Environmental and hormonal control of body-color polyphenism in *Patanga japonica* (Orthoptera, Acrididae): Effects of substrate color, crowding, temperature and (His⁷)-corazonin injection

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Abstract

Patanga japonica (Bolívar) shows various body colors in the field. Most nymphs are green in the summer, but some develop non-green colors, such as yellow, white, brown, reddish, and black, in the fall. Nymphs individually reared in white, yellow-green, and black containers showed green, light-green, white, and reddish body colors, and the substrate color significantly influenced the proportions of green nymphs. A few individuals developed black spots and patterns, and such individuals were most frequently observed in the black containers. Nymphs with distinct black patterns were observed when reared in a group of five individuals per container, and the proportion of such individuals varied slightly depending on the brightness of the substrate color. Singly kept nymphs that were allowed to see five other nymphs in another container turned darker than those that were only allowed to see an empty container, suggesting that visual stimuli without mechanical stimulation induced black patterns. In outdoor cages, nymphs tended to develop more pronounced black patterns during their last instar when the hatching date was delayed and the temperature during the later stages of development was decreased. The effect of temperature during the late stadia was tested by transferring a group of third-stadium nymphs from outdoor cool conditions to a high temperature, while other nymphs were continuously maintained outdoors. Markedly melanized individuals were observed in the outdoor cage, whereas the appearance of such individuals was strongly suppressed at a high temperature. Green nymphs injected with synthetic [His7]-corazonin developed black patterns after ecdysis to the following instars and to the adult stage, and some looked indistinguishable in body color from group-reared nymphs. Nymphs injected with this hormone developed black patterns even at a high temperature. Adults looked similar in body coloration with some variation. Their hindwings turned reddish after overwintering. These results demonstrate that P. japonica exhibits body-color pholyphenism.

Keywords

darkening, green-brown polyphenism, homochoromy, phase polyphenism, temperature-dependent darkening

Introduction

Body-color variation is widespread in the Orthoptera (Ichikawa et al. 2006, Murai and Ito 2011). This is particularly common in grasshoppers and locusts (Rowell 1971, Pener 1991, Pener and Simpson 2009). Body color is determined genetically in some species and mainly determined environmentally in other species, referred to as polymorphism and polyphenism, respectively (Dingle 1996). For example, the steppe grasshopper Chorthippus dorsatus (Zetterstedt, 1821) shows body-color polymorphism in which body color is genetically controlled, and three autosomal loci are involved in the control of body color in different parts of the body (Winter et al. 2021). In contrast, the desert locust Schistocerca gregaria (Forskål, 1775) exhibits body-color polyphenism, and nymphal body color is influenced by various factors including temperature, crowding, and substrate color (Husain and Mathur 1936, Hunter-Jones 1958, Ellis 1964, Lester et al. 2005, Tanaka et al. 2012, 2016a, Tanaka and Nishide 2012).

Three kinds of body-color polyphenism are known in acridid species: phase-color polyphenism, green-brown color polyphenism, and homochromy (Faure 1932, Rowell 1971, Pener 1991). Nymphs at a high population density exhibit phase-color polyphenism in which they develop black patterns or dark colors under crowded conditions. Nymphs growing at a low population density often show green-brown color polyphenism; green morphs are green or light green in color, whereas brown morphs are brown or other non-green colors (Pener 1991). In some locusts, such as the desert locust *Schistocerca gregaria* (Forskål. 1775) the migratory locust *Locusta migratoria* (Linnaeus, 1758), and the grasshopper *Gastrimargus determinatus* (Walker, 1871), nymphs at a low population density develop various body colors depending on the substrate color of their habitat, which is the third type of polyphenism: homochromy (Faure 1932, Rowell 1970, Pener

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1991, Pener and Simpson 2009, Tanaka et al. 2016b). Homochromic coloration is an adaption that allows the insect to match the substrate color of the surrounding habitat.

Two major hormones—juvenile hormone (JH) and [His⁷]corazonin (CRZ)—have been suggested to control various body colors in some acridid species (Pener 1991, Pener and Yerushalmi 1998, Tanaka 2001, 2006, Tanaka et al. 2016b, Pener and Simpson 2009). JH induces green and yellow colors in several species (Joly and Joly 1954, Joly and Meyer 1970, Hasegawa and Tanaka 1994, Tanaka 2004b, Sugahara and Tanaka 2018, 2019), whereas CRZ induces black or various dark colors (Tawfik et al. 1999, Tanaka 2000, 2004b, Yerushalmi and Pener 2001, Sugahara et al. 2015, 2016).

Patanga (also known as Nomadacris) japonica (Bolívar, 1898) is one of the largest grasshoppers occurring in the Asian countries of India, Vietnam, China, Korea, Taiwan, and Japan (Ichikawa et al. 2006, Murai and Ito 2011, Cigliano et al. 2022). However, few studies have reported on the specie's body color variation or the mechanisms controlling this variation. Tanaka and Okuda (1996) noted that crowding causes nymphs of this grasshopper to develop black color on the head, wing pads, and legs. However, no detailed information is available about the environmental and hormonal control of body color variation in this grasshopper.

In a preliminary study investigating the body color of P. japonica in the field, we observed various body colors. This observation led us to explore the factors controlling the body-color variation in this grasshopper. We first examined the effects of substrate color on nymphal body color by rearing nymphs individually in containers lined with paper of different colors. As mentioned earlier, nymphs of this grasshopper turned darker when reared under crowded conditions (Tanaka and Okuda 1996). We performed an experiment to confirm this phenomenon and further tested the role of visual stimuli in inducing black patterns. By rearing nymphs in a group outdoors, seasonal variation in black patterns was observed, and the effect of temperature on this variation was tested. Finally, we examined the role of [His7]-corazonin in inducing black patterns in nymphs and adults of this grasshopper by injecting the hormone into green nymphs. In this paper, we describe our results and compare them with those from similar studies of other species.

Materials and methods

Insect.—*P. japonica* ranges from tropical to temperate regions in Asia (Cigliano et al. 2022). It has a univoltine life cycle and overwinters as an adult. In central Japan, nymphs molt six or seven times before the adult stage (Tanaka 2023). In this study, we use 'stadium' to describe the nymphal stages counted from hatching and 'instar' to describe the penultimate or last nymphal stages that can be identified by the characteristic wing pads (Tanaka 2023). Therefore, in the field census described below, last instar nymphs could be at the sixth or seventh stadium. Adults were reared in pairs of a female and a male, allowed to lay egg pods in moist sand, and eggs hatched at 30°C according to the method of Tanaka (2023).

Variation in body color in the field.—The number of *P. japonica* nymphs and their body color were recorded weekly in a grassy area in Tsukuba, Ibaraki, Japan (36.1°N, 140.1°E) from July 29 to October 28 in 2021. As already reported (Tanaka 2023), one of the purposes of this weekly census was to observe the seasonal changes in nymphal development at the study site that was approximately 200 m long and 2 m wide. *P. japonica* hatchlings started appearing in early July and emerging as adults in mid-September. More than 50% emerged as adults by mid-October. In the present study, data on

seasonal changes in body color and number of nymphs were based on the same census previously published (Tanaka 2023). Although some nymphs occurred until late November, few individuals were observed at the study site; this was partly because the grass was cut short by the government of Tsukuba City on October 30, causing most grasshoppers to disappear. Therefore, the present study presents data on variations in nymphal body color from July 29 to October 28. Variations in body color were also recorded by photographing nymphs and adults. As commonly observed in other grasshoppers and locusts (Pener 1991), *P. japonica* shows green-brown polymorphism or polyphenism. The proportions of green and nongreen nymphs were determined during the growing season.

Effect of substrate color.—To determine whether P. japonica nymphs changed body color in response to the substrate color of their growing environment, newly hatched nymphs were individually housed in transparent plastic containers (volume: 340 cm³) according to the method described for S. gregaria (Tanaka et al. 2012). In brief, the bottom and inside wall of each container was lined with a piece of color paper (3 × 2.5 cm; Joyful Honda Co., Tokyo, Japan), and the container had a transparent lid with 10 holes (diameter, 2 mm each) for ventilation. Nymphs were supplied with pieces of the Japanese millet Echinochloa crus-galli (L.) P. Beauv. leaf (about 2 cm in length each) as food every day. The containers were placed near a window in a room where direct sunshine was avoided. The temperature in the room was recorded every 60 min during the experimental period (May 31-August 15; mean = 25.1 °C, SD = 5.3 °C) with a temperature recorder (Ondotori, T & D Co., Nagano, Japan). The body color of nymphs two or three days after ecdysis to the last instar was recorded using the methods described below. The yellowgreen, white, and black papers used were the same as previously described (Tanaka et al. 2012): The x, y, and Y (brightness) values for the paper of each color were as follows: 0.379, 0.4865, and 65.75 for yellow-green; 0.3709, 0.3709, and 97.56 for white (ivory); and 0.3554, 0.3308, and 5.51 for black. Values of x and y represent chromaticity, and Y represents color value. Color value refers to the relative brightness of the color as perceived by the human eye. Body color at the last instar was recorded using the scoring methods described below. In S. gregaria, maternal crowding conditions affect offspring body color (Hunter-Jones 1958). This phenomenon was not observed in *P. japonica* because the body color of hatchlings derived from females kept in isolation or in a group was consistently green (data not shown). In the present study, however, all nymphs used were obtained from female adults kept singly after mating.

Effects of crowding and substrate color.—Five newly hatched nymphs were held in white, yellow-green, and black containers and reared at room temperature, as described above, until the last nymphal instar from May 31 to August 15. The body color of nymphs at two or three days after ecdysis to the last instar was recorded using the methods described below. Data were collected only from those containers in which four or five nymphs survived. Data obtained in each color container were pooled for analysis.

Role of visual stimuli.—To determine whether visual stimuli induced black patterns or not, 20 newly hatched nymphs were individually housed in yellow-green containers and allowed to see five nymphs housed in a white container after ecdysis to the second stadium according to the method described for *S. gregaria* (Tanaka and Nishide 2012). The inside wall was lined with a piece of yellow-green or white paper, except for a small window (2.5 × 3 cm) used as a skylight. Ten holes were made in the window for ventilation. As shown later, the

transparent lid of the plastic container holding five nymphs of similar ages was placed against the lid of another container housing a test nymph so that each test nymph could see five nymphs kept in a white container through the two layers of transparent lids. Another 20 nymphs were kept in yellow-green containers individually and allowed to see an empty white container as controls after ecdysis to the second stadium. The experiment was carried out from July 15 to August 25 (mean = 28.1° C; SD = 5.1° C). Body color at the last instar was recorded using the black patterning grades described below.

Seasonal variation in black patterning.—Three groups of 50 newly hatched nymphs were reared in nylon screen cages ($40 \times 16 \times 40$ cm) under outdoor conditions where the cages were exposed to sunshine during the day. They hatched from two or three egg pods on June 14, July 21, and August 12, 2021. They were fed the leaves and stalks of *Echinochloa crus-galli* put in a water bottle, which were changed every other day. Within several days after ecdysis to the last instar, they were scored as described above.

Effect of a high temperature on the induction of black patterns.— Nymphs reared in an outdoor cage from hatching on September 7, 2022, to the third stadium were divided into two groups of approximately 30 individuals and housed in separate wood-framed cages ($27 \times 14 \times 27$ cm) on September 28. One cage was incubated at 34°C under photocycles of LD 12: 12h, and the other cage was kept outdoors until the nymphs attained the last instar. Temperature was monitored, and body color at the last nymphal instar was recorded as described above.

Effect of CRZ injections.-Newly hatched nymphs were individually reared in yellow-green containers, and six green nymphs were injected with CRZ (synthesized by Eurofins, Japan; 1 nmol in 2 µl of rapeseed oil) or oil alone (J-OIL MILLS, Japan) as controls the day after ecdysis to the fourth stadium. Injections were performed with a sharpened calibrated capillary tube (Wiretrol 1-5 µl, Drummond Scientific Co., PA, USA) through an incision made between the second and third abdominal sternites of each nymph. Six similarly prepared green nymphs were injected with 1 nmol CRZ at days 1, 3, and 5 of the fourth stadium. All nymphs were scored two days after ecdysis to the fifth (penultimate instar) and sixth stadium (last instar). They were reared at room temperature until the injections and then incubated at 30°C under photocycles of LD 12:12 h. In another experiment, single injections of CRZ (1 nmol) were made into fourth stadium nymphs that were singly kept at 34°C until the last instar.

Scoring of body color.—The visible colors of solitary-reared nymphs were scored at the last instar into four categories: green, light green, white (or whitish), and pink (or pinkish; Fig. 1A). Black patterning was evaluated for solitary- and crowd-reared nymphs and categorized into five grades (Fig. 1B): grade 1, no black patterns and only brownish spots or patterns on the abdomen; grade 2, black patterns on the abdomen and some or no brown spots on the thorax; grade 3, distinct black patterns both on the thorax and abdomen but the lateral sides of pronotum without black spots; grade 4, as in grade 3 but the lateral sides of pronotum with distinct black spots; and grade 5, as in grade 4 but the lateral sides of pronotum with black



Fig. 1. Examples of the different body colors of the last instar nymphs of *Patanga japonica* observed during experiments. A. Variation in background color; **B.** Black patterning grades; **C.** Nymphs with green and reddish legs. In **B**, grade 1, no black patterns and only brownish spots or patterns on the abdomen; grade 2, black patterns on the abdomen and some or no brown spots on the thorax; grade 3, distinct black patterns both on the thorax and abdomen but the lateral sides of pronotum without black spots; grade 4, as in grade 3 but the lateral sides of pronotum with distinct black spots; grade 5, as in grade 4 but the lateral sides of pronotum with black markings.



Fig. 2. Variation in body color of *Patanga japonica* last instar nymphs. Photographs were taken on September 1 (A), October 11 (B), October 21 (C), November 11 (D), September 29 (E), and September 27, 2021 (F).

markings. The hind legs of some solitary-reared nymphs turned reddish in color (Fig. 1C), and the proportion of such individuals was also determined. Preliminary observations suggested that no significant sex difference was observed in body color. Therefore, data for the two sexes were combined for analysis.

Statistical analyses.—The proportions of individuals with different body colors were analyzed using a χ^2 test. Mean grades of black patterning were compared using the Steel–Dwass test. Pearson's correlation coefficient was used to analyze the relationship between the proportions of brown morphs and collection dates after log transformation of the proportions + 1. Mean temperatures were compared using Tukey's multiple comparison test. These analyses were performed using a statistics service available at http://www. gen-info.osaka-u.ac.jp/MEPHAS/kaiseki.html, Descriptive Statistics (Excel, Microsoft Office 365), or StatView (SAS Institute Inc., NC, USA). Differences were judged as significant when p < 0.05.

Results

Body color in the field.—Various background body colors, including green (Fig. 2A), yellow (Fig. 2B, F), brown (Fig. 2C, E), and whitish colors (Fig. 2D), were observed during the observation period. Some nymphs developed black patterns on the head, thorax, abdomen, legs, and antennae (Fig. 2D–F). However, most individual morphs were green with a black line and spots on the hindlegs, and brown (non-green) morphs appeared only later in the season with an increasing tendency toward the end of the growing season (r = 0.93; N = 13; p < 0.001; Fig. 3). Brown morphs were observed in the last three nymphal stadia (data not shown).

Effects of substrate color.—The proportion of nymphs in different body colors was significantly different among the three treatments ($\chi^2 = 14.50$, p < 0.01; Fig. 4A). The proportion of green nymphs was significantly higher in the yellow-green containers than in the white ($\chi^2 = 5.83$, p < 0.05) and black containers ($\chi^2 = 13.46$, p < 0.01), although no significant difference was observed between the white and black containers ($\chi^2 = 1.29$, p > 0.05). It appeared that these differences were not correlated with the brightness of substrate color. In contrast, reddish nymphs were more abundant in the black



Fig. 3. Proportions of green and non-green *Patanga japonica* last instar nymphs observed at a study site in Tsukuba in 2021.



Fig. 4. Effects of substrate color on the frequencies of *Patanga japonica* last instar nymphs with different background body colors (A), with reddish legs (B), and in different black patterning grades (C). Nymphs were reared individually in black, yellow-green (Y-green) and white containers from May 31 to August 15 at room temperature (25.1° C on average). Different letters in **B** indicate significant differences in proportions by a χ^2 test at 5%. For body colors and black patterning grades, see Fig. 1.

containers (17.8%) than in the green (4.3%; $\chi^2 = 4.19$, p < 0.05) and white containers (2.7%). The nymphs with reddish legs also occurred more frequently in the black containers (64.4%; Fig. 4B) than in the yellow-green (23.9%, $\chi^2 = 15.17$, p < 0.01) and white containers (32.4%, $\chi^2 = 8.32$, p < 0.01). The proportion of nymphs with black spots or patterns (grades 2 and 3) in the black containers was significantly higher than in the yellow-green ($\chi^2 = 13.46$, p < 0.01) and white containers ($\chi^2 = 5.83$, p < 0.05). These results suggest that the substrate color significantly influenced the body color of *P. japonica*, and the differences in body color were not related to the brightness of the substrate color under solitary conditions.

Effects of crowding and substrate color in containers.—Unlike the above results, all nymphs reared in a group of 5 per container developed black patterns after ecdysis to the second stadium (Fig. 5), suggesting that crowding induced black patterns. Yellow background body color was observed during the last two nymphal instars. At the last nymphal instar, no significant difference was observed in the mean of black patterning grade among the three treatments with different substrate colors (Steel-Dwass test, p > 0.05; Fig. 6). However, the proportions of individuals in grades 2+3, 4, and 5 were significantly different among the three treatments $(\chi^2 = 6.87, p < 0.05)$. The proportion of grade-5 nymphs decreased as the brightness of



Fig. 5. Body color of *Patanga japonica* nymphs reared in a group at room temperature. Black patterns appeared at the second stadium onward. The penultimate and last nymphal instars were identified based on wing pad size.



Fig. 6. Effects of crowding on the frequencies of *Patanga japonica* last instar nymphs in different black patterning grades in black, yellow-green (Y-green), and white containers. Five nymphs were reared in each container from May 31 to August 15 at room temperature (25.1°C on average). For black patterning grades, see Fig. 1.

the substrate increased, although a significant difference was observed only between the black and white containers ($\chi^2 = 6.25$, p < 0.05). Fig. 7 shows typical body colors (A, B) when the nymphs were reared singly and in a group in green cups, respectively.

Effects of visual stimuli.—Nymphs that could see but not touch five other nymphs from the second stadium to the last nymphal instar (Fig. 8A) developed some black patterns. The proportion of individuals that developed few black patterns in grade 1 was significantly reduced when compared to the control nymphs that could see an empty white container ($\chi^2 = 11.8$, p < 0.01; Fig. 8B, C). All nymphs were generally very light in body color, probably because they were allowed to see the white container (Fig. 8C).

Seasonal changes in black patterns.—Nymphs that hatched in June, July, and August 2021 developed different degrees of black patterns at the last nymphal instar (Fig. 9). The proportion of nymphs in the darkest grade was 0% in the June-hatching group, which was the earliest, and it increased to 19.0 and 57.2% in the July- and August-hatching groups, respectively. The mean grade was 2.6 in the June-hatching group and increased to 3.7 and 4.5 in the July- and August-hatching groups, respectively, and significant differences were observed in mean grade between the three groups (Steel-Dwass test, p < 0.05). As described below, darkening was further pronounced when nymphs hatching on September 7 were reared under outdoor conditions in 2022: 67.7% were in grade 5, with the mean grade being 4.6 (N = 31). The growing period from hatching to the time of scoring at the last nymphal instar varied with the time of hatching. The mean temperature during that period in Tsukuba was the highest in the July-hatching group (25.3°C) followed by the June-hatching group (24.4°C) and the August-hatching group (22.0°C; Japan Meteorological Agency



Fig. 7. Solitary-reared (A, grade 1), group-reared (B, grade 5), and CRZ-injected (C, grade 5) last instar nymphs of *Patanga japonica* at 30° C and individual reared in a group at 34° C (D, grade 2). The individual in C was reared in isolation and green when injected with 1 nmol CRZ at the fourth stadium. For black patterning grades, see Fig. 1.



Holes

Fig. 8. Effects of visual stimuli from five nymphs on the induction of black patterns in isolated-reared nymphs of *Patanga japonica*. **A.** Experimental setup; **B.** Frequencies of last instar test nymphs in different black patterning grades; **C.** Body colors in the three grades observed. For black patterning grades, see Fig. 1.



Fig. 9. Frequencies of *Patanga japonica* last instar nymphs that hatched on June 14, July 21, and August 12 and reared in a group in outdoor cages. For black patterning grades, see Fig. 1.

2022), suggesting that the seasonal changes in black patterns could hardly be explained by the changes in mean temperature during the entire period of nymphal development. In contrast, the mean temperature experienced during the second half of the growing period decreased with delays in the time of hatching: it was 26.1°C, 24.1°C, and 20.2°C in the June- (July 18–August 19), July- (August 17–September 9; Tukey's multiple comparison test, *p* < 0.05), and August-hatching groups (September 10–October 19; Tukey's multiple comparison test, *p* < 0.05), respectively. These results suggest that temperatures experienced during the second half of the nymphal stage may play an important role in inducing black patterns at the last nymphal instar. This possibility was tested in 2022 as described below.

Effect of CRZ injections on black patterning.—Green nymphs injected with CRZ once or three times during the fourth stadium showed a yellow background color with black patterns on various body parts in the following stadium (penultimate instar), whereas control nymphs injected with oil alone remained green with few black patterns (Table 1, Fig. 10). At the last nymphal instar, all individuals in the former developed conspicuous black patterns (grade 4 or 5), whereas those in the latter remained in grade 1 (Table 1, Fig. 10A). Though kept in isolation throughout the experimental period, the CRZ-injected individuals looked indistinguishable from those observed under crowded conditions (Fig. 7B, C), suggesting that the black patterns were likely induced by CRZ in this grasshopper.

Table 1. Number of *Patanga japonica* last instar nymphs in different grades at 30°C after injections with CRZ or oil alone at the fourth stadium. All nymphs were singly reared.

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	Corazonin injection		Oil-injected
Grades	1 nmol	1 nom × 3	control
Penultimate instar (fifth stadium)			
1	0	0	6
2	0	0	0
3	5	0	0
4 + 5	1	5	0
Last instar (sixth stadium)			
1	0	0	6
2	0	0	0
3	0	0	0
4 + 5	6	4	0

Unlike the oil-injected adults, nymphs injected with CRZ emerged as dark-colored adults, except for horizontal whitish stripes on the thorax and forewings (Fig. 11). One similarly dark-colored adult was observed on June 18 among intact old adults kept in a group under outdoor conditions (Fig. 11, bottom).

Effect of high temperature on black patterning.-To examine the effect of temperature on the induction of black patterns, third stadium nymphs that had been reared outdoors were incubated at 34°C on September 28, and their body color at the last nymphal instar (the sixth stadium) was compared to that of third stadium nymphs continuously maintained outdoors as a control. The control nymphs were strongly melanized, and most individuals were categorized as grade 4 or 5 (Fig. 12A). Their background body color was yellow, except for one individual with a reddish color (Fig. 12B-E). In the control, 50% and 100% of individuals attained the last nymphal instar on October 26 and November 4, respectively. On average, the temperature in the cage from September 28 to October 26 was 19.0°C. In contrast, those exposed to 34°C after September 28 developed only a few black patterns on the lateral sides of the pronotum with a bright yellow background color, and most of them were categorized in grade 2 or 3 (Figs 7D, 12A, F-H), suggesting that high temperature strongly suppressed the induction of black patterns on the thorax. However, the other areas, including the posterior part of the head capsule, midline of the abdomen, and legs, remained melanized in those individuals.



Fig. 10. Body and face color of *Patanga japonica* penultimate (top, middle) and last (bottom) instar nymphs after injections with oil alone (A) and with 1 nmol CRZ (B) at the fourth stadium singly kept in yellow-green containers (30°C). Black patterning grades are based on Fig. 1.



Old female reared outdoors

Fig. 11. Body color of *Patanga japonica* adults that were injected with oil alone (left) or 1 nmol CRZ (right) at the fourth stadium and a darkened uninjected old female observed on June 18 in an outdoor cage (bottom).

In a preliminary test, four fourth stadium nymphs were removed from $34^{\circ}C$ during the above experiment, injected with 1 nmol CRZ (October 1), and reared individually in yellow-green containers until the last instar (sixth stadium) at the same temperature. They all developed intense black patterns at the last instar (grade 5, N = 4; Fig. 12I), identical to some of those nymphs continuously kept outdoors (Fig. 12C, D).

Fig. 13 shows typical examples of exuviae shed by last instar nymphs after the various treatments described above. The exuviae in field-collected or singly reared green nymphs had no color, with only a thin black line on the hind femurs (A), whereas exuviae were strongly melanized with a yellow background color in crowdreared nymphs (B) and corazonin-injected solitary-reared nymphs at 30°C or 34°C (C). The exuviae of nymphs exposed to 34°C were slightly melanized with a bright yellow background color, except for the rear portion of the head capsule and legs (D).

Adult body coloration.—Most adults of *P. japonica* in the field looked similar, with a brown background color, a characteristic dark marking under the compound eyes, brownish forewings, and horizontal white stripes (Fig. 14A). However, some individuals showed an orange color on the pronotum and pterothorax (Fig. 14B). A few



Fig. 12. Effect of high temperature on black patterning in *Patanga japonica*. Frequencies of last instar nymphs in different grades after transfer from outdoor conditions to 34°C and LD 12:12h at the third stadium (September 28, 2022) or kept outdoors continuously as a control (A). Examples showing body color variation at the last nymphal instar under outdoor conditions (B–E) and at 34°C (F–H). Last instar nymph that was injected with CRZ at the fourth stadium and then singly kept at 34°C (I). Black patterning grades are based on Fig. 1.



Fig. 13. Examples of exuviae shed by *Patanga japonica* last instar nymphs after various treatments. Exuviae had no color with only a thin black line on the hind femurs in singly reared green nymphs (A), black patterns with a yellow background color in crowd-reared nymphs kept at room temperature (B), and in corazonin-injected nymphs kept at a high temperature (C), and had a few black areas with a bright yellow background color in the thoracic area in nymphs reared at a high temperature (D).

individuals had no markings or stripes on the pronotum (Fig. 14C). An old male collected on July 7 in 2021 had an exceptional body coloration: it was almost completely black except for the legs and abdomen (Fig. 14D). As described above, nymphs exposed to 34 °C developed few black patterns with a yellow background color. After adult emergence, they assumed a slightly lighter body color (Fig. 14E) than field-collected adults (Fig. 14A–C) but developed a brownish body color with dark markings similar to those collected in the field. Adults looked cryptic against leaf litter (Fig. 14F). The hindwings of adults before winter were not pigmented (Fig. 14G, left), whereas those of adults collected in February onward showed a reddish color in both sexes (Fig. 14G, right).

Discussion

The present study demonstrated that *P. japonica* nymphs exhibit body-color polyphenism in response to environmental factors. Green-brown polyphenism was recognized in the field. As mentioned earlier, the brown morphs include all individuals with nongreen colors. In *P. japonica*, the majority of nymphs were green, but a small number of nymphs with yellow, brown, and whitish colors appeared later in the season. At late instars, a few individuals had black patterns on the thorax and abdomen. It has been suggested that, depending on the species of grasshopper, green-brown polyphenism is influenced by the substrate color of the habitat, temperature, light, food quality, and humidity (Faure 1932, Hunter-Jones 1962, Rowell 1970, Tanaka 2004b, Tanaka and Nishide 2012, Tanaka et al. 2012). In P. japonica, this polyphenism was found to be influenced by the substrate color of rearing containers: green individuals were observed more frequently in green containers than in black or white containers. It was also noticed that nymphs with a white body color appeared most frequently in white containers, and those with black patterns occurred most commonly in black containers. These observations suggest that this grasshopper shows homochromy and may explain why brown morphs mainly appeared later in the season, as the leaf color of host plants such as the kudzu, Pueraria montana var. lobata (Willd.) Sanjappa & Pradeep, gradually changed from green to yellow or brown in the fall (Fig. 2C).

The black patterning was observed not only in the field where the population density was low (<1 / m^2 ; Tanaka 2023) but also in solitary-reared nymphs in the laboratory. Therefore, the appearance of black patterns was not caused by crowding. Black nymphs or



Fig. 14. Body color of *Patanga japonica* adults. A–D. Variation in body color in field-collected adults; E. Adult reared at 34°C; F. Adults on leaf litter (yellow arrows); G. Non-pigmented and reddish hindwings observed in adults before (left) and after overwintering (right). Black male adult in D was collected on July 7, 2021. Scales are adjusted to make body sizes approximately equal except for F.

nymphs with black patterns under low population density or solitary conditions have also been reported for *L. migratoria* (Faure 1932, Tanaka 2000) and *S. gregaria* (Pener 1991; Tanaka et al. 2010, 2012).

In *P. japonica*, the appearance of brown morphs at a low population density might be an adaptive response to the substrate color of the growing environment for camouflage against predators such as tree frogs, birds, and lizards. At the study site, such predators were frequently encountered (Tanaka 2023). How important they are as predators of *P. japonica* populations has yet to be investigated.

More intense black patterns were observed in P. japonica nymphs reared in a group than in those singly reared, demonstrating the presence of density-dependent body-color polyphenism. The induction of black patterns by crowding is known in L. migratoria (Faure 1932), S. gregaria (Husain and Ahmad 1936), Schistocerca americana (Drury, 1770) (Tanaka 2004a, Gotham and Song 2013), and several other species (Sword 1999, Lecog et al. 2011, Pocco et al. 2019, Foquet et al. 2021). In S. gregaria, in addition to temperature (Husain and Ahamad 1936), mechanical and visual stimuli are involved in the induction of black patterns (Lester et al. 2005, Tanaka and Nishide 2012, Tanaka et al. 2012, 2016b). In P. japonica, we noticed that nymphs were considerably inactive and did not show physical interactions involving touching and kicking one another under crowded conditions. Therefore, we tested the role of visual stimuli (five nymphs) and observed that visual stimuli without mechanical stimulation induced some black patterns, but intense black patterning was not observed in P. japonica. In this experiment, the possible involvement of olfactory stimuli was not completely excluded. However, it has been shown that constant exposure to the smell of 150 gregarious nymphs has no effect on the induction of black patterns in isolated S. gregaria nymphs (Tanaka and Nishide

2012). Further studies should be carried out to determine the role of olfactory stimuli in the black patterning of *P. japonica*.

Temperature was documented as an important factor in the induction of black patterns in group-reared P. japonica. Intense black patterns on the thorax (grades 4 and 5) were observed in the nymphs exposed to low temperatures but not in those exposed to high temperatures. Interestingly, nymphs reared at 34°C developed a bright yellow background color, as observed in S. gregaria (Sugahara and Tanaka 2018, 2019). In S. americana, rearing density moderately influenced black patterning at 30°C, and black patterns appeared even among singly reared nymphs (Tanaka 2004a). In this grasshopper, black patterns observed at 30°C became rare at 34°C and disappeared at 38°C and 42°C under crowded conditions (fig. 6 of Tanaka 2004a). This observation was consistent with the measurements of luminance in various body parts (fig. 7 of Tanaka 2004a). Gotham and Song (2013) did not pay attention to these responses and concluded that temperature was not a major factor in the induction of black patterns in this grasshopper. However, they provided no experimental evidence at different temperatures to support their statement, and suggested that the different conclusions between the two studies were related to the rearing densities adopted. Gotham and Song noted that they used a 'small' cage (73,899 cm³) to rear 200 nymphs to stimulate high-density conditions, whereas Tanaka reared 30 individuals in cages of 10,206 cm³. However, the average space available (cm³ divided by number of nymphs) for the nymphs in the two studies was nearly equivalent, at 369 versus 340 cm³ or slightly smaller (i.e., more crowded) in the experiment by Tanaka, suggesting a lack of support for Gotham and Song's criticism that the density treatments that Tanaka used were not sufficient enough to induce

density-dependent color plasticity. It is important to examine body-color polyphenism at different temperatures as well as in the field (or under outdoor conditions) to understand the controlling mechanism as well as the ecological and evolutionary significance.

In P. japonica, the degree of black patterning gradually increased as the season progressed under crowded conditions. This phenomenon was likely a response to temperature. However, it was difficult to explain it by the mean air temperature experienced during the whole period of nymphal development. In contrast, the mean temperature during the second half of the growing period showed a seasonal pattern that might explain this phenomenon well: nymphs developed more black patterns as the mean temperature decreased. This hypothesis was supported by the experiment results in which the black patterns were strongly suppressed in nymphs kept at a high temperature at the third stadium onward compared with nymphs continuously exposed to outdoor low temperatures. The rapid increase in the frequency of dark-colored nymphs in the fall might be an adaptive thermoregulation response facilitating efficient heat absorption in a gradually cooling environment. At a low population density, however, only a few individuals developed dark body color in the fall, which might suggest that the homochromic response for camouflage is selectively more important than thermoregulation through temperature-dependent body-color polyphenism. In S. gregaria, the black patterns in gregarious late instar nymphs are often suppressed under hot and sunny conditions in the field (Stower 1959), suggesting that their response is likely to reduce heat absorption under such circumstances.

The role of CRZ in inducing black patterns has been demonstrated in locusts and grasshoppers (Tanaka 2001, 2006, Pener and Simpson 2009). This neuropeptide is synthesized in the lateral neurosecretory cells in the brain and secreted from the corpora cardiaca to the hemolymph. In this study, injections of synthetic CRZ into P. japonica nymphs caused them to develop black patterns after ecdysis to the penultimate instar, and all individuals developed intense black patterns at the last instar, becoming visually indistinguishable from some of the individuals reared in a group. These results suggest that CRZ is involved in the control of body color polyphenism in this grasshopper. Body color on the thorax and abdomen became lighter in nymphs exposed to a high temperature, but dark color was restored when they were injected with CRZ, suggesting that the secretion of CRZ rather than the synthesis of pigments may be suppressed at a high temperature. It is also likely that the sensitivity to CRZ was only reduced locally at a high temperature because some body parts, such as legs, remained black. A similar phenomenon has been reported in L. migratoria (Tanaka 2003) and S. gregaria (Sugahara and Tanaka 2018).

In this study, a bright yellow color manifested in P. japonica nymphs reared at a high temperature. A similar phenomenon has been observed in S. gregaria, in which yellowing was stimulated by high temperature, crowding, and JH (Pener 1991, Nishide and Tanaka 2012, Sugahara and Tanaka 2018). Yellow protein, also known as yellow protein of the takeout family (YPT), is responsible for yellowing in the adult stage (Sas et al. 2007), and the expression of the gene for YPT during the last two nymphal instars is also enhanced by high temperatures and JH in S. gregaria (Sugahara and Tanaka 2018, 2019). According to absorption spectral analyses of the purified yellow fraction extracted from sexually mature S. gregaria males, YPT may bind to β-carotene (Wybrandt and Andersen 2001), as hypothesized by Goodwin and Srisukh (1949). However, absorption spectral analyses of YPT with and without carotenoids revealed that this protein binds to lutein instead of β-carotene (Sugahara et al. 2020). As observed in S. gregaria (Sugahara and

Tanaka 2018), the exuviae shed by the crowd-reared last instar nymphs of *P. japonica* were stained yellow and black, particularly at a high temperature. Unfortunately, the molecular mechanisms behind the yellow body color in *P. japonica* are unknown.

In this study, all *P. japonica* adults had similar body coloration with some variation. This uniformity is probably related to their behavior: they often bask in the sun on leaf litter from fall to spring (Tanaka 2023). They are considerably cryptic against the leaf litter background (Fig. 14F). The reddening of wings observed in overwintered female and male adults might be related to sexual maturation, as observed in *Patanga succincta* (Uvarov 1966). The relationship between color change and sexual maturation and its underlying mechanism awaits further study.

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