & Mauchamp 1998; Leonardi et al., 2001; Sun et al., 2012; Sahan et al., 2019). Therefore, it is important to determine the cause of and find solutions to this syndrome in silkworms.

Detailed examination of the developmental stages of silkworms for determination of the cause of non-spinning syndrome have shown that biochemical composition of silkworm hemolymph is an important factor for silkworm development and growth (Zhou et al., 2015; Shen et al., 2016; Dong et al., 2017). Several studies found that the change of urea concentration and urease activity in the hemolymph in the fifth instar is associated with the onset of spinning (Hirayama et al., 1997, 2000a). Urease hydrolysis urea to carbon dioxide and ammonia which is the main nitrogen source for silk protein synthesis in the spinning larvae (Hirayama et al., 1997; Sirko & Brodzik, 2000). Silkworms are host plant-dependent insect as it cannot synthesize urease (Hirayama et al., 1997, 2000a). In addition to protein, amino acids and other necessary substances, silkworms obtain urease from mulberry leaves (Jyothi et al., 2014). While mulberry leaves are external sources for changing urea level in hemolymph, L-arginine is the main amino acid for production of the urea internally in the insect body (Cochran, 1985; Mobley et al., 1995). In light of this information, it is possible to hypothesize that changes urea and urease concentrations may cause the non-spinning syndrome. However, there has been no studies on the relationship between these parameters and non-spinning syndrome.

In our laboratories, we observed that some silkworm larvae cannot spin their cocoons and complete their life cycle. Our prefeeding trials conducted with the leaves obtained from different regions showed that larvae could not spin their cocoons based on the source of mulberry leaves used for feeding. Given that the metabolite composition of silkworm hemolymph is highly influenced by nutrient composition of the mulberry leaves, it seems likely that mulberry leaves used to feed silkworms probably caused this problem.

The main aim of this study was to analyze biochemical parameters that are important for onset of spinning including urea concentrations in the hemolymph of spinning (those fed on mulberry leaves obtained from Örencik Village) and non-spinning (those fed on mulberry leaves obtained from Hürriyet Campus) silkworm larvae. Additionally, L-arginine concentrations and urease activities in each spinning and non-spinning larvae and mulberry leaves obtained from the same regions were measured to detect relationship of non-spinning syndrome and urea metabolism.

Materials and Methods

This study was conducted between April and December 2018 in Kozabirlik (Cocoon Cooperatives Union) silkworm breeding laboratory on the campus of Bursa Directorate of Provincial Agriculture and Forestry, which has a large mulberry planting of about 3 ha.

Materials

Monovoltin hybrid *B. mori* larvae were used for this experiment. Hybrid silkworm eggs (1 g = 2000 eggs) of (M-Chinese X N-Japan) were obtained from Kozabirlik and were incubated at 25-28ºC and 80-85% RH for 11 days.

Silkworm rearing

Newly hatched larvae were separated randomly into two groups and brushed with a feather into wooden rearing trays (70 x 100 cm). At the bottom and for covering of tray, paraffin papers were used to avoid humidity loss during rearing. Silkworm larvae were fed with fresh mulberry leaves under a 12:12 h L:D photoperiod. Mulberry leaves were obtained two different areas. The first group larvae were fed with mulberry leaves obtained from Kozabirlik Campus where larvae had previously been determined to not spin cocoons. The second group of larvae were fed with mulberry leaves collected from a silkworm producer in Örencik Village where it had previously been determined that silkworms could spin cocoons. This study examined the fifth instar of *B. mori* silkworm larvae which lasts about 8 days. Silkworm larvae were reared at optimum rearing conditions for each instar. During the first instar, silkworm larvae were reared at 28ºC and 85-90% RH. After this period, temperature and RH were decreased by 1ºC and 5%, respectively for in each instar. Silkworm larvae were fed three times each day during the first three instars, and four and five times at the fourth and fifth instars. Paraffin paper was used during the first three instars except molting so as to prevent withering leaf and to maintain humidity. The rearing trays were cleaned at the end of each instar with unconsumed leaves, excreta and dead larvae removed (Krishnaswami et al., 1973).

Collection of the hemolymphs

For the determination of urease activity on day 8 of the fifth instar (just before spinning stage) hemolymph samples of silkworm larvae were pooled into three eppendorf tubes containing for 5-7 samples per tube. To determine the urea concentration of hemolymph on days 5 and 7 of the fifth instar, hemolymph samples were collected into the eppendorf tubes as above. Hemolymph samples were collected by making an incision through one of the prologs and transferred to the eppendorf tubes containing 4 µL of thiourea (0.2 M) to prevent the sample blackening. The samples were then centrifuged (Allsheng Mini10K Mini Centrifuge, Hangzhou, Zhejiang, China) for 10 min in 10,000 rpm and upper phase was transferred to new tubes, and stored at -20ºC until analysis of urease activity and urea level.

Determination of the urease activity and urea in hemolymphs samples

Urease activities of the samples were assayed calorimetrically (Mannheim Boehringer Photometer 4010, Roche Diagnostics, Rotkreuz, Switzerland) in the laboratory of the Department of Pharmacology in Faculty of Medicine of using a commercial kit (Sigma-Aldrich Chemicals, St. Louis, MI, USA) as indicated in user guide. Urease activities of the samples were expressed as enzyme units per unit volume. Urea concentrations of the samples were determined by a spectrophotometric (Jasco FP-750 spectrophotometer, Easton, MD, USA) method provided by a central laboratory of Uludağ University Faculty of Medicine as weight of urea N per unit volume of hemolymph.

Determination of urease activity and L-arginine concentrations in mulberry leaves

For determination of urease activity in mulberry leaves, they were washed with distilled water and then dried with paper towel. About 5 mg of leaf pieces were homogenized in phosphate buffer (0.1 M, pH 7.5). After centrifugation (Allsheng Mini10K Mini Centrifuge) at 12 000 rpm for 10 min, supernatants were used for determination of urease activity with the same kit as used for hemolymph samples.

L-arginine concentrations were measured in dried and powdered samples. Specifically, prewashed mulberry leaves were dried under 80ºC for 24 h and then were powdered in a glass mortar. About 100 mg of dried samples were transferred into the glass flasks containing 100 ml of 0.1 N HCl. The flasks were placed on a shaker and L-arginine was extracted for 12 h. At the end of extraction period, 1 ml of each samples were taken into the eppendorf tubes and centrifuged (Allsheng Mini10K Mini Centrifuge) at 12 000 rpm for 10 min. Upper phases (50 µl) were derivatized and then analyzed for L-arginine with a HPLC system. For determination of L-arginine in hemolymphs samples, samples (200 µl) were first acidified with $HClO_4$ (final concentration of 0.4 N), vortexed (BioSan Vortex, Riga, Latvia), then centrifuged at 12 000 rpm for 15 min and upper phase (50 µl) were derivatized before HPLC analysis.

L-arginine in mulberry and hemolymphs samples were determined by a high-performance liquid chromatography (HPLC) after derivatization with diethyl ethoxymethylenemalonate (DEEMM) as indicated by Megias et al. (2015). Specifically, 3 ml of borated buffer (1 M, pH 9.0, containing 2 µl of DEEMM) was added into the standard and samples. After mixing with vortex, all samples were incubated at 50ºC for 50 min. Samples were centrifuged at 12 000 rpm and then injected (50 µl) into the HPLC system. HPLC system (HP 1100 series, Hewlett-Packard, Palo Alto, CA, USA) consisted of a quaternary pump (HP-G1311A, Hewlett-Packard, Palo Alto, CA, USA), a UV detector (1049A, Hewlett-Packard) and a solvent module (G1322A, Hewlett-Packard, Palo Alto). L-arginine was separated on a reversed phase C18 column (Macherey-Nagel GmbH, Duren, Germany) with mobile phase A (25 mM glacial acetic acid in water) and mobile phase B (acetonitrile). During the first 3 min of elution, the ratio of the A mobile phase was 96%, which was decreased to 69% by the end of the chromatogram (model PU-980 liquid chromatography pump; Jasco). Column was maintained at room temperature and flow rate was adjusted to 0.9 ml/min. Chromatograms were detected at 280 nm and L-arginine concentrations were calculated by comparing peak heights of the samples with L-arginine standards processed together with the samples.

Statistical Analysis

The data obtained were analyzed using the GraphPad software, version 8.0 (GraphPad Software, Inc., La Jolla, CA, USA) and expressed as the mean ± SEM Statistical analyses were performed using the Student's t test. $P < 0.05$ was considered significant.

Results

Urease activity in the hemolymph samples was 24.9 ± 5.8 units/l in spinning larvae (those fed with mulberry leaves from Örencik Village) and 10.9 ± 2.4 units/l ($t = 3.36$, $df = 4$, $P < 0.05$) in non-spinning larvae (those fed with mulberry leaves from Hürriyet Campus). Urease activity in mulberry leaves were 4.0 ± 0.3 units/g, and 2.1 ± 0.9 units/g ($t = 3.26$, $df = 4$, $P < 0.05$) in samples from Örencik Village and Hürriyet Campus, respectively. Thus, as shown in Figure 1, urease activities in both hemolymph samples and mulberry leaves fed for spinning larvae are almost two times higher than the non-spinning larvae and mulberry leaves used.

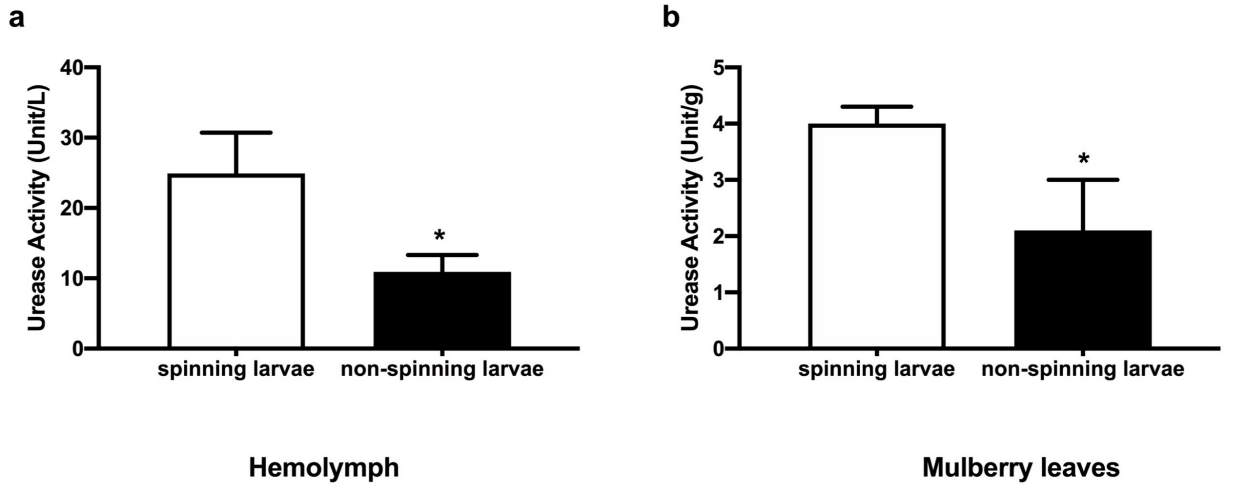

Figure 1. Urease activities in hemolymph samples of *Bombyx mori* (a) and mulberry leaves (b) for spinning larvae (fed with mulberry leaves from Örencik Village) and non-spinning larvae (fed with mulberry leaves from Hürriyet Campus). *P < 0.05, significantly different from spinning larvae group.

It was also observed that spinning larvae started to spin their cocoons on day 8 of the fifth instar (Figure 2a), however, non-spinning larvae became dauer larva and lay down without spinning their cocoon (Figure 2b). In addition, in non-spinning larvae, the fifth instar was prolonged to 18 days and they turned into darker larvae and died without spinning their cocoons (Figure 2c).

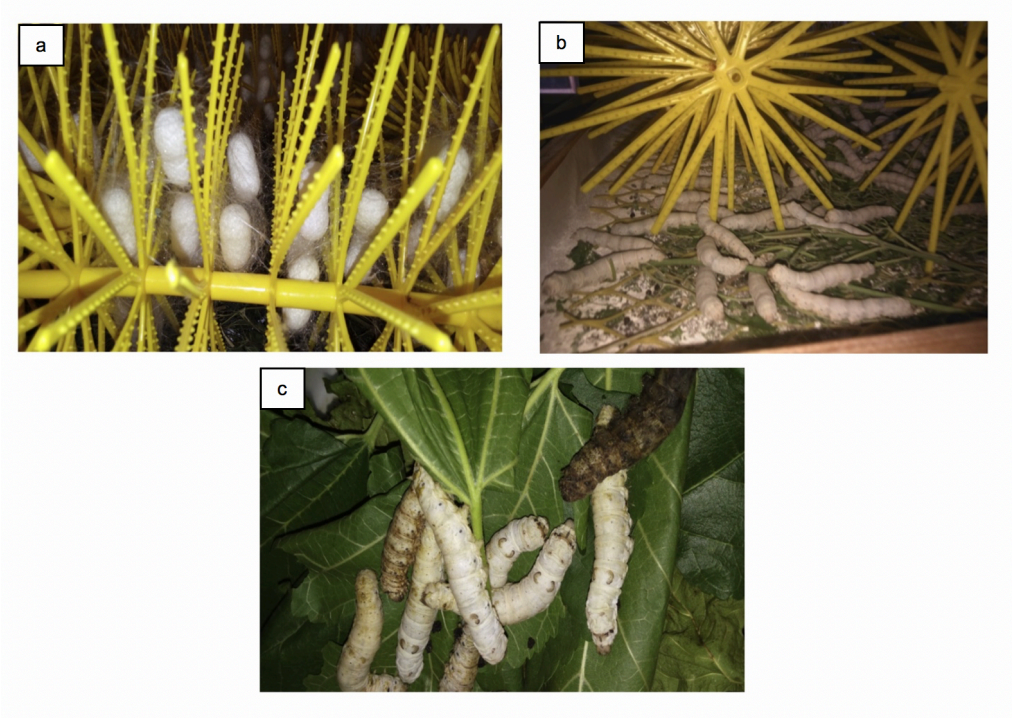

Figure 2. The picture of spinning larvae (a) on day 8 of the fifth instar and non-spinning larvae on day 9 (b) and 15 (c) of the fifth instar of *Bombyx mori.*

Changes in the urea concentrations on days 5 and 7 of the fifth instar in hemolymph samples taken from the non-spinning and spinning larvae were given in Figure 3. Urea concentrations in the spinning larvae hemolymph decreased to 97 ± 17 mg/l on day 7 of the fifth instar from its day 5 value 137 ± 13 mg/l ($t = 3.95$, $df = 12$, $P < 0.01$). Urea concentrations in non-spinning larvae hemolymph, however, did not decline as seen in spinning larvae, but showed a tendency to increase during the same period (from 149 ± 19 mg/dL to 166 ± 4 mg/l).

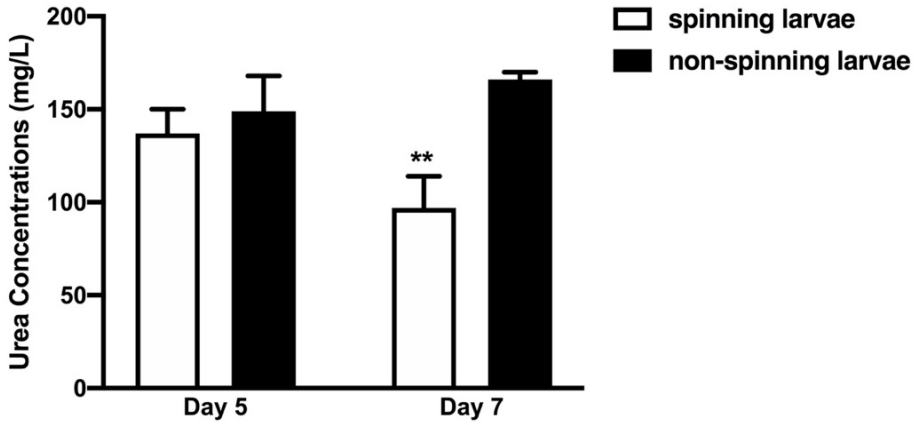

Figure 3. Urea concentrations in hemolymphs samples from spinning and non-spinning larvae of *Bombyx mori* on days 5 and 7 of the fifth instar. **P < 0.01, significantly different from spinning larvae group on day 5.

L-Arginine concentrations in mulberry leaves and hemolymph samples are presented in Figure 4. L-arginine concentrations in mulberry leaves from Hürriyet Campus fed to non-spinning larvae had significantly higher concentrations than mulberry leaves fed to spinning larvae (788 ± 59 nmol/g vs 129 ± 41 nmol/g; t = 9.17, df = 12, P < 0.01). L-Arginine concentrations in the hemolymph were almost twice as high in non-spinning than spinning larvae (4.55 ± 0.48l vs 2.72 ± 0.45 mM; t = 2.80, df = 12, P < 0.01).

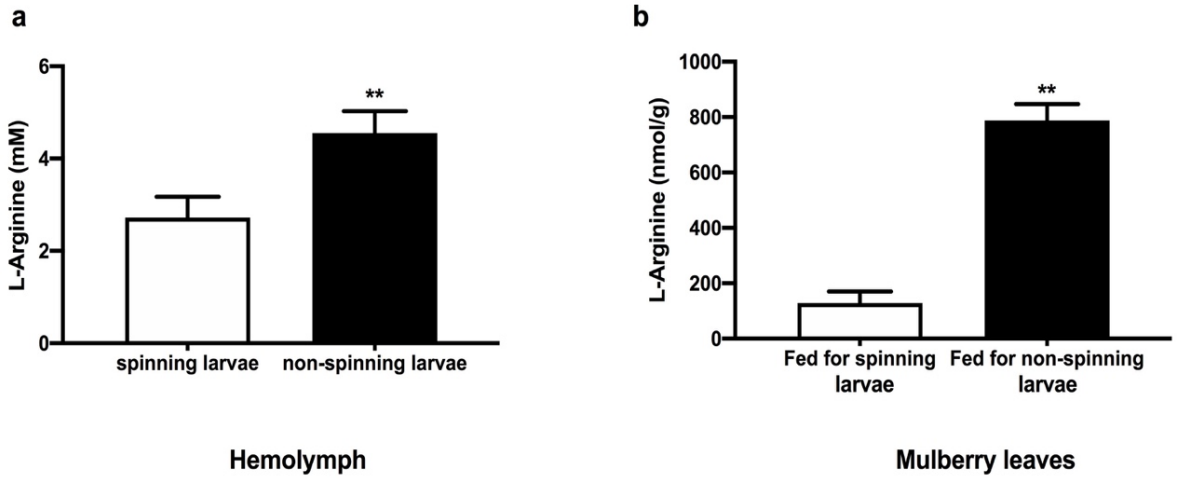

Figure 4. L-Arginine concentrations in a) hemolymphs samples at spinning and non-spinning larvae of *Bombyx mori* and b) mulberry leaves used to feed spinning (Örencik Village) and non-spinning (Hürriyet Campus) larvae. **P < 0.01, significantly different from spinning larvae group.

Discussion

Non-spinning syndrome threatens not only the production of breeding cocoons and eggs, but also affects commercial cocoon production in Turkey and other countries such as Iran, China and Brazil (Monconduit & Mauchamp, 1998; Leonardi et al., 2001; Sun et al., 2012; Sahan et al., 2019). Additionally, both native and foreign sourced silkworm lines are under great risk because of this syndrome. Exact mechanism of the non-spinning syndrome is not known, but many factors seem to be involved in both egg and commercial cocoon crops. Non-synchronous spinning movement and solidification of the silk, very high temperatures in the spinning room, improper handling of cocoons during molting and industrial pollution can cause the non-spinning syndrome. It has been demonstrated that some of these factors change biochemical composition of hemolymph in different developmental stages of silkworms (Etebari et al., 2007; Malik & Malik, 2009).

Several studies have revealed changes in amino acid, lipid and some enzyme concentrations in spinning and non-spinning larvae (Etebari et al., 2007; Zhou et al., 2015), but there has been relatively work on urea metabolism. The sharp change in urea concentrations and urease activity at the onset of spinning indicates the need for investigating the role of urea metabolism in non-spinning syndrome. Thus, our study was mainly focused on urea and L-arginine concentrations and urease activities in hemolymph samples obtained from non-spinning and spinning larvae. Given that *B. mori* uses mulberry leaves as a source of urease and L-arginine, we also determined urease activities and L-arginine concentrations in mulberry leaves fed to these larvae.

It is known that urease of mulberry leaves is involved in urea hydrolysis, and nitrogenous molecules occurred from this hydrolysis are used for production of silk proteins and to complete the insects's life cycle (Kurahashi et al., 2005). Additionally, transport of the mulberry leaf urease from the midgut into the hemolymph is selective and larval-stage specific. When hemolymph is collected just before the spinning stage, no urease activity could be determined even if the larvae were fed on mulberry leaves (Hirayama et

al., 2000b; Sugimura et al., 2001). In present study, we observed that urease activity in hemolymph of the spinning larvae is almost twice as high as in the hemolymph of non-spinning larvae. To our knowledge, this remarkable phenomenon is reported here for the first time, and this significant decline in urease activity in hemolymph of non-spinning larvae may be a critical factor in non-spinning syndrome. In addition to hemolymph samples, we also measured urease activities in mulberry leaves. As shown in Figure 1b, urease activity in mulberry leaves fed to non-spinning larvae was significantly lower than in the leaves fed to spinning larvae, suggesting that low urease activity in non-spinning larvae hemolymph may be cause by the mulberry leaves they consume.

It has been shown that hemolymph of silkworm larvae reared on mulberry leaves contains a considerable quantity of urea (Yamada et al., 1983; Sumida et al., 1990). Urea concentration in the hemolymph increases until days 4 and 5 of the fifth instar, but then decreases sharply to day 7, probably as a result of the sufficient sequestration of urease derived from the ingested mulberry leaves (Sumida et al., 1993). Consistent with these findings, we determined that urea concentrations in spinning larvae hemolymph decreased significantly between days 5 and 7 of the fifth instar. In contrast, urea concentrations in non-spinning larvae hemolymph did not decline but rather increased during the same period, supporting the conclusions being drawn about the reduced urease activity in non-spinning larvae. Consistently, it has been previously reported that high urea concentration in hemolymph of the non-spinning larvae is probably a result of the insufficient transport of leaf urease into the silkworm hemolymph (Sumida et al., 1995).

In the present study we also observed that L-arginine concentrations were significantly higher in both non-spinning larvae hemolymph and the mulberry leaves the consumed (Figure 4). L-arginine is known as a main source of the urea in silkworms. Given that the nitrogen released by urease is used for silk production, this increase in L-arginine concentrations could be considered as an advantage for silk formation (Chakrabarty & Kaliwal, 2012), if there was no decline in urease activity. We did not know if the high L-arginine present in non-spinning larvae comes from the high L-arginine in mulberry leaves or why these mulberry leaves contain higher L-arginine; this needs to be determined by additional studies. It is noteworthy that L-arginine also has the ability to inhibit urease. Due to this property of L-arginine, urease-based ion-selective field effect transistors biosensor for arginine determination have been developed (Sheliakina et al., 2014). Indeed, we determined that L-arginine can inhibit urease in a concentration dependent manner (data not shown; the lowest concentration of L-arginine tested was 10 μM and caused a 20% decline in soybean urease activity).

In summary, based on the data presented, it is suggested that biochemical alterations, such as a decrease in urease activity and increase in L-arginine concentrations in both hemolymph and mulberry leaves, and increase in urea in hemolymph may cause or contribute to non-spinning syndrome in silkworms. Although the reason for this change in the urea metabolism of silkworm larvae may originate from the leaves they consume, there are no studies in the literature on this possible reason for the change in the urease activity of mulberry leaves. Further research needs to be done to investigate these reasons.

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