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Doctoral dissertation

Phase polyphenism in the desert locust, *Schistocerca gregaria* (Orthoptera: Acrididae): Physiological adaptations to crowding and maternal effects on progeny characteristics

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博士論文

サバクトビバッタの相変異：
混み合いに対する生理的適応と子に及ぼす母親の影響

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General Introduction

The desert locust, *Schistocerca gregaria* Forskål has a very wide range of distribution from Africa to Middle East and Asia (Uvarov, 1928, 1966, 1977). This locust is potentially the most destructive pest in the world, because it undergoes outbreaks under favorable conditions and migrates in a swarm over a long distance. The crop loss caused by locust swarms aggravates problems of food shortage in the infested areas. Numerous studies have been conducted to control locust outbreaks. Although it has been thought that uncommon weather such as heavy rain after drought is responsible for the outbreaks (Stige et al., 2007), the capability of locusts to accomplish rapid population growth or outbreaks is not well understood. The goal of this study is to elucidate the physiological mechanism of this phenomenon.

The outstanding characteristics of locusts are their ability to change their behavior, morphology and physiology in response to population density. This phenomenon is called density-dependent phase polyphenism, which was first proposed for the migratory locust, *Locusta migratoria* by Uvarov (1921). This theory was also extended to *S. gregaria* (Uvarov, 1928). Individuals at low population density (solitarious phase) are called solitarious locusts and those at high population density (gregarious phase) gregarious locusts. Intermediate characteristics between solitarious and gregarious phases are observed in locusts which are exposed to intermediate population density or during a transient phase from a solitarious (or gregarious) to a gregarious (or solitarious) phase. In *L. migratoria*, solitarious nymphs are sedentary and assume various body colors matching the background of their habitat, whereas gregarious nymphs actively move in bands and develop conspicuous body color with black patterns with an orange or yellow background color (Pener, 1991). *S. gregaria* shows similar responses to population density. In the laboratory, locusts with their properties similar to solitarious and gregarious forms can be obtained by rearing them under isolated or crowded conditions, respectively.

A shift from one phase to another may occur within the life of an individual or over generations (Uvarov, 1966). A complete phase shift does not take place in one

generation, but takes several generations, because phase change is a cumulative process that crosses from one generation to the next (Uvarov, 1966; Pener, 1991). Thus, the typical phase-specific characters are expressed by locusts which have experienced low or high population density continuously for several generations. This phenomenon is often called phase accumulation. Although this phenomenon has been known for several decades, exactly how phase-specific characters are accumulated across generations is yet to be understood.

Phase-dependent differences can be found in hatchling characteristics. In *S. gregaria*, small and green hatchlings are produced by solitarious adults, whereas large and black hatchlings are produced by gregarious adults. Hatchling characteristics are controlled by the population density experienced by the mother (Faure, 1932; Hunter-Jones, 1958). However, it has not been well understood how the maternal density determines the progeny characters such as egg size, egg-pod size and hatchling body coloration, how the progeny (or hatchling) characteristics influence subsequent development and reproduction at different rearing densities, and what would be the significance of such variation. The main objective of the present study was to find the answers to these questions in *S. gregaria.* This thesis consists of 6 chapters: the first three chapters focus on the physiological adaptations of *S. gregaria* to crowding and the last three concern with the mechanism of maternal effects on the progeny characters.

To study phase polyphenism, it was essential to produce many hatchlings of *S. gregaria* with different phase-related characteristics. During this study, nymphs and adults of a solitarious line were kept in isolation and each female was kept with a male for only one or two days for mating to minimize the crowding stimuli received from the male. This procedure was important, because crowding or the number of adults per cage greatly influences the hatchling characters (Hunter-Jones, 1958). Preliminary observations indicated that a sexually mature male had greater “gregarizing” effects on the progeny of his mating partner than did an immature male. Apparently, there seemed to be some differences in sexual behavior or sexual activity between them. To establish

an experimental system to produce hatchlings with different phase-dependent characteristics, I firstly made some observations on sexual behavior of *S. gregaria* using a gynandromorph in Chapter 1.

Locust nymphs show body-color polyphenism (Faure, 1932; Rowell, 1971; Dearn, 1990). Body coloration during nymphal development may directly influence the progeny fitness, because nymphs with conspicuous body coloration against their habitat background color are likely to be found more easily by predators (Isely, 1938). In *S. gregaria,* remarkable differences in body coloration occur at hatchling, but nymphs can modify the body coloration in response to environmental conditions. No detailed studies have been reported about how phase-dependent differences in hatchling body coloration would influence the body-color polyphenism at a late nymphal stage. To solve this problem, the effects of hatchling characters on body coloration at the last nymphal stadium of *S. gregaria* were investigated in Chapter 2.

Among various phase-dependent characters, progeny size would be most important in terms of the survival of hatchlings which may often encounter crowding and a temporary shortage of food. In general, large progeny can often better withstand severe environmental stresses such as crowding, starvation, desiccation and nutritional stresses as compared with small one (Godfray et al., 1991; Fox et al., 1997). Although conspicuous differences found in progeny size have been suggested to be correlated with the number of nymphal stadia in locusts (Hunter-Jones, 1958), no detailed studies focusing on the influence of body size at hatching on nymphal growth have been carried out under controlled conditions. Rapid development would reduce the time of exposure to predators, but it will expense adult body size by a rule of trade-off. In general, adult body size directly influences the fecundity: large females produce more progeny than do small ones (Fox & Czesak, 2000). Developmental and reproductive performance of locusts would be directly related to rapid population growth observed during outbreaks. In Chapter 3, I investigated the effects of hatchling body size on several developmental and reproductive traits of the locusts at different rearing densities to elucidate the

physiological adaptations to crowding.

The maternal control of hatchling characters has received much attention in locusts, although relatively little is understood about the genetic and physiological controls (Dale and Tobe, 1990; Tanaka, 2001, 2006). Body color mutations such as albinism occur in locusts (Hunter-Jones, 1957; Verdier, 1965; Hasegawa and Tanaka, 1994; Yerushalmi et al, 2000). In *S. gregaria*, the albinism is a recessive trait controlled by a single Mendelian unit (Hunter-Jones, 1957). While the genetic background for color mutation in locusts has received much attention, no information is available about the genetic relationships among different types of mutant. During a study of phase polyphenism in *S. gregaria*, I found reddish brown hatchlings in a crowd-reared laboratory colony. Preliminary observations showed that they develop reddish brown patterns instead of black ones under crowded conditions, whereas they assume green color like normal solitarious hatchlings under isolated conditions. Because our laboratory had an albino strain of *S. gregaria* (Schoofs et al., 2000), the occurrence of this mutant provided us with an opportunity to determine the genetic mechanisms controlling these mutants by crossing them. The results of crossing experiments between the reddish-brown mutant and a normal strain as well as between this mutant and the albino mutant strain will be described in Chapter 4.

Based on a series of studies by the Oxford research group, Simpson and his colleagues suggested that hatchling body color is determined after egg deposition by a water-soluble pheromonal factor produced by the accessory gland of the female parent (Islam et al., 1994a, b; McCaffery et al., 1998; Simpson et al., 1999; Hägele et al., 2000). They suggested that the foam plugs deposited by gregarious adults contained a pheromonal factor responsible for the induction of black patterns characteristics of gregarious forms in the hatchlings. Washing or separation of presumptive gregarious eggs from egg pods of crowd-reared females prevented darkening of the hatchlings. Although their foam hypothesis could well explain the process of development of phase-dependent body coloration in hatchlings, their studies did not consider the

phase-dependent variation in hatchling body size. It has been widely accepted that solitarious and gregarious hatchlings are produced by small and large eggs, respectively. Because such differences in hatchling body size seemed extremely unlikely to occur after oviposition, I re-examined the role of this pheromonal factor to elucidate the physiological mechanism controlling progeny characteristics in *S. gregaria*. After these results were published, Simpson and his colleague (2007) wrote a review article in which they pointed out two possibilities to explain the differences between their results and ours. I then decided to test these possibilities to substantiate my conclusion. Therefore, Chapter 5 consists of two parts.

The results obtained from Chapter 5 cast doubt on the validity of the foam hypothesis proposed by Simpson and his colleague (Tanaka and Maeno, 2006). This leads to the question of exactly where the phase-dependent body size and color of hatchlings are determined. I reached a conclusion that the determination of hatchling characteristics occurs in the ovary rather than in the oviduct or after oviposition (Simpson and Miller, 2007). While the phenomenon that adult density influences the progeny size and number in locusts is well known (Norris, 1950, 1952; Uvarov, 1966; Injeyan and Tobe, 1981; Pener, 1991), the physiological mechanisms controlling this process remains unclear. For example, it is not known if the age of the mother at deposition would influence the progeny characteristics including egg size and number as well as hatchling body coloration and how quickly female adults would modify these progeny characters after a change in population density. The purpose of the last chapter is to clarify these problems. One of the most important findings in Chapter 6 is that locusts of this species tend to produce a mixture of hatchlings with different body sizes and different colors in the first egg pod compared with the egg pods produced later. This discovery not only reveals a new aspect of phase polyphenism in locusts but also provides an explanation for the discrepancy in results between the Oxford research group and ours (Chapter 5).

Review of literatures

Phase polyphenism

Locusts show phase polymorphism in which they change behavior, morphology and physiology in response to population density. Behaviors and appearance of gregarious locusts are conspicuously different from solitarious ones. In the case of the migratory locust, *Locusta migratoria*, solitarious and gregarious locusts had been regarded as separate species, i.e, *L. danica* and *L. migratoria*, respectively. Uvarov (1921) proposed the phase polymorphism theory and suggested that only a single species is involved, but this species can shift from one phase to another depending on the population density. This theory was also extended to the desert locust, *Schistocerca gregaria* and other species of locusts (Uvarov, 1928).

Phase-dependent characteristics, reflecting differences between solitarious and gregarious locusts, are found in behavior, body color, morphology, development, reproduction, physiology, biochemistry, molecular biology and cytology (Uvarov, 1966; Pener, 1991; De Loof et al., 2006). To characterize the phase state of individuals, aggregation behaviors, activity, body color, morphometrical and reproductive data are most frequently used as parameters.

Locusts cannot transform from solitarious phase to gregarious phase or *vice versa* in a single generation (Uvarov, 1966; Pener, 1991). When the effects of parental density are combined with those of the rearing density of their progeny, they express a greater degree of phase variation (Hunter-Jones, 1958). Thus, isolated-reared progeny from isolated-reared parents have typical solitarious morphometrics, while some of the crowd-reared progeny from crowd-reared parents have more extreme gregarious morphometrics than those from isolated-reared parents (Hunter-Jones, 1958; Hoste et al., 2002a,b; Tanaka et al., 2002; Maeno et al., 2004). Phase characteristics such as behavior, ovariole numbers, morphometrics, production of pheromone and flight ability are known to accumulate over generations, and several generations are required to reach the extreme phase-dependent characteristics (Uvarov, 1966; Michel, 1980; Pener, 1991). This phenomenon is called ‘phase-accumulation’, but the mechanism involved in this

phenomenon has not been elucidated completely.

Phase-dependent behavior

Behavior is the first phase characteristic to change in response to population density (Ellis, 1951; Roffy and Popov, 1968; Roessingh & Simpson, 1994; Simpson et al., 1999). Solitarious nymphs are sedentary and tend to avoid each other, whereas gregarious ones actively move and form a group (Uvarov, 1966, 1977). To characterize phase-dependent behaviors, aggregation or grouping and activity are often used as a parameter. Ellis (1951, 1962) is a pioneer in this field and recent studies by the Oxford research group confirmed her results by using multiple logistic regression analysis (Roessingh et al., 1993, 1998; Roessingh & Simpson, 1994; Bouaïchi et al., 1995; Lester et al., 2005).

Aggregation is one of the gregarious behaviors. In *L. migratoria*, nymphs isolated during the 1st nymphal stadium and then crowded after the next molt exhibited a tendency to aggregate 24 hours later. After 4 days of crowd-rearing, they aggregated as well as hoppers kept crowded since hatching (Ellis, 1962). On the other hand, when crowd-reared nymphs were isolated, even after 8 days there was only a small reduction in the numbers of aggregating individuals when tested, suggesting that hoppers are easily conditioned to aggregate, but that such conditioning is less easily lost. A comparison of aggregation ability between *L. migratoria* and *S. gregaria* suggested that hatchlings of the former did not aggregate until they were 3 days old, while those of the latter showed signs of aggregation only 6 hours after hatching, and 1 day was sufficient to achieve the maximum aggregation (Ellis, 1962). In *S. gregaria*, 4 hours were sufficient to bring solitarious 4th stadium nymphs to the level of crowded line, but in *L. migratoria* this was not achieved even after 48 hours of crowding (Ellis, 1962; Ellis & Pearce, 1962). In the former, even 30 minutes of crowding had striking effects. Nymphs of this locust form aggregation much more quickly, and lose it less rapidly when isolated again, than those of *L. migratoria*. These results indicate that the mechanism to

control aggregation behavior is different between species.

Activity level is apparently greater in gregarious nymphs than in solitarious ones. This is partly due to their mutual stimulation, but there is also an intrinsic difference between the two phases. Gregarious nymphs show a concerted movement in large bands, as called marching. This marching behavior was reproduced in the laboratory by using special cages (Ellis, 1951). Nymphs placed in such a cage marched round the floor in a circle. This activity was particularly pronounced if the hoppers were starved for several hours, but occurred also if food was present. The marching of nymphs was interrupted by periods of rest and feeding. To observe marching behavior, Ellis (1951) conducted an experiment in which 5 marked nymphs were placed in a cage with 20 nymphs and observed for 8 hours. As a result, gregarious nymphs rapidly increased their activity and finally spent over 60 % of the time in marching, while the solitarious nymphs remained inactive longer and never marched for more than 20 % of the time. In the presence of food in the cage, nymphs of both phases showed reduced marching, but the quantitative difference between them remained. There was also a difference in the average marching speed, which was 3.27 cm per second in gregarious nymphs and 1.33 in the solitarious ones. To find the critical population density to start marching, Buhl et al. (2006) recorded locust's motion for 8 hours and analyzed after different numbers of 3rd stadium nymphs, ranging from 5 to 120 insects (densities of 12.3 to 295 locusts/m^2), were kept in a ring-shaped arena. The marching behavior increased depending on locust density. Nymphs hardly showed marching behaviors at low density, whereas they showed a strong tendency of marching at high population density. At intermediate population density, the incidence of marching insects increased rapidly.

Once the locusts gather, pheromones play a role in keeping the aggregations together. Guaiacol and phenol are the predominant electrophysiologically active compounds released from feces of 5th stadium nymphs and adults (Loher, 1990; Whitman, 1990; Ferenz and Seidelmann, 2003, Hassanali et al., 2005). Interestingly, guaiacol and phenol are synthesized by bacteria, *Pantoea agglomerans*, in the locust gut

(Dillon et al., 2002).

The site of mechanosensory input causing phase change has been studied by repeated localized touch of different body parts of solitarious nymphs over 4 hour periods and then recording the behavior of the insects (Simpson et al., 2001). A significant switch from solitarious to gregarious behavior occurs when the outer surface of a hind femur is stimulated but not when other body parts are similarly stimulated. However, this observation was limited only in behavior as a phase characters, so whether the hind femur is primary organ to receive physical stimuli causing gregarization in nymphal body coloration or morphology remain unknown. Lester et al. (2005) reported that physical contact was not needed to induce gregarious black patterns, but yellow background body coloration was only fully induced when locusts received conspecific physical stimuli. My preliminary experiments showed that even crowd-reared nymphs, which amputated with their hind legs at the 2nd nymphal stadium, developed intensive black patterns with yellow background body color typical of gregarious phase at the last nymphal stadium, indicating that hind legs should not be a primary mechanosensory site (Maeno & Tanaka, unpublished). Antennae play an important role in receiving environmental stimulations. Removal of the antennae from crowd-reared nymphs of S. gregaria at the 3rd nymphal instar causes the latter to develop solitarious green body coloration at the 5th nymphal stadium and solitarious morphology in the adult stage (Mordue (Luntz), 1977). Hind tarsal amputation has no such effect, indicating that antennal chemoreception may be critical for sustaining the gregarious phase.

Little is known about the ecological significance of marching behavior of gregarious form. It was suggested that marching might be an escape behavior from adverse conditions such as high temperature (Uvarov, 1977), but this claim has not been accepted widely. Physiological conditions, especially a period of starvation, might be involved in marching behavior. In nymphs of *L. migratoria*, marching activities increased with a decrease of food quantity (Ellis, 1951). There is a possibility that a

shortage of food plants and their low water content may contribute to the amount of marching, its speed and the distances traveled, but there are no quantitative data on these points (Uvarov, 1977). Locusts do not have a group leader like queen ants. Nevertheless, they can ensure marching band and synchronize in the same direction and at the same speed. Kennedy (1954) called this phenomenon as gregarious inertia. However, little is known about the mechanism to socialize in a large group. To elucidate the mechanism controlling locust behaviors furthermore, we would have to pay attention to their circadian rhythms in behavior such as feeding, settling and marching. The physiological state of insects is also an important factor, because activity of nymphs fluctuates in intensity even within the same nymphal stadium. Even gregarious locusts tend to avoid each other and become quiescent just before and after molt. Further comprehensive studies considering physiological traits of locusts are required to understand locust behavior.

Phase-dependent body coloration

Locusts show body color polyphenism (Faure, 1932; Uvarov, 1966; Rowell, 1971; Fuzeau-Braesch, 1985; Dearn, 1990; Pener, 1991). In *S. gregaria* and *L. migratoria*, solitarious nymphs develop cryptic or camouflaged green or brown body color with few or no black patterns. On the other hand, gregarious nymphs assume bright yellow or orange body color with intense black patterns. Three types of polyphenism are related to body coloration (Pener, 1991). One is so-called homochromy, which is an adaptation of the color to that of the background. Green-brown polyphenism is a second type in which individuals in a population are either green or brown nymphs, or as both nymphs and adults. The third type is phase or density-dependent body color polyphenism in which population density or crowding mainly controls body color. Nymphs of *S. gregaria* exhibit the last two polyphenisms, while those of the other locust show the three.

Nymphal body coloration of *S. gregaria* is affected by environmental factors including humidity, moisture content of food, temperature, light and radiation,

concentration of carbon dioxide and population density (Husain and Ahmad, 1936; Hunter-Jones, 1962; Uvarov, 1966; Rowell, 1971; Fuzeau-Braesch, 1985; Dearn, 1990; Pener, 1991). It is well known that nymphs of *S. gregaria* do not show homochromy, but isolated-reared nymphs kept under dry conditions show a wide range of color variation like homochromy including green, yellow-orange, olive green, shades of brown, and other colors (Hunter-Jones, 1962). Thus, there is a possibility that nymphs of *S. gregaria* can express some degree of homochromy under dry conditions at low population density.

Humidity influences the green-brown polyphenism in locusts. Low humidity causes green nymphs to turn brownish in later nymphal stadia. High humidity results in a high incidence of green nymphs but only at a low population density. However, crowding inhibits development of the green body color even at high humidity. Though green and brown individuals are observed within solitarious nymphs, they differ in their tolerance to different humidity levels. In *L. migratoria*, nymphs with brown body color are more resistant to starvation at low humidity than those with green, whereas the latter are more resistant to starvation at high humidity than the former (Albrecht, 1965).

Body color polyphenism is also observed in hatchlings. The color differences between solitarious and gregarious hatchlings are correlated with egg size and these differences are related to the maternal density. Green hatchlings appear from small eggs produced by solitarious female adults, whereas crowd-reared females deposit large eggs that produce black hatchlings, thought intermediate hatchlings also appear in both phases (Faure, 1932; Gunn & Hunter-Jones, 1952; Hunter-Jones, 1958; Pener, 1991; Islam et al., 1994; Bouaïchi et al., 1995; McCaffery et al., 1998; Bouaïchi & Simpson, 2003). Hatchling characters are affected by density during the adult stage but nymphal stage in *L. migratoria* and *S. gregaria* (Hunter-Jones, 1958). A single egg pod often produces a mixture of these hatchlings, but how and when such egg pods are produced has not been revealed.

The endocrine factors controlling phase-related characters have been studied,

particularly about the role of juvenile hormone (JH). JH is an important hormone that induces green body color often observed in solitarious individuals of locusts (Fuzeau-Braesch, 1985; Dale & Tobe, 1990; Pener, 1991; Pener & Yerushalmi, 1998; Tanaka, 2001; Breuer et al., 2003). Chemical allatectomy that destroys the source of JH production (corpora allata) in solitarious nymphs causes the green body color to fade away, but does not induce black patterns with an orange background color typical of gregarious nymphs. Therefore, it has been suggested that JH is not a major hormone in the control of body-color polyphenism (Pener et al., 1992; Applebaum et al., 1997).

The presence of a dark-color inducing factor in the corpora cardiaca was suggested by Nickerson (1956) who concluded that it was a steroid, but this substance had never been isolated. Recently, it was discovered that an albino strain of *L. migratoria* is deficient in a hormonal factor responsible for the induction of dark color (Tanaka, 1993). Implantation of corpora cardiaca, brain or thoracic ganglia taken from normal (pigmented) locusts into albino nymphs caused the latter to develop dark color. This factor was heat-stable, but lost its biological activity if incubated with a protainase, suggesting that it is a peptide (Tanaka and Pener, 1994). A similar dark-color inducing factor was also suggested to be present in *S. gregaria* by transplanting corpora cardiaca of this locust into albino nymphs of *L. migratoria* (Tanaka and Yagi, 1997). The dark-color inducing factors of the two species were determined to be identical to a neurohormone, [His^7]-corazonin (Tawfik et al., 1999). It had been isolated from an acridid, *S. americana*, without known function (Veenstra, 1991) and known to be present in the brain and corpora cardiaca of locusts and grasshoppers (Veenstra, 1991; Tanaka, 1993, Tanaka and Pener, 1994; Tanaka, 2004b; Schoofs et al., 2000; Roller et al., 2003). [His^7]-corazonin has recently received much attention as an important hormone controlling phase-related body coloration and morphogenesis (for reviews, see Tanaka, 2001; Breuer et al., 2003; De Loof et al., 2006). In *L. migratoria*, various non-green dark colors observed in solitarious forms are also induced by [His^7]-corazonin (Tanaka, 2000a, b).

Phase-dependent developmental traits

Isolated-reared (solitarious) adults have 5 or 6 nymphal instars, whereas crowd-reared (gregarious) ones have 5 in *S. gregaria* (Uvarov, 1966; Pener, 1991). In general, locusts have reduced numbers of nymphal instars under crowded conditions (Uvarov, 1966; Pener, 1991). Hatchling characters also influence the number of ecdyses during nymphal development (Hunter-Jones, 1958). In the filed, 6th stadium nymphs appear when body color at hatching is green and the population density is low throughout their development (Stower, 1959). In general, adults with 6 nymphal stadia appear from isolated-reared lines in the laboratory (Injeyan and Tobe, 1981a, Hoste et al., 2002a; Maeno et al., 2004). The proportion of adults with 6 nymphal instars increased rapidly in one generation under isolated conditions and this level was maintained throughout 6 generations (Injeyan & Tobe, 1981a).

The possibility that food may have some influence on nymphal development has been studied by rearing *S. gregaria* on lucerne (Uvarov, 1966). This food generally slows nymphal development, and causes abnormal development passing only 4 nymphal stadia, instead of the normal 5. The resulting adults were much smaller than normal, and their forewings were only half the normal length, not reaching the end of the abdomen. They, however, matured normally, copulated and laid eggs.

The mechanism controlling the number of nymphal stadium has not been elucidated completely. Molting and metamorphosis are controlled by hormonal factors and the difference in the number of nymphal stadia between solitarious and gregarious locusts may reflect a somewhat different programming of related endocrine events (Pener, 1991). Implantation of prothoracic glands, which secrete ecdysone, into 1st and 2nd stadium nymphs caused reduction in the number of nymphal instars in *L. migratoria* (Staal, 1961). Implantation of corpora allata (CA), which secrete JH, into 2nd stadium nymphs increased the number of nymphal stadia from 5 to 6, but a decrease in the number of 6 nymphal stadium locusts was observed after implantation of extra CA into

young 5th stadium nymphs. The critical period determing the number of nymphal stadia remains unknown.

Phase-dependent morphometric characters

Adult morphology shows conspicuous differences between solitarious and gregarious locusts. In gregarious locusts, the head is wider, the pronotum is shorter and more constricted with its crest somewhat depressed than solitarious ones. Additional features are larger compound eyes, which are also more widely separated on the vertex, shorter hind femur and longer fore wings. Thus, gregarious adults have smaller F/C (hind femur length / head width) and larger E/F (fore wing length / hind femur length) ratios than solitarious ones. Phase-dependent differences are observed in the types and number of spins (probably sensilla) on the hind femur in *S. gregaria* (Roonwal and Bhanotar, 1959). Two types of femoral spines, i.e. large (height, 0.037-0.111 mm) or small (height, 0.009-0.055 mm) are distinguishable. Solitarious adults have both types of spins, whereas gregarious ones have only the small type. The former have a mean of 8 (6-11) small spins, but the latter 18 (17-21). The number of hind-tibial spins also varies among individuals in *S. gregaria* (Roonwal, 1946). Although no significant difference in the number of the spins was observed between solitarious and gregarious phases, the former show a greater degree of variation than the latter. The number of spins did not show significant correlation with that of the eye-stripes (number of nymphal stadium). The number of spines did not increase during post-embryonic development, indicating that the number has already been determined at hatching. These morphometrical differences are induced by rearing density during the nymphal stage (Uvarov, 1966; Pener, 1991), and the extent of the change increases with hopper density (Gunn & Hunter-Jones, 1952). If some of the crowded hoppers are isolated during the successive instars, the adult morphometrics reflect the duration of crowding or isolation (Hunter-Jones, 1958).

Gunn & Hunter-Jones (1952) demonstrated that *L. migratoria* kept crowded and

exposed to sharp day and night fluctuations of temperature (exact data were not shown) approached the extreme morphometrics of wild gregarious populations more closely than under constant conditions. The general failure to obtain extreme expression of phase in the laboratory may well be due to the established belief that constant temperature rooms are absolutely essential for all experimental work, because no extreme gregarious locust has been obtained in the laboratory (Gunn & Hunter-Jones, 1952; Hunter-Jones, 1958).

[His^7]-corazonin causes a shift in morphometric ratios towards the values typical for the gregarious phase when injected into solitarious nymphs in *S. gregaria* (Hoste et al., 2002b; Maeno et al., 2004) and *L. migratoria* (Tanaka et al., 2002). In both species, the earlier the time of injection during the nymphal stage the larger the 'gregarizing' effect on the adult morphology (Maeno et al., 2004). Recently, it has also been reported that [His^7]-corazonin affected the development of antennal sensilla in *L. migratoria* (Yamamoto-Kihara et al., 2004) and *S. gregaria* (Maeno and Tanaka, 2004). In the latter, solitarious locusts injected with [His^7]-corazonin during the nymphal stage developed significantly fewer basiconic type A and coeloconic sensilla on the observed 2^{nd}, 8^{th} and 14^{th} antennal segments in the adult stage than the oil-injected counterparts. The earlier the time of injection of [His^7]-corazonin during the nymphal stage the greater the degree of reduction in the number of antennal sensilla at the adult stage. These results suggest that [His^7]-corazonin is an important factor for the control of phase-related morphogenesis in locusts. However, the fact that the phase-related morphometric changes can be observed in the albino strain that lacks [His^7]-corazonin suggests that the presence or absence of this hormone alone does not explain the whole phase polyphenism (Tanaka et al., 2002). Recently, Tanaka (2007) demonstrated that injection of extract of albino corpora cardiaca (CC) induced morphometric gregarization in isolated-reared albino in *L. migratoria*. This indicates that the albino CC contains a factor similar to [His^7]-corazonin, although it has no dark-color inducing activity. The factor has not been identified.

Phase-dependent reproductive characteristics

The reproductive potential or fecundity is higher in solitarious females than in gregarious females (Norris, 1950, 1952; Uvarov, 1966; Pener, 1991). Rearing density apparently influences the reproductive performance, but the differences may be caused by the variation in numbers of ovarioles. The number of ovarioles is highest in locusts kept under isolation for several successive generations (Injeyan & Tobe, 1981a). Injeyan & Tobe (1981a) reported that the average number of ovarioles was 110 in crowd-reared females of *S. gregaria*, and it increased to 130 and 154 in the 2^{nd} and 4^{th} generations, respectively. However, despite the differences in the number of ovarioles, biomass calculated by the number of eggs per pod and hatchling size (Hunter-Jones, 1958) and the average vitellin content per ovary (Injeyan & Tobe, 1981a) are approximately equal in crowd- and isolated-reared females, because the eggs of the latter are smaller. Thus, the number of eggs per pod laid by isolated-reared females is larger at the expense of egg size and vitellin content per egg than crowd-reared females. However, these studies did not pay attention to differences in adult body size. In general, larger organisms are more fecund than small ones (Fox et al., 2000). In locusts, body size is larger in solitarious locusts than gregarious ones (Uvarov, 1966). A comparison of reproductive potential by considering female body size has not been conducted.

The mechanism controlling hatchling characters has not been understood fully. The physiological state of female parents at the time of sexual maturation must be considered. Hatchlings with green, black and intermediate body colors often appear from the same egg pod (Husain and Ahmad, 1936; Gunn & Hunter-Jones, 1952; Hunter-Jones, 1958). Husain and Ahmad (1936) reported that hatchlings from one egg pod might be all black, all green or a mixture of black and green, black, intermediate and green, black and intermediate, or green and intermediate. Either gregarious or solitarious females sometimes produce such egg pods containing a mixture of hatchlings. There is an obvious need for a systematically designed study to uncover the

physiological and hormonal background of this phenomenon.

Recently, factors influencing hatchling characters have been reported. McCaffery et al. (1998) indicated that hatchlings produced by isolated-reared females turned black when treated with saline extracts of foam plugs produced by gregarious females on the day of oviposition, whereas hatchlings produced by crowd-reared parents turned green when the eggs were washed with water (McCaffery et al., 1998). In addition to the above factor present in the egg pod foam, another gregarizing factor has also been suggested to be present in the accessory glands of crowded females that promote gregarious behavior in even hatchlings obtained from isolated-reared females (Hägele et al., 2000). Malual et al. (2001) indicated that aggregating behavior was enhanced in hatchlings when the eggs produced by solitarious adults had been incubated in sand previously used for oviposition by gregarious females. They identified three ketones ((Z)-6-Octen-2-one, (E,E)-3,5-Octadien- 2-one, and (E,Z)-3, 5-Octadien- 2-one) in the egg-pod foam (and in the gregarious female accessory glands) as gregarizing factors. It is very unlikely that these ketones are identical to the factors extracted with water by McCaffery et al. (1998), because ketones are not water-soluble.

Internal factors influencing the hatchling characters are also known. Some reproductive traits are controlled by the corpus allata (CA) or JH (Pener, 1991). JH induces vitellogenin production in the fat body and mediates vitellogenin uptake by the developing oocytes (see review for Pener, 1991). Thus, JH is necessary to induce and maintain the reproduction in females, implying that more active CA and/or higher JH titers may be responsible for the higher fecundity of isolated-reared females. Implantation of extra CA into crowded female adults of *S. gregaria* resulted in a decrease of hatchling weight and a shift toward a green hatchling color (Cassier and Papillon, 1968). A similar phenomenon is also obtained for *L. migratoria* (Cassier, 1965; Lauga, 1976b). Furthermore, unilateral allatectomy of isolated mothers results in an increased weight of the hatchlings (Cassier, 1966a). Unilateral allatectomy combined with severance of the allatal nerve yields similar results and also induces darkening in

some hatchlings (Cassier, 1966b). Exogenous JH treatment of eggs obtained from crowded *S. gregaria* females causes disturbances in embryogenesis and hatching, but does not induce solitarious characteristics in the hatchlings (Injeyan et al., 1979). Density may alter the responsiveness of the target tissues (fat body and/or oocytes) to the JH (Injeyan & Tobe, 1981b), and reproductive traits may be changed accordingly. As mentioned above, [His^7]-corazonin is present in *S. gregaria, L. migratoria* and other orthopterans (Tawfik et al., 1999; Tanaka, 2001). In preliminary observations, repeated injections of the peptide into solitarious *S. gregaria* females during the nymphal and adult stages (1 nmol ×4 or 5) failed to induce dark color in their hatchlings (Tanaka, 2000c, 2001). Hatchlings obtained from crowd-reared female adults developed black patterns, whereas those from isolated-reared adults remained green color even after injection with [His^7]-corazonin (Tanaka, 2001). So, darkening may be controlled by some other factor(s) different from [His^7]-corazonin (Tanaka, 2001). Another hormone, bursicon, was suggested to induce melanization and sclerotization in crowded *S. gregaria* hatchlings (Vincent, 1972; Padgham, 1976a,b), although it has not been characterized. The hormone seems to be also present in green hatchlings, but it only induces sclerotization in them (Padgham, 1976a,b). The production site of bursicon has not been revealed in locusts.

Chapter 1

Morphological and behavioural characteristics of a gynandromorph of the desert locust, *Schistocerca gregaria*

Abstract

Morphological and behavioural characteristics were investigated for a gynandromorph of the desert locust, *Schistocerca gregaria* that appeared under isolated rearing conditions in the laboratory. It had both male and female external reproductive organs bilaterally. The body size and dimensions were similar to a normal male. Morphometric traits, *F/C* ratio and *E/F* ratio (*F*, hind femur length; *C*, maximum head width; *E*, fore wing length), of the gynandromorph were typical for the values of solitarious locusts. When the gynandromorph was placed into an arena holding ten sexually mature gregarious females, it showed a distinct male behaviour: it jumped on a female and tried to mate with her. When kept together with males, males recognized this gynandromorph as a female because some of them tried to mount, although no successful copulation was observed. The results suggest that the gynandromorph might have had a female-specific pheromone. Dissection revealed that the gynandromorph had no testis but abnormal ovaries containing vitellogenic oocytes. These observations indicate that the gynandromorph obtained has a mixture of male and female morphological characteristics and behaves like a male but is recognized as a female by conspecific males.

1. Introduction

The gynandromorphy or sexual mosaic has both male and female characteristics and is known in various orders of insects (Wigglesworth, 1972). Although the occurrence of the gynandromorphy is very rare, several examples have been reported in locusts and grasshoppers (Potter, 1940; Severin, 1943, 1955; Morales Agacino, 1957; Dirsh, 1957; Pener, 1964; Uvarov, 1966). In most cases, external characters are male and female bilaterally, but dorso-ventrally in some cases. In a few cases, the internal morphology such as reproductive organs is also involved. Little information is available about behavioral characteristics of gynandromorphs.

During a study of phase polyphenism in the desert locust, *Schistocerca gregaria* (Forskål)(Orthoptera: Acrididae), an adult looking like a gynandromorph was found in the Tsukuba laboratory colony. The external reproductive organs of this individual have both male and female characteristics bilaterally. Although the general body structures are similar between the two sexes in locusts and grasshoppers, sexual dimorphism is observed in some internal and external morphological traits as well as body size (Uvarov, 1966). In the present study, several body dimensions of the gynandromorph are compared with those of normal males and females, and the internal morphology of the gynandromorphy is observed.

Locusts have sex-specific sexual behaviors and pheromones in both sexes (Uvarov, 1966, 1977; Hassanali *et al*., 2005). In *S. gregaria*, sexual behavior of a gynandromorph has been observed by Pener (1964). He described two gynandromorphs: both had ovaries but one had a complete set of male accessory glands and the other only the left (male side) set of male accessory glands. Both showed distinct male sexual behaviors, but with their aging this behavior disappeared. That result indicates that a sexual mosaic occurs in behavior as well as morphology. However, it remains unknown how normal males would have responded to the gynandromorphy. In the present study, the sexual behavior of a gynandromorph is observed when introduced to sexually mature males and females.

2. Materials and methods

2.1. Insects and rearing conditions

The colony of *S. gregaria* used has been described previously (Maeno & Tanaka, 2004) and isolated-reared locusts are called solitarious and crowd-reared ones gregarious. A gynandromorph appeared from a 3rd generation of a solitarious line. Solitarious nymphs and adults were reared individually in small cages (28 × 15 × 28 cm) except for a short period for mating and gregarious ones were kept in group of about 100 individuals in large cages (42 × 22 × 42 cm) at 32 ± 1 ℃, LD 16:8 h and 50-70% relative humidity, as described previously (Maeno *et al*., 2004). They were fed leaves of orchard grass and cabbage together with wheat bran.

2.2. Measurements of body dimensions

To determine the body dimensions, hind femur length (*F*), maximum head width (*C*) and fore wing length (*E*) were measured by using digital calipers (SC-15S, Mitsutoyo Co., Japan). Under isolated conditions, this locust has 5- or 6-nymphal stadia (Hunter-Jones, 1958). The gynandromorph obtained had 5-nymphal stadia. Because the number of ecdyses affects body size and classical morphometric ratios of *F/C* and *E/F* (Maeno *et al*., 2004), only individuals with 5-nymphal stadia were used for comparison.

2.3. Behavioral observations

To observe sexual behavior of the gynandromorph, two experiments were carried out 20 days after adult emergence. The gynandromorph was put into a polyethylene arena (20 x 28 x 10 cm) housing ten sexually mature gregarious females (ca. 3 weeks old) at 30 ℃. Only yellowish females were used for the experiments, because gregarious locusts change the body color from pinkish beige to yellow when they are sexually mature (Norris, 1954). The bottom of the container was covered with a sheet of brown paper and the top was covered with a clear acrylic plate to observe the behavior.

Mounting behavior was regarded as a male-specific sexual behavior (Uvarov, 1966, 1977). In each experiment, a crowd of ten locusts was first tested by introducing a single sexually mature normal female or male in the arena for 3 min to determine if any male-female or same-sex mounting behavior would occur. This preliminary test was repeated with three different individuals each. After the above tests, the gynandromorph was introduced to the crowd of ten females in the first experiment and their behavior was observed for 3 min. This procedure was repeated for a total of 5 times with a 1-min interval between observations. In the second experiment, basically the same procedure was repeated except that the gynandromorph was introduced to a crowd of 10 sexually mature males instead of 10 females. These males were derived from mounting male-female pairs under crowded conditions.

2.4. Statistical analysis

Inter-sex comparisons of body dimensions including *C*, *F* and *E* were made by a *t*-test using StatView 6. *F/C* and *E/F* ratios of isolated-reared and crowd-reared locusts were analyzed by the Mann-Whitney *U*-test.

3. Results and Discussion

3.1. External morphology

Figure 1B shows a ventral view of the abdominal tip of a gynandromorph together with a normal female (Fig. 1A) and normal male (Fig. 1C) of *S. gregaria* obtained under isolated conditions. The female has ovipositor valves separated along the mid-line at the abdominal tip, which are sclerotized to dig the ground for oviposition. The male sternite is more or less incurved and cerci projected from the both lateral sides. In the gynandromorph, the right half of the abdominal tip apparently represented the female structure (left side in Fig. 1B) and the left one the male (right side in Fig. 1B).

The general body shape was similar between normal males and females of *S. gregaria* (Fig. 1D and F). In general, body size is smaller in males than in females of

locusts and grasshoppers (Uvarov, 1966). The gynandromorph was smaller in body size than normal females, but similar to normal males (Fig. 1E). Head width was significantly smaller in males than in females (Fig. 2; *t*-test; $P<0.001$). The same was true for hind femur (Fig. 2A; *t*-test; $P<0.001$) and fore wing lengths (Fig. 2B; *t*-test; $P<0.001$). In the gynandromorph, a small difference was found in hind femur and fore wing lengths between the right and left half pairs. The scatter plots of head widths and hind femur lengths indicated that both hind femur length and head width of the gynandromorph were within the range for males (Fig. 2A). The same results were obtained for head width and fore wing lengths (Fig. 2B). These results may indicate that the external body dimensions of the gynandromorph showed male characteristics.

Locusts change their morphological characters depending on the population density (Faure, 1932; Uvarov, 1966). Solitarious locusts have a higher *F/C* ratio and a lower *E/F* ratio than gregarious ones in *S. gregaria* (Dirsh, 1951, 1953). In the present study, the *F/C* and *E/F* ratios of solitarious female locusts were significantly different from those of gregarious ones (Fig. 3A; Mann-Whitney *U*-test; $P<0.001$ each), as reported previously for the same strain (Maeno *et al*., 2004). Similar results were also obtained for males (Fig. 3B; Mann-Whitney *U*-test; $P<0.001$). The gynandromorph had a higher *F/C* ratio and a lower *E/F* ratio than did gregarious normal adults, but these ratios were similar to the values for solitarious individuals of both sexes (Fig. 3A and B).

3.2. Sexual behavior

Twenty days after adult emergence, the sexual behavior of the gynandromorph was observed by introducing the individual to sexually mature gregarious locusts. In the preliminary tests, it was observed that sexually mature gregarious females used were attractive to the male and did not show homosexual behavior. One hour after the preliminary tests which showed usual courtship behaviors, the gynandromorph was introduced to 10 females. It jumped on the back of one of the females within a few

seconds and after a few trials succeeded to mount a female just like a normal male (Fig. 4A). The gynandromorph was kicked hard by the female, but vibrated the hind legs and tried to copulate. The gynandromorph bent its abdomen to connect to the female's genitalia from either the right or left side, although it had male genital organs only on the left side (Fig. 4A). The observations were repeated 5 times with the same result, but no successful copulation occurred. These results indicated that the gynandromorph behaved as a male, as reported previously by Pener (1964).

After a 1-h interval, the second experiment using 10 sexually mature gregarious males was carried out. It has been reported that gregarious males of *S. gregaria* often mount on the back of other males if there is no female around (Loher, 1959) and is fairly common in the laboratory even when both sexes are present (unpublished observations). These results apparently indicated that at least some of the males used were sexually active. One hour after preliminary trials that indicated male-female but male-male interactions, the gynandromorph was introduced. Some males tried to jump on the back of the gynandromorph in this case and one of the males mounted on it within 3 min (Fig. 4B). The gynandromorph kicked hard the mounting male with the hind legs to shake him off. Some mounting males were actually shaken off, but others managed to stay on the gynandromorph, vibrated their hind legs and tried to copulate by bending the abdomen toward the genitalia of the gynandromorph. This behavior of mounting is referred to as a male-specific sexual behavior (Loher, 1959). In the experiment, mounting was observed in all 5 replications, but no copulation was successful. These results indicated that the gynandromorph was recognized as a female by males.

Normal females often show digging behavior related to oviposition when they are sexually mature even if they are virgin. In the present study, the gynandromorphy did not show such behavior.

After the behavioral observations, the gynandromorph was kept with two sexually mature gregarious virgin females in a small cage to determine if copulation would take

place. The gynandromorph jumped on the back of one of the females as soon as they were put together. When checked every 2 or 3 h, the gynandromorph was found mounting on the same female during the first two consecutive photo-phases, indicating that mounting probably lasted for one and half days. However, no genital connection between them was observed throughout the observations. Unlike another locust, *Locusta migratoria*, in which mounting occurs before copulation (Zhu & Tanaka, 2002 ; Tanaka & Zhu, 2003), males of *S. gregaria* usually display post-copulatory mounting to guard the female from rivals, because the last copulated male's sperm are used for fertilization (Hunter-Jones, 1960). In the present study, prolonged mounting by the gynandromorph was obviously pre-copulatory. This behavior was either abnormal or induced because copulation had never been realized. In the preliminary experiment, normal males also displayed prolonged 'pre-copulatory mounting' when kept together with females whose genitalia was coated with glue to prevent copulation (unpublished observation).

In locusts and grasshoppers, it has been demonstrated that visual, acoustic, chemical and tactile cues are used for mate finding and recognition (Whitman, 1990). In *S. gregaria*, acoustic signals are not important for the male response, but visual stimuli play a role in finding females, although visual stimuli alone are not enough to induce sexual behavior in males (Inayatullah *et al*., 1994; Ferenz & Seidelmann, 2003). *S. gregaria* uses sex pheromones for mate finding (Whitman, 1990; Byers, 1991; Ferenz & Seidelmann, 2003; Hassanali *et al*., 2005). At a low density, solitarious females emit a sex pheromone to attract males, and the pheromone enhances the meeting chance of the sexes (Inayatullah *et al*., 1994). In the present study, gregarious males jumped on the isolated-reared gynandromorph almost immediately after the latter was introduced to the arena. Therefore, it is possible that the gynandromorph had a female-specific sex pheromone. Another possibility is that the gynandromorph had no phenylacetonitrile (PAN) which is usually produced by sexually mature gregarious males as a courtship inhibition pheromone against rivals (Seidelmann & Ferenz, 2002; Seidelmann *et al*.,

2003). This pheromone is not produced by females and isolated-reared males (Seidelmann *et al*., 2000). It was likely that PAN was absent in the gynandromorph, either because it was an isolated-reared locust, and/or it was partially a female. Therefore, the possibility that the sexually mature gregarious males mounted the gynandromorph due to lack of PAN can not be ruled out. To examine this possibility, one sexually mature isolated-reared male (3 weeks old) was introduced into a group of ten sexually mature gregarious males, as it was done in the preliminary tests reported using crowd-reared individuals. The results showed that no mounting behavior was observed in any of the three trials using different isolated-reared males (data not presented). Therefore, lack of PAN does not seem to explain the present case.

After the behavioral observations, the body color of the gynandromorph turned yellow partially. Crowd-reared males turn yellow when they are sexually mature, but isolated-reared ones never do so even after they are sexually mature (Norris, 1954). The yellow color of the gynandromorph was probably induced by crowding experienced during the behavioral observations.

3.3. Internal morphology

The gynandromorph died naturally on the 28th day of adult emergence and it was dissected immediately. The autopsy revealed that this individual had no testis but a pair of ovaries. In *S. gregaria*, the ovary of a normal female has a total of 85 – 145 ovarioles and is connected to the oviduct (Uvarov, 1966). In the gynandromorph, however, the left ovary was vestigial with no ovarioles and the right half was also small with only 11 ovarioles. Only the latter was connected to the oviduct. Two ovarioles contained small oocytes with vitellogenin. The gynandromorphy had female accessory glands, but neither the female spermatheca nor male accessory glands were found. Thus, the internal reproductive organs of this individual were female, but the structure was abnormal. According to Pener (1964), a gynandromorph which had both male and female external genetalia bilaterally also had no testis but many ovarioles containing

full size eggs. That gynandromorph also showed distinct male sexual behaviors in response to normal females. However, he did not expose the gynandromorph to a normal male so that it remains unknown whether the gynandromorph would have been recognized by females or not. In the present study, the gynandromorph behaved like a male against females, but it was recognized as a female by conspecific males.

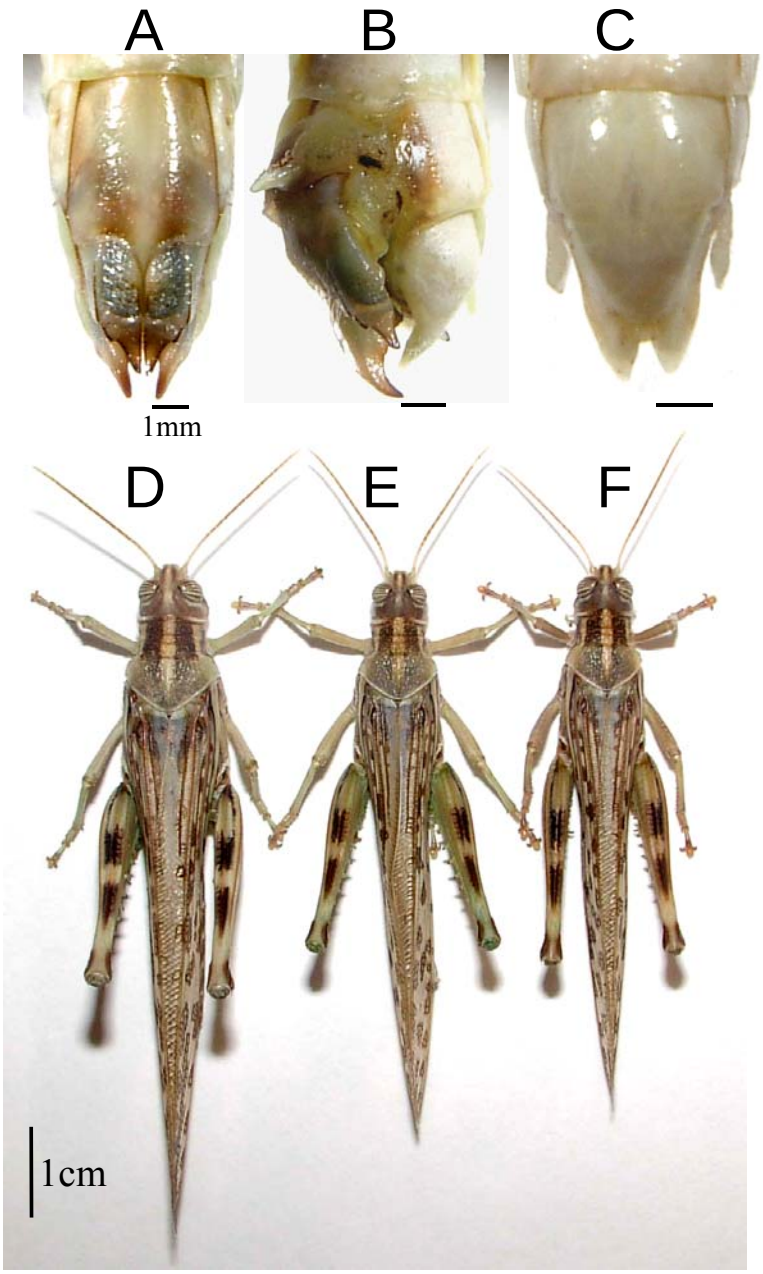

Fig. 1. Ventral view of the abdominal tip and body sharp of a normal female (A, D), a normal male (C, F) and a gynandromorph (B, E) of *Schistocerca gregaria*.

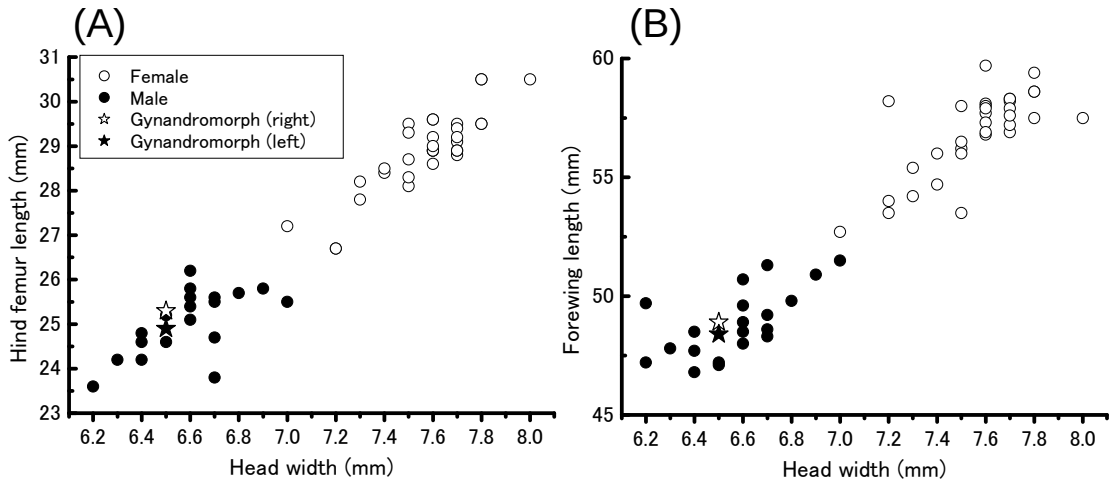

Fig. 2. Scatter plots of head widths and hind femur lengths (A) or fore wing lengths (B) of isolated-reared normal females (○), males(●) and gynandromorph (right, ☆; left, ★) of *Schistocerca gregaria*.

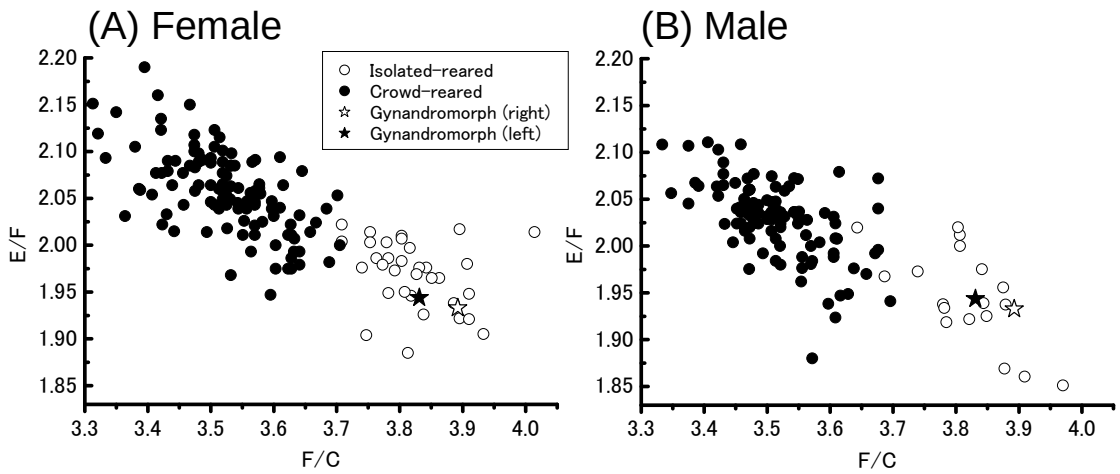

Fig. 3. Scatter plots of *F/C* and *E/F* ratios (*F*, hind femur length; *C*, maximum head width; *E*, fore wing length),of isolated-reared (○) and crowd-reared (●) females (A) and males (B), and an isolated-reared a gynandromorph (right, ☆; left, ★) (A, B) of *Schistocerca gregaria*.

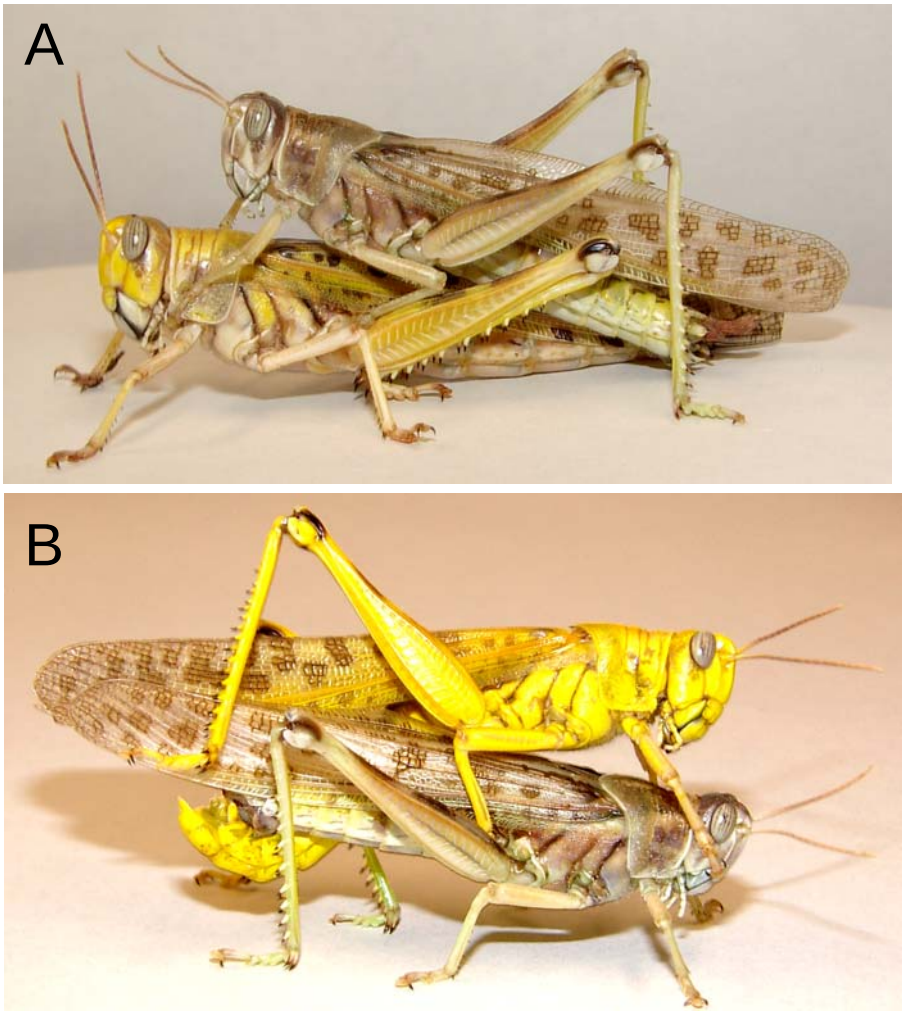

Fig. 4. Sexual behavior of a gynandromorph of *Schistocerca gregaria*. Note that the gynandromorph (top in A) mounted on a female (bottom in A), whereas it (bottom in B) was mounted by a male (top in B).

Chapter 2

Effects of hatchling body color and rearing density on body coloration in last-stadium nymphs of the desert locust, *Schistocerca gregaria*

Abstract

The influences of hatchling character and rearing density on body color at the last-nymphal stadium are investigated for the desert locust, *Schistocerca gregaria*. Hatchlings are divided into five groups based on the darkness of the body color and reared either under isolated or crowded conditions. Two types of body color variation at the last-nymphal stadium are separately analyzed, i.e. the background color and black patterns. Under isolated conditions, the background body color is either greenish or brownish. Most individuals are greenish and the highest percentage of brownish insects is obtained from hatchlings with the darkest body color. Under crowded conditions, the background color is yellow or orange and the percentage of yellowish nymphs tends to decrease when they are darker at hatching. The intensity of black patterns differs depending on the body color at hatching and subsequent rearing density. Most isolated-reared nymphs exhibit few or no black patterns, but nymphs with some black patterns also appear, particularly among those that had been dark at hatching. Under crowded conditions, the black patterns become more intensive when they are darker at hatching. Therefore, last-stadium nymphs with typical solitarious or gregarious body coloration appear when they have the phase-specific body coloration at hatching as well. The present results demonstrated that both body color at hatching and rearing density during nymphal development influenced the body coloration at the last- nymphal stadium.

1. Introduction

The body-color polyphenism is common in locusts and grasshoppers (Faure, 1932; Rowell, 1971; Dearn, 1990). In the desert locust, *Schistocerca gregaria* (Forskål)(Orthoptera: Acrididae) and the migratory locust, *Locusta migratoria,* solitarious nymphs assume cryptic or camouflaged green or brown body color with few or no black patterns. Such body coloration seems to be adaptive, because nymphs with conspicuous body colors against the habitat background color are likely to be found easily by predators (Isely, 1938). On the other hand, under crowded conditions, gregarious nymphs display bright yellow or orange body color with intense black patterns. Such body coloration is often suggested to be an aposematic or warning signal because nymphs sometimes eat toxic plants (Key, 1957; Sword, 2002). Predators such as birds or lizards which experience unpalatable insects may then correctly identify and subsequently avoid attacking such prey. However, gregarious nymphs of *L. migratoria* develop black patterns with an orange background color, but there is no evidence that they feed on toxic plants. The intensive black patterns might be related to thermoregulation, because locusts with a dark body color absorb heat more efficiently than those with a light body color (Pepper & Hastings, 1952; Joern, 1981). It also seems possible that gregarious body color may serve as a visual signal for nymphal aggregation or group cohesion during marching, but experiments using albino locust failed to support this hypothesis (Gillett, 1973).

Body color at hatching is influenced by the rearing conditions of the parents in *S. gregaria* and *L. migratoria* (Faure, 1932; Hunter-Jones, 1958; Islam *et al*., 1994). Hatchlings of *S. gregaria* show a conspicuous body color variation with intensive black patterns ranging from pale green to entirely black, and a similar tendency is also observed in *L. migratoria*, although no green hatchlings occur in this species (Husain & Ahmad, 1936; Gunn & Hunter-Jones, 1952; Hunter-Jones, 1958; Stower, 1959; Islam *et al*., 1994; McCaffery *et al*., 1998; Bouaichi & Simpson, 2003). In both species, the density of parents as nymphs does not affect hatchling body color, but their density as adults does (Hunter-Jones, 1958).

Gregarious characteristics of hatchlings such as body color and gregarious behavior of *S. gregaria* have been suggested to be influenced by a pheromonal factor derived from the

accessory gland of the female parent (McCaffery *et al*., 1998; Simpson *et al.,* 1999; 2005). According to their studies, this factor is a small hydrophilic compound (<3 kDa), present only in gregarious females and secreted into the foam plug of egg pod. It is effective only during a short time of deposition and washing presumptive gregarious eggs with saline or even separating them without washing at deposition causes them to turn into solitarized hatchlings. Although these results may well explain the generational cumulative effect of populations that are either continuously solitarious or gregarious, recent studies by Tanaka and Maeno (2006) have completely failed to reproduce these results and demonstrated that presumptive gregarious eggs separated even before the foam plug deposition produce dark-colored hatchlings, casting doubts on the presence of the recently suggested pheromonal factor on the body color of the hatchlings.

It has been reported that the effect of rearing density during the nymphal stage masks the inherited effect in the nymphal body color, so that isolated-reared nymphs develop the solitarious coloration and crowded ones gregarious coloration, regardless of the parental treatment in *S. gregaria* but not in *L. migratoria* (Hunter-Jones, 1958). However, no detailed studies have been reported to document this phenomenon. In this study, the effects of the maternal factor, i.e. hatchling body color, are separated from the rearing density during nymphal development on the body coloration at the last-nymphal stadium in *S. gregaria*. The present paper discusses the significance of the results in relation to phase polyphenism.

2. Materials and methods

2.1. Insects and rearing conditions

The colony of *S. gregaria* used in the present study was derived from a stock that had been maintained at the International Center for Insect Physiology and Ecology (ICIPE), Nairobi, Kenya. The latter was originally derived from the Desert Locust Control Organization for Eastern Africa in Addis Ababa, Ethiopia. Nymphs and adults were reared in either large (42 × 22 × 42 cm) or small cages (28 × 15 × 28 cm) at 32 ± 1 ℃, LD 16:8 h and 50-70% relative

humidity, as described previously (Maeno *et al*., 2004). The bottom of each cage was covered with a sheet of brown paper to remove their faeces easily. Locusts were provided with leaves of orchard grass and cabbage as well as wheat bran. The grass was inserted into water jars (250 mL) and changed every other day. A plastic cup (8-cm diameter; 10-cm high) filled with moist sand was put in each cage for oviposition, and the egg pods laid in the cup were removed from the cage every day. The egg pods in cups were kept at 30 ℃ or 32 ℃ to control the time of hatching. A gregarious line was kept in group (about 100 individuals per large cage) for at least 20 generations in the Tsukuba laboratory. To establish a solitarious line, hatchlings produced by crowd-reared parents (gregarious line) were held individually in Petri dishes (9 × 2 cm) within 24 h of hatching and provided with leaves of orchard grass, cabbage and wheat bran. Upon ecdysis to the 2nd stadium, they were reared individually in small cages. Adults of the solitarious line were also kept in isolation except for a few days for mating to minimize the crowding effect from the male, because crowding or the number of adults per cage influences the body color of their progeny at hatching (Hunter-Jones, 1958). Most hatchlings of the 2nd solitarious generation were green in color. Some isolated-reared female adults were kept either in isolation or in groups to obtain hatchlings with different colors. Twenty days after adult emergence, each of sexually mature isolated-reared females was paired with a sexually mature male for various lengths of time. The duration of pairing ranged from 10 h to 10 days. Hatchlings tended to have more extense black patterns as the duration of pairing of their parents had been longer (data not shown). The 1st, 2nd, 5th and 6th solitarious generations were used for the present experiments. Hatchlings from each egg pod were equally divided into two groups that were then reared under isolated or crowded conditions.

2.2. Scoring of hatchling coloration

Hatchling body coloration was scored on the day of hatching using five color grades, ranging from entirely green to heavily black, according to the classification of Islam *et al*. (1994); 1, background color uniformly green with no black pattern; 2, background color green with some black markings (not more than 30% of the body surface); 3, background color

green or background color almost obscured by black markings (more than 30% of the body surface), prominent femoral black stripes and light-colored eyes; 4, pale background color almost obscured by black markings (60-80% of the body surface) and dark-colored eyes; 5, background color entirely obscured by black markings (more than 80% of the body surface). In the present study, these five categories will be called hatchling color groups (HCGs). To evaluate how accurately the darkness of the body would represent the body color of an individual, another method was adopted to quantify the darkness of body color according to Tanaka (2003, 2004a, b); after a hatchling was immobilized on ice, it was photographed using a scanner (Epson ES 2000, Japan) connected to a personal computer (Epson, Type-MA, Japan) using commercial software, Photoshop 7.0 (Adobe Systems Incorporated, San Jose, California). Each nymph was placed with one side down on the glass table of scanner for photographing. The image type used was 24-bit color at a resolution of 400 d.p.i. Using the histogram function, luminance of the head and femur was measured. Because the mean and median values were almost identical (Tanaka, 2003), the mean luminance was recorded for each individual.

2.3. Assessment of coloration of last-stadium nymphs

To examine the effects of hatchling color and rearing density on the body coloration at the last-nymphal stadium, the last-stadium nymphs (5^{th} or 6^{th}) obtained were photographed as described above, and the background color of the body was recorded. It was either greenish or brownish under isolated conditions, whereas it was yellow or orange under crowded conditions (Fig. 1). The distinction between the yellow and orange body types was made based on the color on the thorax and legs. Isolated-reared individuals attained the last-nymphal stadium after 4 or 5 moults. However, the number of ecdyses before reaching the last-nymphal stadium did not cause any significant difference in results, e.g. for HCG 1 ($\chi^2 = 1.53$, d.f. = 1, $P > 0.05$; n=140). Under crowded conditions individuals reaching the last (6^{th}) nymphal stadium after 5 moults were also observed, but the numbers were small and the data for 5^{th} and 6^{th} stadium individuals at the last-nymphal stadium were combined. Insects were also

categorized into 3 grades based on the intensity of black patterns on the head and pronotum according to the classification of Hunter-Jones (1958) and Stower (1959). Examples representing these grades are given in Figure 2. These black pattern grades will be abbreviated as BPGs. Females and males were pooled, because no sex difference was found; e.g. no significant sex difference was found in the percentage of individuals in different BPGs for HCG 5 ($\chi^2 = 0.23$, d.f. = 1, $P > 0.05$; n=85). Locusts with their antenna lost or injured during nymphal development were discarded, because a loss of body parts is known to affect the body color (Mordue (Luntz), 1977).

3. Results

3.1. Body coloration of hatchlings

Hatchlings produced by female adults kept with a male for various lengths of time were categorized into five HCGs and their luminance values of the head and femur were measured to evaluate the relationship between HCGs and luminance values (Fig. 3). The mean luminance value of the head was significantly different among different HCGs (Scheffe's-test, $P < 0.05$; Fig. 3A); the mean ± SD for HCGs 1-5 was 118.2±6.1, 95.2±8.4, 68.1±8.1, 44.6±8.9 and 22.9±5.7 (n=30 each), respectively. Hatchlings of HCG 1 had the highest mean (or the brightest), and those of HCG 5 the lowest (or the darkest), although the variation was continuous. Similar results were obtained for the femur (Scheffe's-test, $P < 0.05$; Fig. 3B); the mean ± SD for HCGs 1-5 was 134.3±8.3, 107.0±10.3, 77.9±11.1, 57.5±8.4 and 41.1± 10.0 (n=30 each), respectively. These results demonstrated that the darkness of hatchling body color greatly varied among individuals, and that HCGs would give reliable groupings of hatchlings closely correlated to the darkness of body color.

3.2. Background colors of last-stadium nymphs

Figure 4 summaries the percentages of last-stadium nymphs with different background colors under isolated and crowded conditions in nymphs from different HCGs. Under isolated conditions, the background color was either green or brown, and the percentage of greenish

individuals was 95, 96, 100, 88 and 66% in nymphs from HCGs 1, 2, 3, 4 and 5, respectively (Fig. 4A). These proportions were significantly different from one another (χ^2 = 28.24, d.f. = 4, P < 0.001). The percentage of brownish individuals was the highest in the nymphs from HCG 5. Under crowded conditions, either yellow or orange background body color was observed (Fig. 4B). The percentage of yellowish individuals was 50, 39, 50, 31 and 11% in nymphs from HCGs 1, 2, 3, 4 and 5, respectively, and was significantly different among HCGs (χ^2 = 43.84, d.f. = 4, P < 0.001) and tended to be smaller when the body color at hatching was darker. These results indicated that the background color of last-stadium nymphs was influenced not only by the rearing density during nymphal development but also by the body color at hatching and the latter exerted its influence more strongly under crowded conditions than under isolated conditions.

3.3. Black patterns of last-stadium nymphs

Under isolated conditions, nymphs from all HCGs developed head coloration of either BPG 1 or 2 (Fig. 5A). Most nymphs had a light-colored head (BPG1), although the percentage of such individuals was significantly different among different HCGs (χ^2 = 52.70, d.f. = 4, P < 0.001). The percentage of BPG 2 heads was highest in individuals from HCG 5. Similar differences related to HCGs were obtained for the pronotum (χ^2 = 45.85, d.f. = 4, P < 0.001; Fig. 5B). Under crowded conditions, on the other hand, heads were relatively dark and only BPGs 2 and 3 were observed (Fig. 5C). The percentage of different grades was significantly different among HCGs (χ^2 = 146.71, d.f. = 4, P < 0.001). The percentage of nymphs with blackish heads, i.e. BPG 3, was larger when they had been darker at hatching. Similar differences were obtained for the pronotum (χ^2 = 166.42, d.f. = 4, P < 0.001; Fig. 5D). These results indicated that the body coloration of hatchlings and rearing density influenced the intensity of black patterns at the last-nymphal stadium, and that the former had a significant impact especially under crowded conditions.

4. Discussion

Nymphs of *S. gregaria* have two main types of body-color polyphenism, so-called green-brown polyphenism and phase- or density-dependent body-color polyphenism (for reviews, see Rowell, 1971; Fuzeau-Braesch, 1985; Dearn, 1990; Pener, 1991; Tanaka, 2001, 2006). In *S. gregaria,* the former is expressed only under low-density conditions and observed in solitarious nymphs. The latter is affected by crowding and observed in transient and gregarious nymphs. Hatchlings of *S. gregaria* also show body-color variation that is influenced by the crowding experienced by their mother (Faure, 1932; Hunter-Jones, 1958; Islam *et al*., 1994). Hunter-Jones (1958) suggested that the effect of rearing density during the nymphal stage completely masks any maternal influence on color differences at hatching in *S. gregaria.* However, the present study demonstrates that body color at hatching influences the expression of body coloration at the last-nymphal stadium. The latter consists of two types of polyphenism associated with the background color and the black pattern (Stower, 1959; Pener, 1991), which are analyzed separately. Under isolated conditions, most nymphs are of green type, but a relatively high percentage of brown-type nymphs is obtained from darkest hatchlings (Fig. 4). Under crowded conditions, on the other hand, yellow and orange types appear and the percentage of yellow-type nymphs tends to decrease when they are darker at hatching. These results indicate that not only rearing density but also body color at hatching influences the expression of body color under both isolated and crowded conditions.

The intensity of black patterns at the last-nymphal stadium is also influenced by body color at hatching. Most isolated-reared nymphs exhibit few or no black patterns, but a small proportion of nymphs develop some black patterns. The percentage of such individuals tends to increase when they are darker at hatching. Under crowded conditions, on the other hand, all last-stadium nymphs develop black patterns and some show very intensive black patterns (BPG 3). The head and pronotum of the latter are almost completely black. Such body coloration appears most frequently in individuals from the darkest hatchling group.

The differences in results between the present study and that of Hunter-Jones (1958) are probably caused by the differences in rearing conditions. Hunter-Jones (1958) reared locusts at

relatively low humidity and used different group sizes. In general, high relative humidity is favorable for the development of green-type nymphs, and low humidity brown-type ones (Pener, 1991). The various group sizes used by Hunter-Jones could have induced different degrees of expression of body-color polyphenism, because body coloration of last-stadium nymphs tends to be more gregarious (or darker) with increasing the group size (Gunn & Hunter-Jones, 1952). In the present study, a special care is taken to keep the relative humidity high (>50%) in the cages and to maintain the group size relatively constant (about 80-120 individuals/cage).

The present results thus demonstrate that body color at hatching and rearing density during nymphal development are important factors determining the induction of black patterns at the last-nymphal stadium in *S. gregaria*. Very black last-stadium nymphs typical for gregarious forms are obtained more frequently when they are darker at hatching. Hatchlings with lighter body color may develop black patterns in the last-nymphal stadium under crowded conditions, but the intensity is generally less.

It may be beneficial to discuss the ecological significance of the density-dependent body-color polyphenism in the locusts in relation to the present results. At low density, complete cryptic body color matching their habitat background color on which they live could be required for locusts, because predators might easily find prey with body color contrasting to their habitat (Isely, 1938). It is thought that both hatchlings derived from the solitarious and gregarious phase retain the ability to assume camouflaged body color under low density conditions. On the other hand, it is often argued that gregarious body coloration is an aposematic signal (Key, 1957; Sword, 2002). However, the possible function as a cryptic coloration has not been investigated. Cuthill *et al*. (2005) showed disruptive color effects in which patterns on the body's outline promote concealment and highly contrasting colors enhance survival rate by exposing artificial moth-like targets to birds. This may suggest a possibility that conspicuous black and yellow or orange coloration in *S. gregaria* might provide an advantage to be cryptic in their fluctuating habitats. This hypothesis may be consistent with the occurrence of black patterns with a different background color in other

acridids such as *L. migratoria* (Faure, 1932), the American grasshopper, *Schistocerca americana* (Tanaka, 2004a) and an acridids, *Nomadacris japonica* (S. Tanaka, unpublished) that are not known to consume toxic plants.

Little information is available about how black hatchlings develop more intensive black patterns in later nymphal stages than do green ones under crowded conditions. One possibility is that the difference might be caused by different activity levels of hatchlings. Crowding and tactile stimulation is the most important gregarizing factor in locusts (for reviews, see Uvarov, 1966, 1977; Pener, 1991, 1998; Applebaum & Heifetz, 1999; Simpson *et al*., 1999). First-stadium nymphs with black body color move more actively than those with green color (Islam *et al*., 1994). Therefore, the former may receive more frequent mutual tactile stimulation, which might explain the present results. However, solitarious hatchlings start showing gregarious behavior if they are kept crowded after hatching (Islam, 1996), so that behavioural differences between green and black hatchlings do not account for the present results.

Recently, the mechanism controlling phase polyphenism in locusts has been studied intensively since the discovery of Sca-corazonin ([His^7]-corazonin) as a dark-color inducing factor (for reviews, see Tanaka, 2001, 2006; De Loof *et al*., 2006). This peptide exhibits other physiological activities. A shift in adult morphometrics toward the characteristics typical for gregarious forms is brought about when solitarious (isolated-reared) nymphs are injected with Sca-corazonin in *S. gregaria* (Hoste *et al*., 2002; Maeno *et al*., 2004) and *L. migratoria* (Tanaka *et al*., 2002). The peptide also influences development of the number of olfactory antennal sensilla in the two locusts (Yamamoto-Kihara *et al*., 2004; Maeno & Tanaka, 2004). Injection of the peptide induces the black patterns characteristic of gregarious forms in *L. migratoria* and *S. gregaria* (Tawfik *et al*., 1999; Tanaka, 2001). In the former, various non-green dark body colors observed in solitarious forms are also induced in albino nymphs by injections of Sca-corazonin (Tanaka, 2000a, b). It is highly likely that the physiological condition of hatchlings influences the titer of this neurohormone, resulting in various intensities of black patterns of the last-nymphal stadium in *S. gregaria*. However,

Sca-corazonin induces neither orange nor yellow colors characteristic of gregarious nymphs in *S. gregaria* (Tanaka, 2001). Thus, it has been suggested that another hormone(s) is involved in the induction of these body colors. The present study establishes a method to produce nymphs with typical gregarious body coloration and this method will be useful to identify the unknown factor responsible for the gregarious background color.

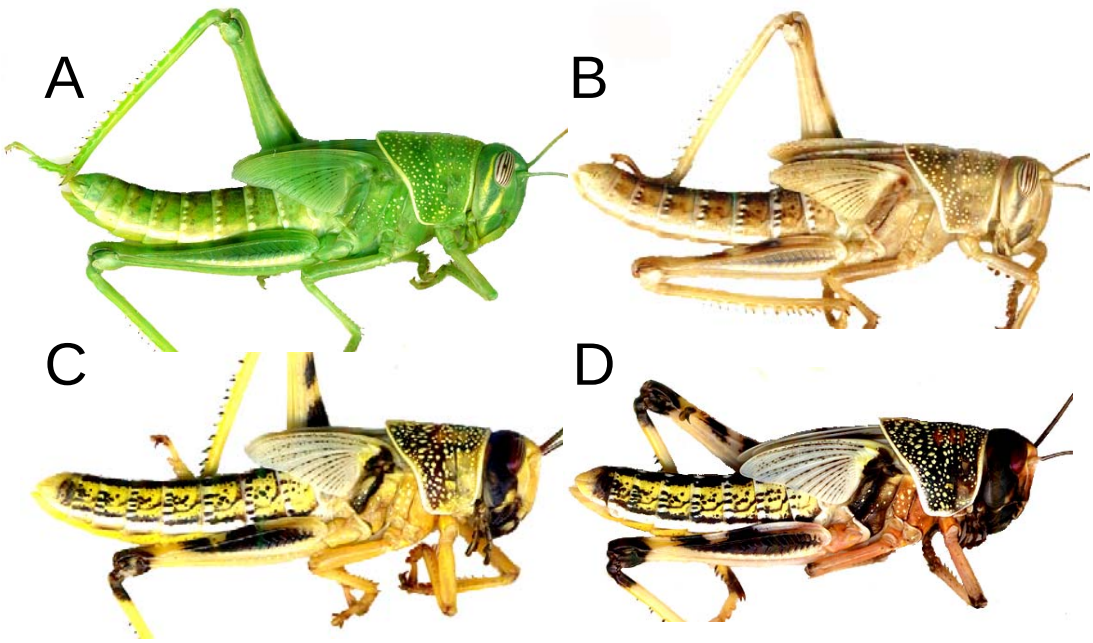

Fig. 1. Photographs showing green (A), brown (B), yellow (C) and orange (D) background body colors in last-stadium nymphs of *Schistocerca gregaria*. Isolated-reared nymphs (A and B) and crowded-reared nymphs (C and D).

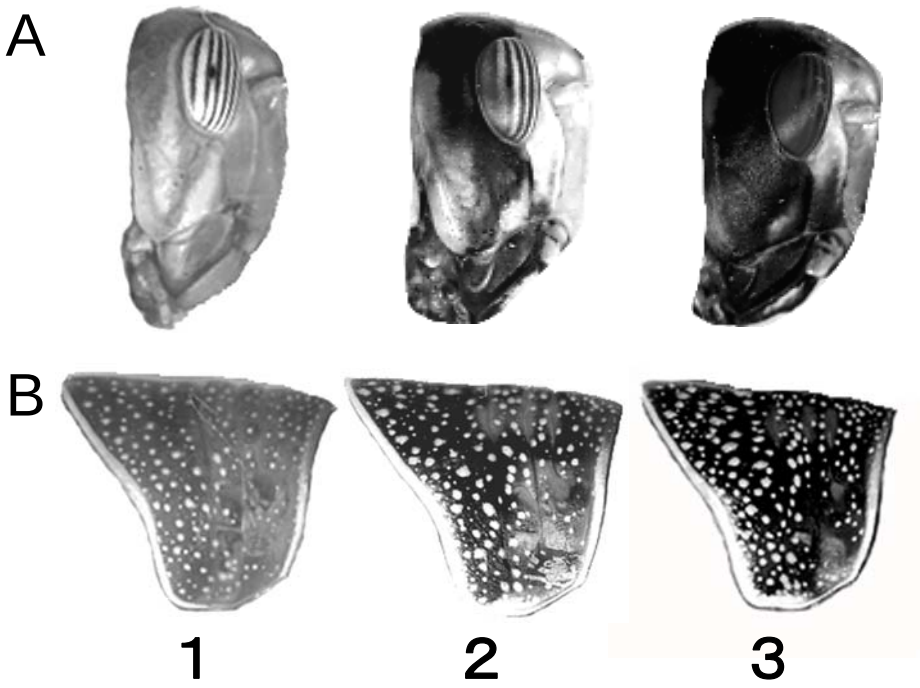

Fig. 2. Black pattern grades (BPGs) of heads (A) and pronota (B) in the last-stadium nymphs of *Schistocerca gregaria*; grade 1, no or few black patterns; grade 3, intensive black patterns; grade 2, intermediate between grade 1 and 3.

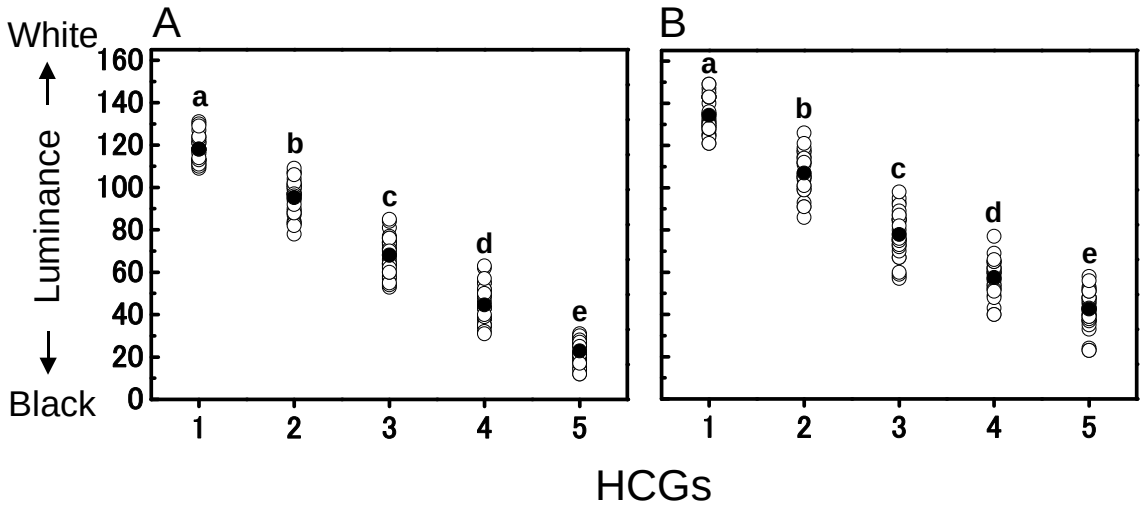

Fig. 3. Mean luminance values for the head (A) and femur (B) of hatchlings of different hatchling color groups (HCGs). Open circles indicate individual datum points and closed ones the means. Different letters in each panel indicate significant differences at $P < 0.05$ by Scheffe's test. n=30 each.

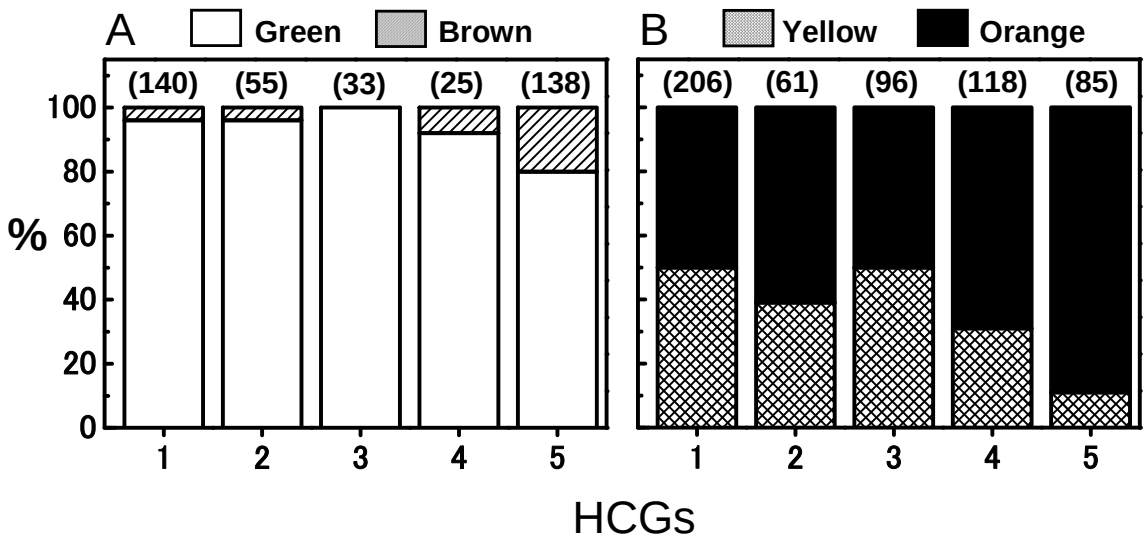

Fig. 4. Percentages of different background colors at the last-nymphal stadium in *Schistocerca gregaria* from different hatchling color groups (HCGs) reared under isolated (A) and crowded conditions (B). The numbers in parentheses indicate *n*. For background colors of last-stadium nymphs, see Figure 1.

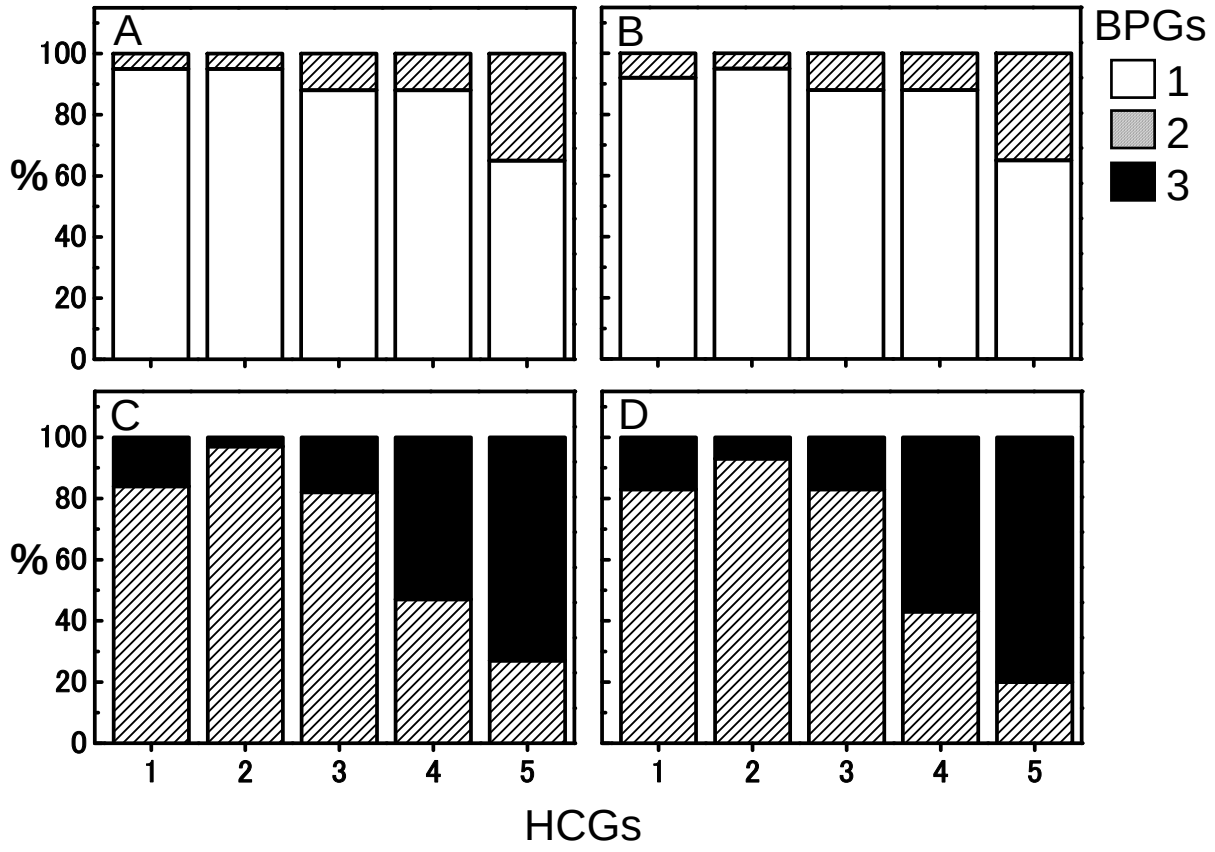

Fig. 5. Percentages of heads (A and C) and pronota (B and D) in different grades at the last-nymphal stadium in *Schistocerca gregaria* from different HCGs reared under isolated (A and B) or crowded (C and D) conditions. For black pattern grades (BPGs) of last-stadium nymphs, see Figure 2. Sample sizes are given in Figure 4.

Chapter 3

Phase-specific developmental and reproductive strategies in the desert locust.

Abstract

Locusts modify developmental and reproductive traits over successive generations depending on the population density. A trade-off between developmental rate and body size and between progeny size and number is often observed in organisms. In this study, we present evidence that this rule is evaded by desert locusts, *Schistocerca gregaria* Forskål, which often undergo outbreaks. Under isolated conditions, large hatchlings typical of the gregarious forms grow faster but emerge as larger adults than do small hatchlings typical of the solitarious forms, except for some individuals of the latter group that undergo extra molting. Under crowded conditions, large and small hatchlings grow at a similar rate, but the former become larger adults than the latter. Small hatchlings show a trade-off between development time and body size at maturation, but this constraint is avoided by large hatchlings. Phase-specific as well as body size–dependent differences are also detected in reproductive performance. As adult body size increases, females of a solitarious line produce more but slightly smaller eggs, whereas those of a gregarious line produce more and larger eggs. Total egg mass per pod is larger in gregarious forms than in solitarious forms. A trade-off between egg size and number is shown by a solitarious line but not by a gregarious line that produces relatively large eggs with similar numbers of eggs per pod. These results suggest that phase transformation involves not just a shift of resource allocation but also an enhanced capability expressed in response to crowding.

1. Introduction

Locusts often undergo outbreaks that last over several generations (Lecoq, 2005; Huis *et al.*, 2007). The desert locust, *Schistocerca gregaria* Forskål, and the migratory locust, *Locusta migratoria*, show density-dependent phase polyphenism in behavioral, morphological and physiological characteristics (Faure, 1932; Uvarov, 1966, 1977; Pener, 1991; Heifetz & Applebaum, 1995; Pener & Yerushalmi, 1998). Although the behavioral aspect has been studied intensively (Simpson et al., 1999; Seidelmann & Ferenz, 2002; Ferenz and Seidelmann, 2003; Tanaka & Zhu, 2003; Hassanali *et al.*, 2005), the morphological and developmental changes are also important in explaining locust outbreaks, because some of these characteristics are directly related to population growth. In this study, I present evidence suggesting that solitarious and gregarious locusts of *S. gregaria* show different patterns of nymphal development and reproduction in response to crowding conditions. That is, solitarious (isolation-reared) nymphs are constrained by a trade-off between developmental time and the final body size attained, whereas gregarious (crowd-reared) nymphs evade this constraint and grow faster or as fast as solitarious ones without getting smaller as adults. In this study, I produced hatchlings of various body sizes ranging from small to large, typical for solitarious and gregarious forms, respectively, and reared them under either isolated or crowded conditions to determine the effects of hatchling body size on nymphal growth and adult body size. Few studies have manipulated progeny size and investigated the influence of juvenile size on growth rates in arthropods (Fox *et al.*, 2000).

In studies to compare reproductive performance, locusts are often kept in small cages individually or in large cages as a group. Although the differences in the reproductive potential between crowded and isolated locusts are well documented, the conclusions are not always consistent. Certain factors such as competition for food and egg-laying space, relating more to the experimental methods than to real phase characteristics, may contribute to these differences (Pener, 1991). In this study, I minimized such differences by rearing all mated females individually in cages of the same size with either two males (crowded conditions) or with no males (isolated conditions). By collecting eggs from females with known body size, I analyzed

the relationships between adult body size and fecundity in terms of egg size, egg number and egg biomass per egg pod between solitarious and gregarious lines.

2. Materials and Methods

2.1. Insects and rearing conditions

The *S. gregaria* colony used in the present study has been described (Tanaka & Yagi, 1997). The rearing method was described elsewhere (Maeno & Tanaka, 2007). Briefly, nymphs and adults were reared at 32 ± 1°C under a light-dark 16:8-h photoperiod and 40–70% relative humidity in a well ventilated room. Locusts were kept either in a group of 100 individuals in a large cage (42 × 22 × 42 cm; crowded conditions) or in isolation in small cages (28 × 15 × 28 cm; isolated conditions). They were supplied with fresh leaves of orchard grass, cabbage and wheat bran. A gregarious line had been maintained at a density of ca. 100 individuals for more than 20 generations, and a solitarious line was established from the gregarious colony by rearing nymphs and adults individually in small cages, except for a short period for mating (Maeno & Tanaka, 2007). Plastic cups (diameter, 9 cm; height, 5 cm) filled with clean moist sand were placed in cages to collect egg pods. All experiments were carried out with 3rd and 4th solitarious generations and with >20th gregarious generations. The grass used was raised by the Field Management Section of NIAS at Ohwashi.

2.2. Hatchling size groups

Body size and color at hatching are closely correlated; for example, hatchlings with darker body coloration are heavier (Hunter-Jones, 1958; Tanaka & Maeno, 2006). I used body color to divide hatchlings into five groups of different body sizes. To obtain hatchlings of various sizes, each of 30 20-day-old sexually mature isolation-reared females was paired with a sexually mature male for various lengths of time ranging from 10 hours to 10 days (Maeno & Tanaka, 2007). Females kept longer with a male tended to produce hatchlings with more extensive black patterns. Hatchling body coloration was scored on the day of hatching using five color grades ranging from entirely green to heavily black, as described (Maeno & Tanaka, 2007). In this

study, these five categories were called hatchling size groups, or HSGs. Hatchlings of each HSG to be reared in isolation had been weighed individually. The mean and SD were 13.0 ± 1.6 mg (n = 50), 14.8 ± 1.3 mg (n = 50), 15.8 ± 0.9 mg (n = 50), 17.2 ± 0.9 mg (n = 50) and 19.8 ± 1.3 mg (n = 50) in HSGs 1, 2, 3, 4 and 5, respectively.

2.3. Determination of the number of nymphal stadia

S. gregaria normally passes through five or six nymphal stadia (Rao & Gupta, 1939; Maeno *et al.*, 2004). Two methods were used to determine the number of nymphal stadia; in one method, the number of nymphal stadia was counted by checking the nymphs every day for ecdysis. In the other method, the eye stripes in the adult stage were counted. Adults with five nymphal stadia have six eye strips, whereas those with six nymphal stadia have seven (Rao & Gupta, 1939). Under crowded conditions, it was difficult to follow the history of ecdyses for each individual, so only the second method was used.

2.4. Measurements of adult body weight

Within 24 hours after the final molt, adults that had not started feeding were weighed to give an estimation of adult size. The weight gain per day was determined by dividing adult body weight by the number of days required for nymphal development. In this case, the hatchling body weight (7–25 mg) was not subtracted from the adult body weight (ca. 1000–2600 mg) because it was small and the differences among individuals were negligible.

2.5. Egg pod collection and measurements of egg size and egg number

Egg pods were collected from female adults of a solitarious line, as described above. Females of a gregarious line were weighed at adult emergence and marked individually with white paint (Pentel, EZL31-W, Japan). They were kept together with males in a group of about 100 individuals in a large cage during the first 12 days of adult life. To obtain egg pods from individual females with known body size, females were removed from the large cage and held individually in small cages with two sexually mature males. In *S. gregaria*, pairing of a female

with a single male induces crowding effects on the progeny that are as strong as rearing her with many males (Hunter-Jones, 1958). Egg pods collected were incubated at 32°C ± 1. Egg length was measured using an ocular micrometer installed in a microscope 2 days after oviposition. A total of 10 eggs were randomly chosen from each egg pod and placed on a piece of moist filter paper (9 cm diameter) to avoid desiccation before measurements. The number of eggs per egg pod was also counted at that time. In my unpublished observations, egg weight (y, mg) was highly correlated with egg length (x, mm) ($y = 2.09x - 7.288$; $r = 0.939$; $n = 200$; $P < 0.001$). Thus, this equation was used to estimate egg weight from egg length. I adopted this method because eggs were often coated with sand particles glued with egg foam and removing them without damaging the eggs was very time consuming. After measurements, eggs were returned to moist sand and incubated at the same temperature until hatching. Only egg pods deposited after 25 days of adult emergence were used.

2.6. Statistics

Data for developmental and reproductive traits were mainly compared by a t-test or ANOVA using Stat View, version 6 (SAS Institute, Cary, North Carolina, USA). The number of eggs that correlated with adult body size was also analyzed by ANCOVA when it was appropriate. Ratios of egg biomass to adult body size were analyzed by the Mann-Whitney *U*-test.

3. Results

3.1. Nymphal development and density

Desert locusts undergo either five or six nymphal stadia, as mentioned above. First, I examined the effects of hatchling body size and rearing density on this trait because it affects the duration of nymphal development. Hatchlings categorized into five different hatchling size groups (HSGs) were reared under either isolated or crowded conditions. HSG 1 consists of smallest hatchlings typical of solitarious forms, whereas HSG 5 comprises largest ones typical of gregarious forms. The incidence of nymphs exhibiting six stadia depended on HSG, rearing

density and sex (fig. 1). The highest incidence was obtained when females derived from HSG 1 (smallest hatchlings) were reared under isolated conditions. No nymphs exhibiting six stadia appeared from HSG 5 (largest hatchlings) in females or from HSGs 4 and 5 in males. The incidence of nymphs exhibiting six stadia within HSG 1 was higher under isolated conditions than under crowded conditions in either females ($\chi^2 = 10.429$; df = 1, 503; $P < 0.001$) or males ($\chi^2 = 5.310$; df = 1, 354; $P < 0.05$). The duration of nymphal development was significantly shorter in locusts with five nymphal stadia (mean ± SD =30.0 ± 2.2 days, $n = 175$ for females and 29.2 ± 2.2 days, $n = 152$ for males) than in locusts with six nymphal stadia (mean ± SD = 33.6 ± 2.5 days, $n = 170$ for females; t = –13.969; df = 343, $P < 0.001$; 33.8 ± 2.3 days, $n = 44$ for males; t = –11.965; df = 194; $P < 0.001$). Within HSG 1, adult body weight was significantly smaller in individuals with five nymphal stadia (mean ± SD =1947 ± 142 mg, $n = 175$ for females and 1394 ± 99 mg, $n = 152$ for males) than in those with six nymphal stadia (mean ± SD = 2166 ± 182 mg, $n = 170$ for females; t = –12.490; df = 343, $P < 0.001$; 1508 ± 107 mg, $n = 44$ for males; t = –6.577; df = 194; $P < 0.001$). Because the developmental performance in each HSG was influenced by the number of nymphal stadia, the following analyses on nymphal development were conducted mainly for locusts with five nymphal stadia.

Rearing density influenced the duration of nymphal development (figs. 2A and B). In all HSGs, nymphal development was faster under crowded conditions than under isolated conditions. Under isolated conditions, nymphs tended to grow slightly faster as hatchlings were bigger (figs. 2A and B), and a negative correlation was found between individual hatchling body weight and developmental time for both sexes (data not shown; $r = –0.211$; $n = 439$; $P < 0.001$ for females; $r = –0.154$; $n = 329$; $P < 0.01$ for males). Under crowded conditions, developmental time did not vary with HSG for either sex ($P > 0.05$ each). However, hatchling body size influenced adult body weight under both isolated and crowded conditions; larger hatchlings attained a larger body weight at adult emergence (figs. 2C and D). Interestingly, rearing density resulted in differences in adult body weight in small hatchlings only. Daily weight gain during the nymphal stage was consistently greater under crowded conditions than under isolated conditions (figs. 2E and F), although the differences for HSGs 1 and 2 in females

were not significant ($P > 0.05$; fig. 2E). In both sexes, larger hatchlings showed greater daily weight gain. Figure 3 summarizes the relationships between development rate (the inverse of the number of days required for nymphal development) and adult body weight for different HSGs. For females (fig. 3A), a negative relationship indicating a trade-off was found in the smallest three HSGs. For males (fig. 3B), a negative relationship was found only in HSG 1. These results indicate that small hatchlings grow faster under crowded conditions than under isolated conditions at the expense of the final body size. Larger hatchlings also grew faster under crowded conditions than under isolated conditions, but without becoming smaller as adults.

3.2. Reproductive traits and density

Female adults from a solitarious (isolation-reared) and a gregarious (crowd-reared) line were housed individually or with two male adults, and the number of eggs, individual egg weight and total egg weight per egg pod were determined (fig. 4). For the solitarious line, adults with five and six nymphal stadia are presented separately, because adult body weight was significantly smaller in the former, as mentioned above. The number of eggs per egg pod increased with adult body weight in both lines (fig. 4A; $P < 0.001$). ANCOVA with adult body weight as the covariate indicated significant differences in the number of eggs between the two groups of solitarious adults ($F_{1, 294} = 7.22$; $P < 0.01$) as well as between the two lines ($F_{1, 527} = 23.69$; $P < 0.001$). Egg weight varied positively with adult body weight in the gregarious line (fig. 4B; $r = 0.269$; $n = 234$; $P<0.001$) but negatively in the solitarious line ($r = –0.128$; $n = 296$; $P < 0.05$). Mean egg weight was significantly greater in the gregarious line (mean ± SD = 8.02 ± 0.63 mg) than in the solitarious one (5.89 ± 0.54 mg; t = 41.66; df = 528; $P < 0.001$). No significant difference was found in egg weight between the two groups within the solitarious line ($P > 0.05$). Total egg biomass per pod calculated based on egg weight and the number of eggs per pod was positively correlated to adult body weight in the two lines (fig. 4C). It was significantly larger in the gregarious line (604.6 ± 134.9 mg; $n = 234$) than in the solitarious line (554.1 ± 13.2 mg; $n = 296$; t = 4.358; df = 528; $P < 0.001$). However, no significant difference was found in this trait between the solitarious females with five nymphal stadia (538.8 ± 131.9

mg; $n = 193$) and those with six nymphal stadia (582.8 ± 122.6 mg; $n = 103$; ANCOVA, $F_{1, 294} = 2.267$; $P > 0.05$) after adult body size was adjusted. Body weight–specific egg production (total egg mass / adult body weight) was relatively constant over a wide range of adult body weights in the solitarious line (fig. 4D; $r = –0.031$; $n = 296$; $P > 0.05$), but rapidly increased with adult body weight in the gregarious line ($r = 0.253$; $n = 234$; $P < 0.001$).

3.3. Trade-off between egg size and number

Figure 5 illustrates the relationship between egg weight and number per pod produced by adults of a solitarious and a gregarious line. The overall correlation involving all eggs produced by the two lines was highly significant ($r = –0.456$; $n = 530$; $P < 0.001$). In the solitarious line alone, the negative correlation was less obvious but still significant for egg pods produced by all adults ($r = –0.273$; $n = 296$; $P < 0.001$) and those produced by adults with five nymphal stadia ($r = –0.286$; $n = 193$; $P < 0.001$) or six nymphal stadia ($r = –0.265$; $n = 103$; $P < 0.01$). Unexpectedly, the corresponding correlation was not statistically significant for the gregarious line ($P > 0.05$).

4. Discussion

In *S. gregaria*, the density experienced by females as adults determines the progeny size (Faure, 1932; Chauvin, 1941; Hunter-Jones, 1958); solitarious females produce small hatchlings and gregarious females large hatchlings. The variation in progeny size caused by this maternal effect influences the number of nymphal stadia (Hunter-Jones, 1958) and thus the development and body size at maturation, as demonstrated in this study. I investigated developmental performance of various sizes of *S. gregaria* hatchlings under isolated and crowded conditions. I confirmed that extra molting occurs only in small hatchlings (Hunter-Jones, 1958) and found that the incidence of extra molting also depends on the rearing density of the nymphs.

Because the number of nymphal stadia influences the duration of nymphal development and body size at maturation, my analysis of developmental traits was conducted mainly for locusts with five nymphal stadia. I found that, irrespective of hatchling body size, nymphal

development was consistently faster under crowded conditions than under isolated conditions. Despite the fact that this locust is potentially the economically most harmful insect pest in the world, few reliable data are available about phase-dependent differences in duration of nymphal development and the information is not consistent. In *S. gregaria* and *L. migratoria*, nymphal growth has been reported to be faster in crowd-reared locusts than in isolation-reared ones in some studies (Kennedy, 1956; Uvarov, 1966, 1977; Pener, 1991; Heifetz & Applebaum, 1995), whereas the reverse conclusion has been reported in other studies (Staal, 1961; Applebaum & Heifetz, 1999). Most studies were conducted without considering the variation in the number of nymphal stadia. Crowding, particularly in late-stadium nymphs, can easily cause a shortage of food, and special care is required to avoid a secondary effect of crowding. In the present study, I analyzed locusts with five or six nymphal stadia separately and changed the grass twice a day for late-stadium nymphs to ensure that they had food *ad libitum* throughout nymphal life.

Rapid development often results in smaller body size. This negative relationship, or trade-off, is well documented in various organisms (Stearn, 1992). In *S. gregaria*, rearing density affects developmental rate and adult body size. I found a trade-off between the two variables for relatively small hatchlings, which grow faster but emerge as smaller adults under crowded conditions than under isolated conditions. However, such a trade-off is not found in large hatchlings, which grow faster under crowded conditions than under isolated conditions without becoming smaller adults (fig. 35). This finding may suggest an important feature of this locust, which often undergoes outbreaks. In solitarious forms, hatchlings are small and take a long time to mature (with low developmental rates) but can attain a large adult body size. At low population density at which food is less likely to be limiting, large adult body size rather than rapid growth may be more important in terms of fitness. Conversely, in gregarious forms, hatchlings are large and grow rapidly. These characteristics are likely to be adaptive under crowded conditions, because large hatchlings are more tolerant to desiccation and fasting than small ones (Albrecht & Blackith, 1960), and rapid development would reduce the time of exposure to predators. Indeed, large hatchlings rarely undergo extra molting even if reared in isolation. With increased nymphal growth efficiency, gregarious locusts can accomplish both

rapid growth and large adult body size by evading the trade-off by which solitarious locusts are constrained.

A comparison of reproductive performance between a solitarious and a gregarious line revealed another important feature of this locust. ANCOVA demonstrated that the number of eggs per pod depends on the body size of the female parent, and confirmed that solitarious females produce more eggs than gregarious ones (Uvarov, 1966). The present study also confirmed that gregarious locusts produce larger eggs than solitarious locusts (Uvarov, 1966). Interestingly, with increased body size of the female parents, egg size tends to increase in gregarious forms, whereas it tends to decrease slightly in solitarious forms. A negative correlation between progeny size and female size is rare (Fox *et al.*, 2000). It is possible that under non-competitive conditions at low population density, selective pressure has favored solitarious females to increase the number of eggs at the expense of individual egg size. The production of smaller eggs by larger females in isolation-reared locusts might be an adaptive response because the environment in which large adults occur is likely to be more favorable for nymphal growth compared with an environment where small adults occur. This would effectively lead to a reduction in the amount of investment to each egg without lowering hatchling survival and a greater investment in egg production. At high population density, on the other hand, large body size in hatchings is likely to impart increased fitness, as mentioned above. Because of such differences in selective pressure between the two phases, a trade-off between egg size and number, which is clearly shown when data for the two phases are combined, may become less obvious within the solitarious forms and non-significant within the gregarious forms.

In conclusion, locusts reared in isolation or in groups with minimal stress from competition for food and egg-laying space exhibit phase-dependent and body size–dependent differences in various developmental and reproductive characteristics. Unlike the solitarious forms in which development and reproduction are constrained by a trade-off, the gregarious forms have acquired capacities to grow faster without reducing the final body size and to produce more and larger eggs as the body size of the female parent increases. The latter is achieved by increasing

the egg-developing capacity relative to body size. Crowding seems to serve as a stimulating signal for locusts to express a set of gregarious characteristics that contribute to rapid population growth during outbreaks.

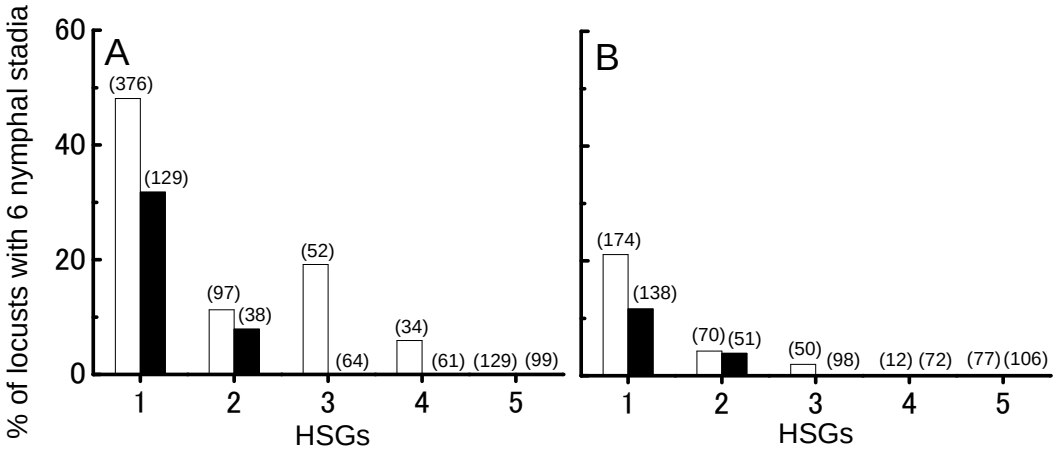

Fig. 1. Proportions of individuals with six nymphal stadia in different hatchling size groups (HSGs) of *Schistocerca gregaria* reared under isolated conditions (open bars) or crowded conditions (closed bars). A, females; B, males. Numbers on bars indicate *n*.

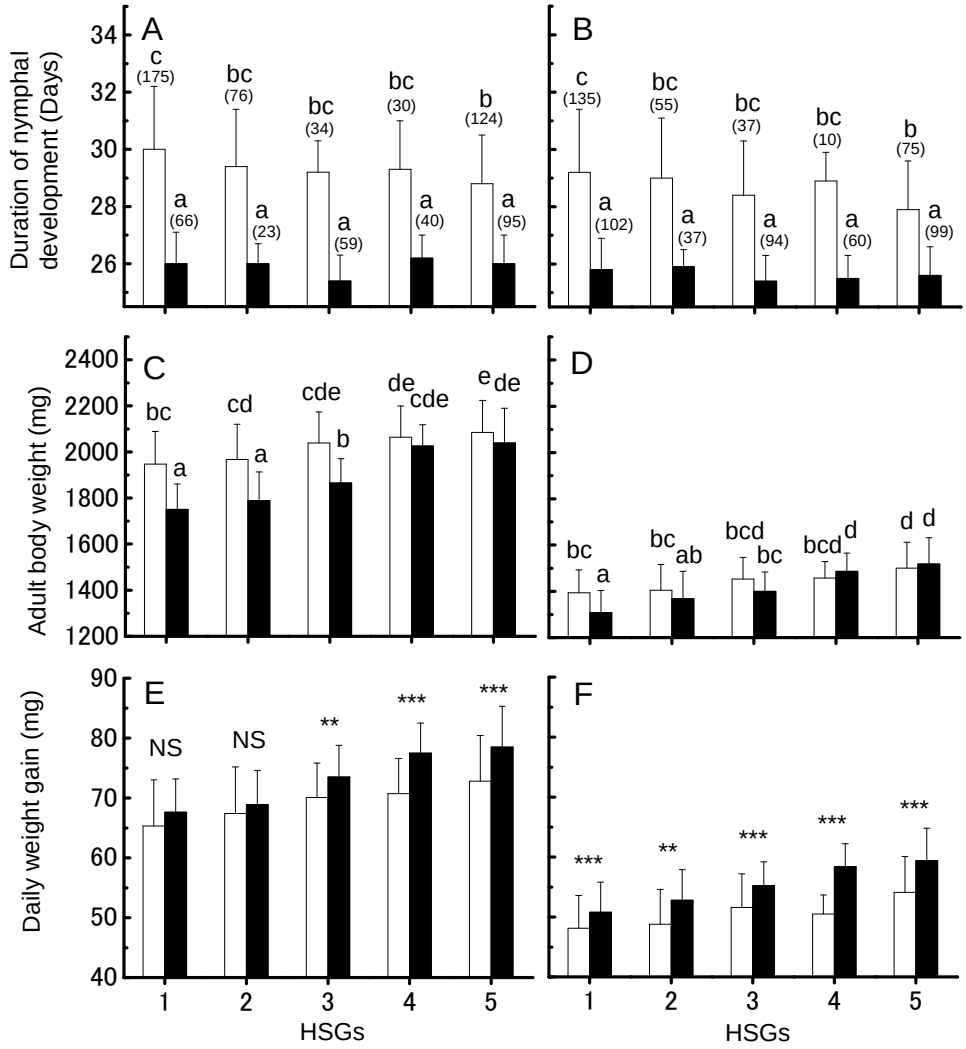

Fig. 2. Effects of hatchling body size on various developmental traits in *Schistocerca gregaria*. Duration of nymphal development (A, B), adult body weight (C, D) and daily weight gain (E, F) in locusts of different HSGs reared under isolated conditions (open bars) or crowded conditions (closed bars). A, C and E, females; B, D and F, males. Means with one side of sd are presented. Numbers in parentheses in panel A and B indicate *n*. Different letters in each panel indicate significant differences at 0.05% by Scheffé's test. Daily weight gain was tested by Mann-Whitney U-test: NS, not significant; **, $P<0.01$; ***, $P<0.001$.

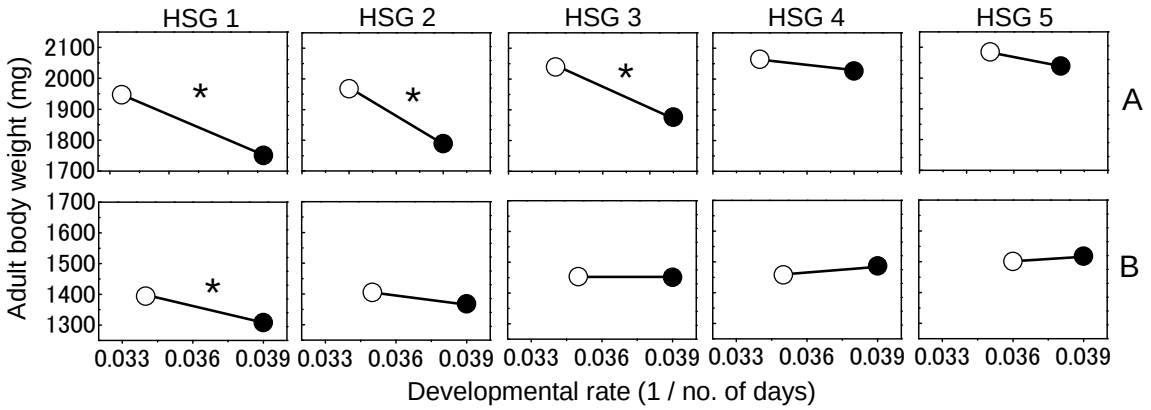

Fig. 3. Relationships between developmental rate of nymphs and adult body weight in different HSGs of *Schistocerca gregaria* reared under isolated conditions (open circles) or crowded conditions (closed circles). Data are based on results in Figure 2. A, females; B, males. Asterisks indicate significant differences in both adult body weight (by t-test) and developmental rate (by Mann-Whitney *U*-test) between the two groups at $P<0.05$. Sample sizes are given in Figure 2.

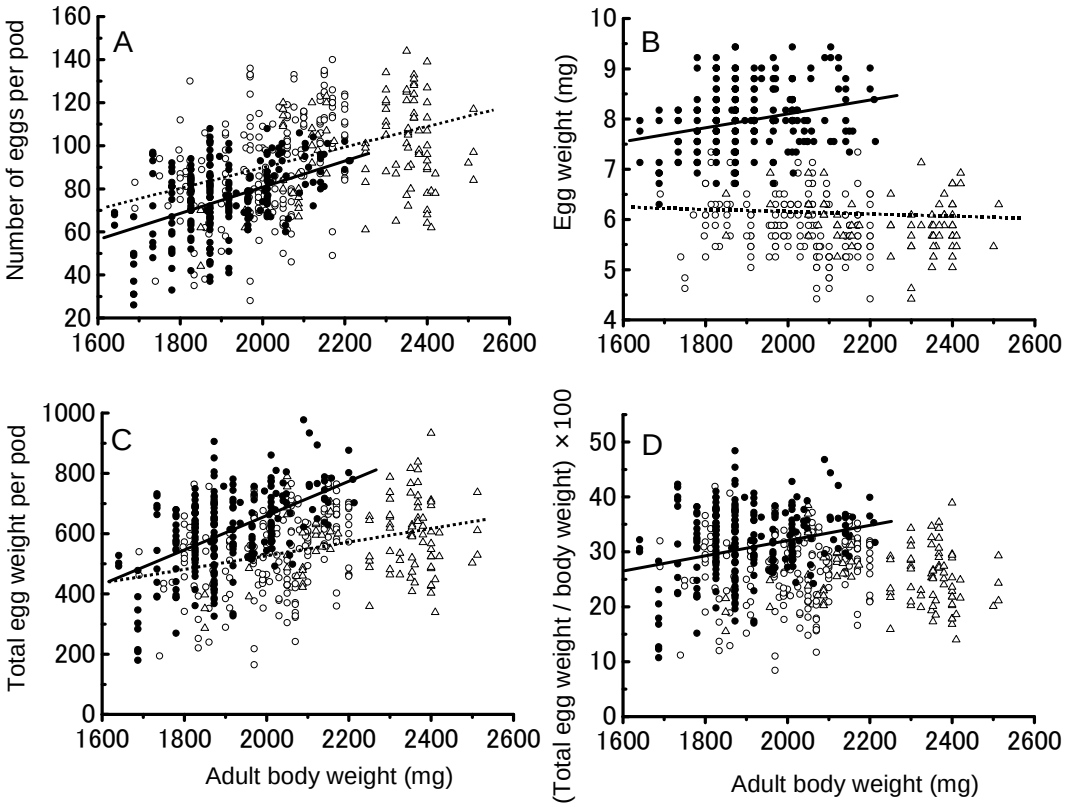

Fig. 4. Relationships between body weight at adult emergence and various reproductive traits in female adults of a solitarious and a gregarious line of *Schistocerca gregaria*. A, the number of eggs per pod; B, egg weight; C, total egg weight per pod; D, total egg weight relative to adult body weight. Eggs produced by solitarious adults with five (open circles) or six nymphal stadia (triangle) are shown separately, together with those produced by gregarious adults (closed circles). Regression lines for the gregarious line are black, and those for the solitarious line are dotted. n = 193 and 103 for solitarious individuals with five and six nymphal stadia, respectively, and n = 234 for gregarious individuals.

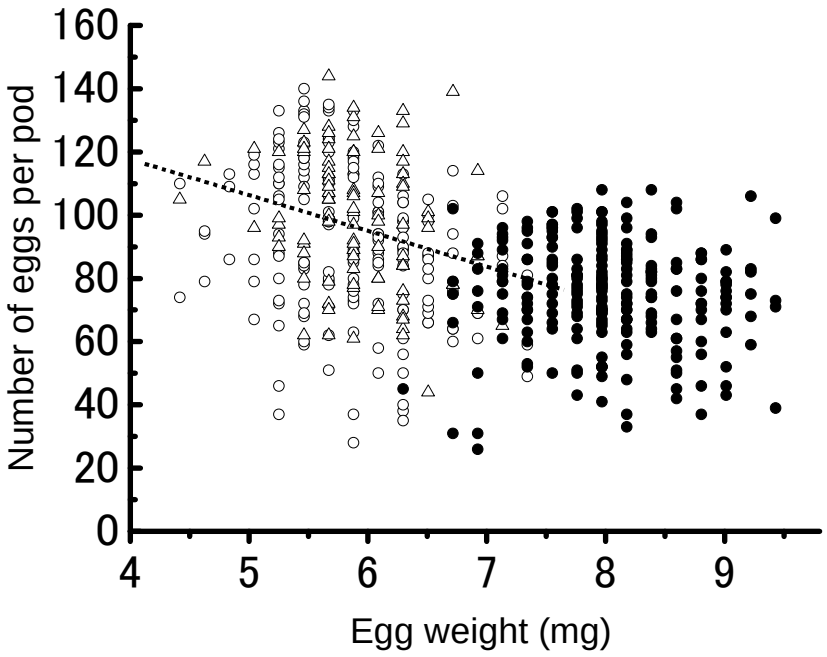

Fig. 5. Relationships between the weight and number of eggs per pod laid by solitarious (isolation-reared) adults with five (open circles) or six nymphal stadia (triangle) and by gregarious (crowd-reared) adults（closed circles）of *Schistocerca gregaria.* A negative correlation is found only in the solitarious line (dotted line). n = 296 in the solitarious line and 234 in the gregarious line.

Chapter 4

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1. Genetic control of body color in a phase-dependent reddish-brown mutant in the desert locust, *Schistocerca gregaria*.

2. Genetic and hormonal control of body color expression in the desert locust, *Schistocerca gregaria*.

Abstract

The genetic and hormonal control of body coloration was investigated for a reddish-brown (RB) mutant of the desert locust, *Schistocerca gregaria* that was found in a laboratory colony. The dark patterns of this mutant are similar to those of normal individuals, but the intensity of the melanization is weaker in the former. Nymphs of this mutant can be visually distinguished from normal individuals under crowded conditions only, because the dark patterns do not appear under isolated conditions. Reciprocal crosses between the mutant and normal strains indicated that the RB phenotype was recessive to the normal phenotype and controlled by a simple Mendelian unit. Reciprocal crosses between the RB mutant and another mutant (albino) produced only normal phenotypes in the F1 generation. In the F2 generation, the normal, RB and albino phenotypes appeared in a ratio of 9:3:4, indicating that two Mendelian units, which control the appearance of dark body color and the intensity of melanization, may be involved in the regulation of body coloration. Injections of [His^7]-corazonin, a neuropeptide inducing dark color in this locust, failed to induce dark color in albino nymphs but showed a dose-dependent darkening in RB nymphs, some of which became indistinguishable from normal individuals. These results may suggest that the RB mutant gene regulates the degree of melanization, possibly through controlling the production and/or release of [His^7]-corazonin.

1. Introduction

Body color mutation occurs in various orders of insects (Fuzeau-Braesch, 1985). Albino mutants which look normal in every respect except for the body color have been reported occasionally for locusts and grasshoppers (Faure, 1932; Hunter-Jones, 1957; Putnam, 1958; Verdier, 1965; Boutheier, 1966; Nolte, 1971; Tanaka, 1993). Albino mutants have been used to study sperm competition (Hunter-Jones, 1960; Zhu and Tanaka, 2002; Tanaka and Zhu, 2003), to test the role of gregarious body color in the formation and maintenance of aggregation and group walking (marching) behavior (Gillett, 1973) and to screen for the presence of a pigmentotropin in various insects (Tanaka, 2000c, 2006).

Locusts show body-color polyphenism depending on the population density (Faure, 1932; Hunter-Jones, 1958; Stower, 1959; Uvarov, 1966; Pener, 1991; Lester et al., 2005; Maeno & Tanaka, 2007). In the desert locust, *Schistocerca gregaria* and the migratory locust, *Locusta migratoria*, solitarious nymphs occurring at a low population density assume cryptic or camouflaged green or brown body color matching the habitat background color. At a high population density, on the other hand, gregarious nymphs display a conspicuous yellow or orange body color with intensive black patterns. Albino locusts of either species do not develop the gregarious body coloration and become whitish under crowded conditions. Under isolated conditions, on the other hand, they are either whitish or green in color (Hunter-Jones, 1957; Verdier, 1965; Hasegawa and Tanaka, 1994), although the brownish color often observed on legs and the ventral surface of the body in normal individuals of *L. migratoria* is not expressed (Tanaka, 2000b).

Another type of body-color polyphenism occurs in hatchlings of locusts (Hunter-Jones, 1958; McCaffery et al., 1998; Tanaka and Maeno, 2006). In *S. gregaria*, dark-colored hatchlings are produced by crowd-reared female adults, whereas green hatchlings are produced by isolated-reared female adults, although hatchlings intermediate between the two forms are sometimes produced together with the other

forms. During a study of phase polyphenism in *S. gregaria*, light-colored hatchlings were found in a crowd-reared laboratory colony. They develop reddish-brown patterns instead of black ones under crowded conditions, but under isolated conditions they are greenish like normal hatchlings. Therefore, this mutant, which will be referred to as RB mutant, can be separated visually from normal individuals only under crowded conditions. A black mutant which remains almost black even under isolated conditions has been reported for *S. gregaria*, and it is recessive to the normal pigmented phenotype (Volkonsky, 1938). In *S. gregaria* (Hunter-Jones, 1957) and *L. migratoria* (Nolte, 1971; Hasegawa and Tanaka, 1994), the albinism is also a recessive trait controlled by a single Mendelian unit. While the genetic background for color mutation in locusts has received much attention, no information is available about the genetic relationships among the different types of mutant. Because we have an albino strain in our laboratory, the occurrence of the RB mutant provided us with a unique opportunity to approach this problem. In the present study, crossing experiments were carried out between the RB mutant and normal strains as well as between the RB and albino mutant strains to understand the genetic basis for this mutant.

The albinism in *L. migratoria* is caused by a deficiency of a hormone normally present in the central nerves system, brain and corpora cardiaca (Tanaka, 1993). The hormone has been found to be identical to [His^7]-corazonin, a neuropeptide originally isolated from the American grasshopper, *S. americana* without known function (Veenstra, 1991). This hormone controls the body-color polyphenism in *L. migratoria* and *S. gregaria* (Tawfik et al., 1999; Tanaka, 2001, 2006). In albino *L. migratoria*, injections of the peptide induce not only black patterns and orange background color characteristic of gregarious forms but also various other body colors characteristic of solitarious forms (Tanaka, 2000a, b). On the other hand, an albino strain of *S. gregaria* appears to have a different mechanism, because this strain owns [His^7]-corazonin (Yerushalmi et al., 2000; Schoofs et al., 2000). The cause for the albinism in *S. gregaria* remains unclear. The role of [His^7]-corazonin in the control of phase-polyphenism has

been studied intensively (Tanaka, 2001, 2006), but the role of the neuropeptide in the transduction pathway and the pigment biosynthesis pathway have been little understood. There is a possibility that the RB mutant is caused by a mutation in the pathway of pigment biosynthesis associated with [His^7]-corazonin, because this neuropeptide controls darkening in locusts. To investigate this possibility, [His^7]-corazonin was injected into nymphs of the RB mutant and its effect on the body color determined. The present study describes these results and discusses the genetic and hormonal mechanisms controlling the expression of body coloration in desert locusts.

2. Materials and methods

2.1. Insects and rearing conditions

The normal strain of *S. gregaria* used has been described previously (Tanaka and Yagi, 1997; Maeno and Tanaka, 2004) and the albino strain was originated from the one previously described (Hunter-Jones, 1957; Yerushalmi et al., 2000; Schoofs et al., 2000). The RB mutant was established in 2006 from reddish-brown nymphs that were found in the normal strain maintained for more than 40 generations in the Tsukuba laboratory. Nymphs and adults were kept in groups of approximately 100 individuals in large cages (42 × 22 × 42 cm) at 32 ± 1 ℃, LD 16:8 h and 50-70% relative humidity, as described previously (Maeno et al., 2004). They were fed leaves of orchard grass and cabbage together with wheat bran.

2.2. Crossing

Newly emerged female adults of each strain were separated from males and reared in a group of about 30 individuals for crossing. Twenty five days after adult emergence females were individually transferred from the large cages to small cages (28 × 15 × 28 cm) in which each female was paired with a sexually mature male (ca. 3 weeks old). Female adults constantly kept with a male as pairs produce hatchlings characteristic of gregarious forms (Hunter-Jones, 1958). To determine the genetic control of the RB trait, two experiments were carried out. In the first experiment, reciprocal crosses were made between the RB mutant and normal strains. Their eggs were incubated at 32℃ and the body color of hatchlings was examined. Hatchlings with reddish-brown patterns and black patterns were regard as RB mutant and normal phenotypes, respectively (Fig. 1A and B). They were raised to adults and crossed to each other to obtain F2 generations or backcrossed either to the pure-bred reddish-brown mutant or normal strain to determine the ratios of body-color phenotypes. It is known that egg pods deposited by crowded females sometimes produce a few small, green hatchlings characteristic of solitarious forms together with dark colored ones (Hunter-Jones, 1958; Tanaka and Maeno, 2006).

In this study, such individuals also appeared in small number, but they were discarded.

In the second experiment, the RB and albino mutants were crossed as in the first experiment, but no backcross was performed. As mentioned, this albino strain shows a Mendelian pattern of inheritance in which the albinism is recessive (Hunter-Jones, 1957). In most crosses, 5-10 pairs were used and 5 egg pods collected from each pair. The eggs obtained were handled as described by Tanaka and Maeno (2006) and incubated at 32℃ until hatching. Egg hatching occurred in 2 weeks. Hatchling body coloration was recorded after 6 h of hatching and classified into three phenotypes based on the darkness of the body coloration. In addition to the RB and normal phenotypes, greenish hatchlings without dark patterns appeared, which were regarded as albinos (Fig. 1C) if they turned whitish after ecdysis to the second stadium.

2.3. Hormonal injection

Injections of [His^7]-corazonin into reddish-brown mutants were made according to the method of Tanaka (2000a), and the procedure will be described only briefly here. Various doses of the peptide (10 pmol–1 nmol) synthesized by Yamazaki Co. (Tokyo, Japan) were mixed with 2 μl of rapeseed oil (Hayashi Chemical Co., Tokyo, Japan) and injected into nymphs through the membrane between the 3rd and 4th abdominal segments with a micro syringe (MS-N50, Ito, Shizuoka, Japan) the day after ecdysis to the 2nd stadium. These doses were known to induce dark color in normal *S. gregaria* and albino *L. migratoria* (Tanaka, 2001). Some nymphs were injected with 2 μl of oil alone or nothing as controls.

2.4. Measurements of body color

To quantify the hormonal effects on the body color, treated nymphs were immobilized on ice after they reached the 3rd stadium and photographed with a scanner (Epson ES 2000, Japan) connected to a personal computer (Type-MA, Japan) using commercial software, Photoshop 7.0 (Adobe Systems Incorporated, San Jose, CA)

according to the methods of Tanaka (2003). The image type used was 24-bit color at a resolution of 400 d.p.i. Using the histogram function, luminance of the head and pronotum was measured. Because the mean and median values were almost identical (Tanaka, 2003), the mean luminance was recorded for each individual.

3. Results

3.1. Genetic control of body coloration

In the first experiment, all F1 locusts from reciprocal crosses between the RB mutant and normal strains showed the normal phenotype (n=515-840), as in crosses between normal adults, whereas RB pairs produced only RB offspring (n=592). This result indicates that the RB phenotype is recessive to the normal one.

Figure 2 summaries the results of F2 generation and backcrosses to the purebred strains. The results indicated that crosses between F1 adults gave rise to values that were not significantly different from the expected ratio (3:1) by the law of segregation by Mendel (A and B in Fig. 2; χ^2-test; $P>0.05$). In backcrosses of F1 locusts to RB mutants, the frequencies of RB and normal progenies were almost equal to each other (C and D in Fig. 2; χ^2-test; $P>0.05$). On the other hand, backcrosses to the normal strain produced only normal phenotypes. These results indicated that the RB phenotype is recessive and controlled by a single Mendelian unit.

In the second experiment, reciprocal crosses were made between RB and albino mutants. All F1 hatchlings showed the normal phenotype (A and B in Fig. 3). In the F2 generation, three phenotypes appeared, and normal, RB and albino hatchlings appeared in a ratio of approximately 9:3:4. The ratio of pigmented individuals (black plus RB) to albino ones was not deviated significantly from 3:1, the ratio expected in the F2 generation by the law of segregation for a simple Mendelian unit (C and D in Fig. 3; χ^2-test; $P>0.05$). Likewise, the ratio of normal to RB phenotypes was also close to 3:1, which was consistent with the involvement of a Mendelian unit (C and D in Fig. 3; χ^2-test; $P>0.05$). These results suggested that the albino phenotype was recessive to the

two pigmented phenotypes and the RB phenotype was dominant over the albino one but recessive to the normal phenotype.

3.2. Hormonal control of body coloration

To investigate the effect of [His^7]-corazonin on the body color in RB locusts, various doses of the peptide (10 pmol – 1 nmol) were injected into 2nd stadium nymphs of the RB mutant and their body color was examined in the 3rd stadium. Mean luminance of the head was significantly higher in RB mutant controls than that in untreated normal individuals (Fisher's PLSD test; $P<0.05$; Fig. 4A; A and C in Fig. 5). However, it decreased gradually as the dose of the injected peptide increased, and no significant difference was found between individuals injected with 1 nmol and untreated normal individuals (Fisher's PLSD test; $P>0.05$; Fig. 4A; B and C in Fig. 5). Similar results were obtained for the pronotum. These results indicated that single injections of [His^7]-corazonin caused RB mutants to turn darker and they became indistinguishable from normal individuals (see B and C in Fig. 5).

4. Discussion

The present study demonstrated that the RB phenotype of *S. gregaria* is recessive to the normal pigmented trait and controlled by a single Mendelian unit. The present study suggested that albino *S. gregaria* lack the capacity for pigmentation but carry some genetic factor that is owned by normal locusts, so that they produce F1 hatchlings with normal phenotype when crossed to the RB strain. In the F2 generation, normal, RB and albino phenotypes appeared in a ratio of about 9:3:4. These results may be explained by assuming the presence of two genes (Fig. 6). One gene, tentatively designated as pigmentation gene *P* or *p*, determines whether locusts become pigmented or not, and the other gene, designated as melanization gene *M* or *m*, determines the degree of melanization. According to this model, albino locusts carry a recessive gene *p*, whereas normal and RB locusts have a dominant gene *P* so that they are both pigmented. Of the

latter, the RB strain carries a recessive gene *m* which expresses only a low degree of melanization compared with normal locusts that carry a dominant gene, *M*. Because F1 hatchlings between the RB (*P, m*) and albino (*p,?*) mutants are strongly pigmented like normal hatchlings, they should carry two dominant genes, *P* and *M*. This means that gene *M* must have come from the albino strain. If this is the case, our albino strain is incapable of developing dark color, because it has a recessive gene *p*, although it carries a dominant gene *M* that should otherwise express intensive melanization like normal hatchlings. In the second experiment, the genotype for albino phenotypes in the F2 generation is expected to be either *p/M* or *p/m*. Indeed, some of these albino locusts produced offspring with RB phenotype only, when backcrossed to pure-bred RB locusts (Maeno and Tanaka, unpublished observations).

At present, the exact function of the pigmentation gene is unknown, but it might be related to the expression of enzymes responsible for pigment synthesis or that of corazonin receptor systems, because injections of a huge dose (50 ng) of [His^7]-corazonin induced a slight darkening in some of the injected albino locusts (Yerushalmi et al., 2000).

In the present study, the effects of injection of [His^7]-corazonin on the body color were investigated for the RB mutant. The peptide induced darkening in a dose-dependent manner, and some individuals injected exhibited intensive black patterns characteristic of normal gregarious forms. The albino bioassay developed by Tanaka (1993) showed that implantation of corpora cardiaca taken from reddish-brown mutants into albino *L. migratoria* induced darkening in the latter (Maeno and Tanaka, unpublished observations). One may argue that the melanization gene, *M/m*, assumed here might regulate the production and/or release of [His^7]-corazonin. However, the present results are based on visual colors, and we know little about the pigments involved. The reddish-brown color may not be caused simply by a reduced intensity of black pigmentation, although both reddish-brown and black pigments stain the exuviae, indicating that they are probably melanins. Therefore, we cannot rule out the possibility

that an injection of [His^7]-corazonin simply masked the reddish-brown color by inducing black pigment in the cuticle. Further studies on the pigments and the function of [His^7]-corazonin will be necessary to understand the mechanism of the body color polyphenism in locusts. The RB mutant may provide a useful tool for studies along this line.

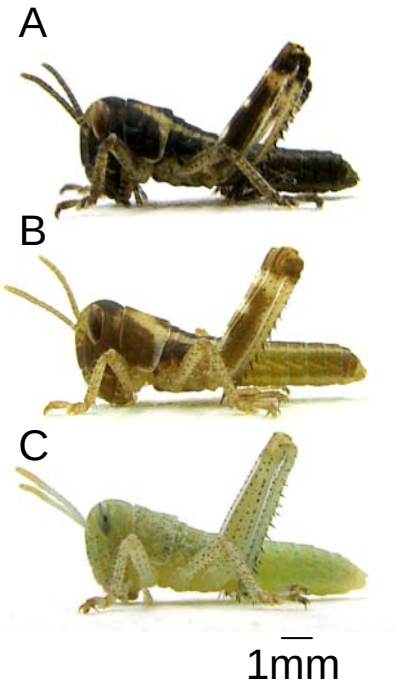

Fig. 1. Body coloration of a normal (A), reddish-brown mutant (B) and albino mutant hatchling (C) of crowd-reared *Schistocerca gregaria*. Note that the dark patterns are lighter in the reddish-brown mutant than in the normal hatchling, while the albino hatchling has no black patterns.

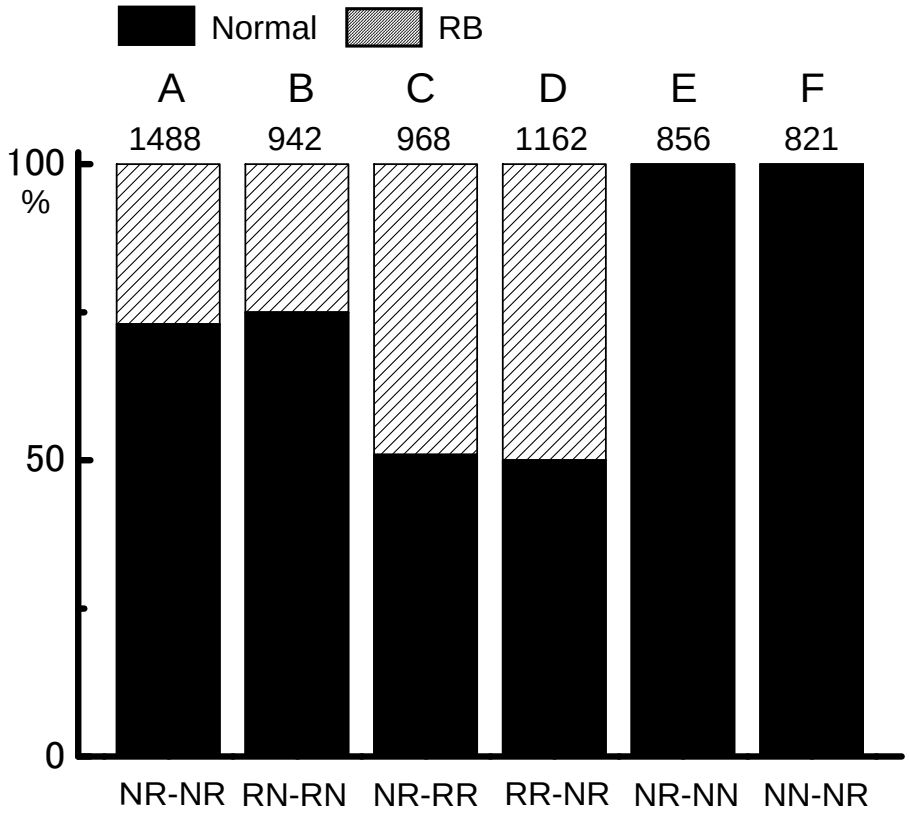

Fig. 2. Proportions of normal and reddish-brown individuals obtained from crosses between F1 locusts (A and B) between the normal (N) and reddish-brown (R) strains of *Schistocerca gregaria* and from backcrosses (C-F). In each cross the female parent is listed first. Numbers on the bars indicate *n*.

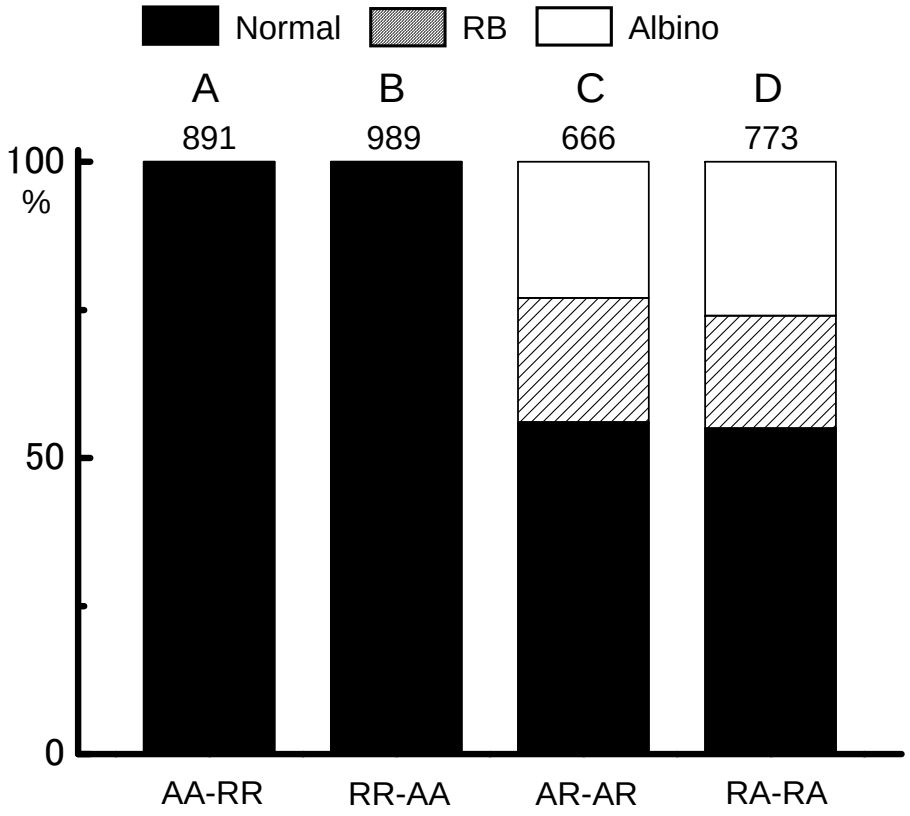

Fig. 3. Proportions of normal, reddish-brown and albino phenotypes obtained from crosses (A and B) between the reddish-brown (R) and albino (A) strains and from crosses (C and D) between F1 locusts of *Schistocerca gregaria*. In each cross the female parent is listed first. Numbers on the bars indicate *n*.

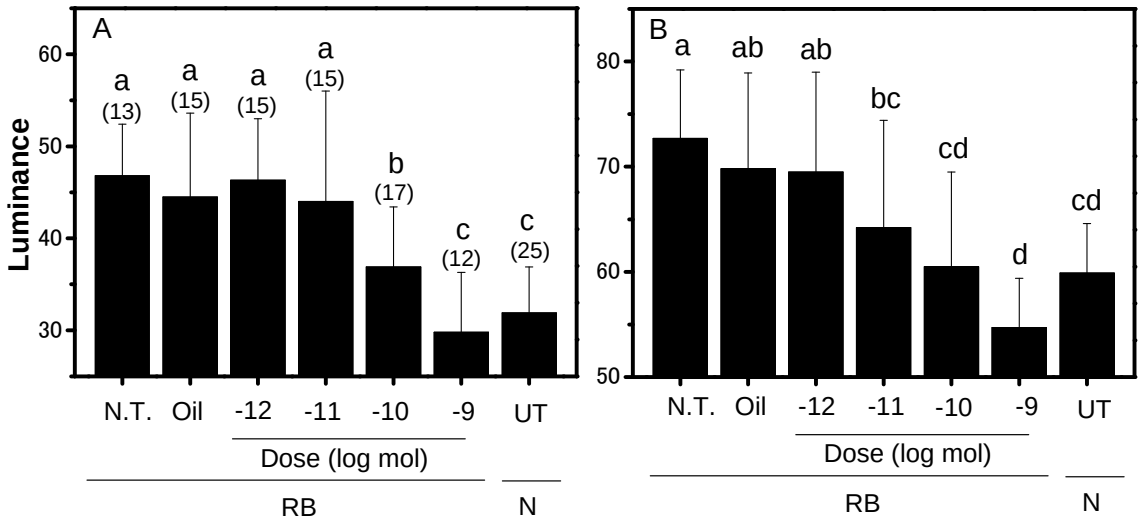

Fig. 4. Average luminance values for the head (A) and pronotum (B) of reddish-brown (RB) mutant of 3rd stadium nymphs of *Schistocerca gregaria* after injection of various doses of [His^7]-corazonin. Different letters on the histograms indicate significant differences at 5% (Scheffe's test). Numbers in parentheses indicate *n*. Controls were either untreated (UT) or injected with oil. Normal (N) individuals were untreated.

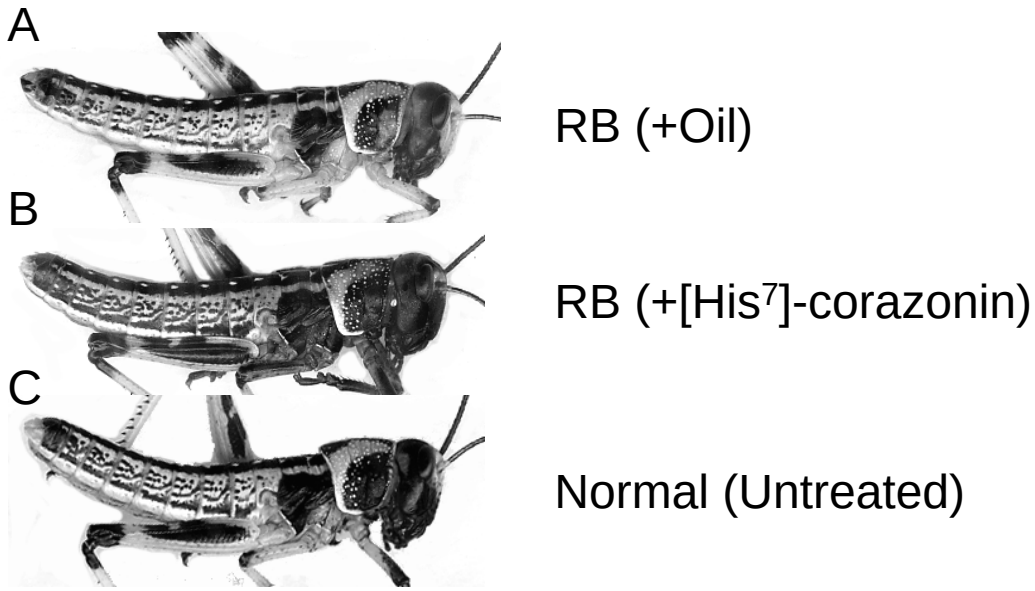

Fig. 5. Photographs of 3rd stadium nymphs of the reddish-brown (RB) strain that were injected with oil (A) or 1 nmol of [His^7]-corazonin (B) as well as an untreated normal nymph (C).

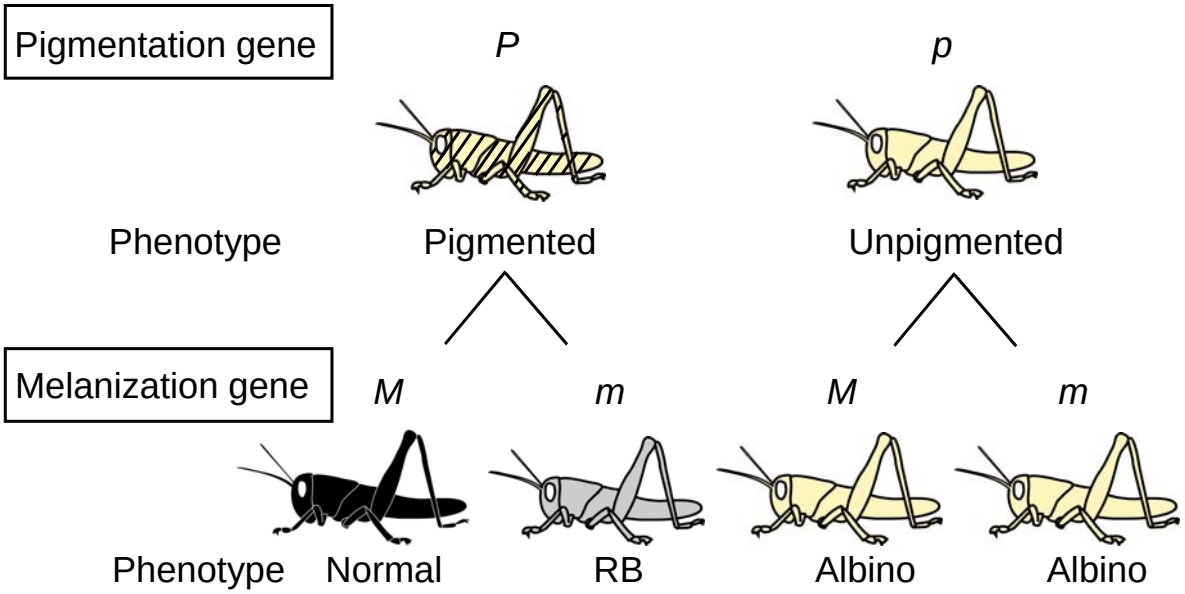

Fig. 6. A model explaining the genetic control of body color expression in *Schistocerca gregaria*. Pigmentation gene (*P*/*p)* determines whether locusts are pigmented or not; melanization gene (*M* / *m*) determines the intensity of melanization. For explanation, see text. RB, reddish-brown.

Chapter 5.1.

Phase-related body-color polyphenism in hatchlings of the desert locust, *Schistocerca gregaria*: re-examination of the maternal and crowding effects

Abstract

The mechanism controlling the body color of hatchlings was studied for the desert locust, *Schistocerca gregaria*. A pheromonal factor secreted by gregarious female adults into the foam plugs of egg pods has been suggested to cause darkening in their progeny. We re-examined the role of this maternal factor by washing or separating eggs at deposition. Eggs produced by crowd-reared female adults were washed with saline or separated individually without being washed immediately after deposition and the body color of the hatchlings from them was compared with that from the eggs unwashed and kept in the egg pod until hatching. Most hatchlings were dark and no significant difference was found in the proportions of dark- and light-colored hatchlings between the treatments and controls. Likewise, eggs separated before the foam plug deposition produced dark-colored hatchlings as in the un-separated controls. These results demonstrated that neither washing nor separation of eggs at deposition affected the hatchling body coloration. The variation in hatchling body color was correlated closely to the body weight at hatching, indicating that hatchling body color had been determined maternally. Green hatchlings reared under crowded conditions remained green until the second stadium at which black patterns were induced. It was concluded that body color at hatching has been determined maternally and crowding during the first nymphal stadium influences nymphal body color but its effect is not manifested until the second stadium. The present study casts doubts on the presence of the pheromonal factor recently suggested.

1. Introduction

Many grasshoppers and locusts show body-color polyphenism (Faure, 1932; Uvarov, 1966, 1977; Dearn, 1990; Pener, 1991; Pener and Yerushalmi, 1998). In the desert locust, *Schistocerca gregaria* Forskål, a wide spectrum of body color variation has been noticed during the nymphal stage and various environmental factors such as crowding, temperature and humidity are known to influence this variation (Faure, 1932; Uvarov, 1966, 1977; Pener, 1991). Under low population density, nymphs are either green or beige in color with few black patterns. Under high density, they develop intensive black patterns with a yellow or orange background color. The phase-related variation in body color is particularly well studied for late stadium nymphs. Crowding is the most important factor inducing black patterns characteristic of gregarious nymphs (Stower, 1959; Gunn and Hunter-Jones, 1952; Hunter-Jones, 1958; Maeno and Tanaka, 2006). The corpora cardiaca have been demonstrated to contain the factor responsible for the induction of black patterns by implantation experiments in which extra CC induced black patterns in green solitarious nymphs when implanted (Tanaka & Yagi, 1997). Recently, this factor was identified by Tawfik et al. (1999) to be [His^7]-corazonin, a neuropeptide first isolated from the American grasshopper, *Schistocerca americana* without known function (Veenstra, 1991). Interestingly, it has been found that this neuropeptide not only induces black patterns of nymphs in various locusts and grasshoppers (Tanaka, 2000a, 2000b, 2004; Yerushalmi and Pener, 2001) but also influences phase-related morphological characteristics of adults in *S. gregaria* (Hoste et al., 2002; Maeno and Tanaka, 2004; Maeno et al., 2004) and the migratory locust *Locusta migratoria* (Tanaka et al., 2002; Yamamoto-Kihara et al., 2004).

The control mechanisms of the body color for hatchlings have also received much attention. In *S. gregaria*, Hunter-Jones (1958) demonstrated that the crowding conditions of female parents as adults determine whether the hatchlings of their progeny develop black patterns or not, although relatively little has been understood about the hormonal control (for reviews, see Dale and Tobe, 1990; Tanaka, 2001, 2006). Recently,

it was suggested that the foam plugs deposited by gregarious adults contained a pheromonal factor responsible for the induction of black patterns and gregarious behavior in the hatchlings of this locust by an Oxford research group (McCaffery et al., 1998; Simpson et al., 1999; Hägele et al., 2000). Washing of presumptive gregarious eggs from egg pods of crowd-reared females prevented darkening of the hatchlings. Saline extracts of egg pod foam plugs contained an active factor which promoted darkening in both hatchlings from eggs of solitarious females and those from eggs of gregarious females which were separated and washed to remove the factor. This factor was suggested to be a small (<3 kDa), hydrophilic substance (McCaffery et al., 1998; Simpson et al., 1999). The solitarizing effect was also obtained by just separating gregarious eggs within 1 hour of deposition, because the factor in or around the eggs was presumably removed by the separation (McCaffery et al., 1998; Simpson et al., 1999). Crowding conditions after hatching also appear to influence the body color in the hatchlings. If hatchlings from eggs of solitarious females make contact with each other for the first few hours, the switch towards the gregarious phase is initiated and the first visible sign is the appearance of blackish cuticular coloration (Islam et al., 1994a; De Loof et al., 2006). This finding is in contrast to unpublished observations reported by Injeyan et al. (1979) that a shift towards gregarious coloration becomes noticeable only after ecdysis to the second nymphal stadium.

During a study of body color polyphenism in locusts, I needed to obtain many green hatchlings and separated gregarious eggs or hatchlings according to the methods reported by McCaffery et al. (1998) and Islam et al. (1994a). However, I could not reproduce their results and decided to re-examine the effects of washing and separation of eggs on the control of body color polyphenism in hatchlings of *S. gregaria*. As a result, I reached a conclusion that neither washing nor separation of gregarious eggs prevented the darkening of the hatchlings obtained from eggs laid by crowded females. My results rather indicated the possibility that the hatchling body color had been determined before egg deposition, casting doubts on the presence of the accessory gland

factor suggested by McCaffery et al. (1998) and Simpson et al. (1999). Furthermore, I observed that rearing density during the first nymphal stadium did not elicit any marked effect on the body color before ecdysis to the second stadium, which was contrary to what was concluded by Islam et al. (1994a). The present paper describes these results and discusses the possible causes for the inconsistency among different studies.

2. Materials and Methods

2.1. Insects

The locusts used in the present study were derived from the same colony as in previous studies (Tanaka and Yagi, 1997; Maeno and Tanaka, 2006). They had been maintained over 20 generations at 32℃ and a photoperiod of LD 16:8 h in my laboratory where approximately 100 individuals were reared in a large cage (42 x 28 x 42 cm), as described previously (Maeno and Tanaka, 2004). Green hatchlings were obtained from female adults that had been maintained under isolated conditions except for a short period of mating with a male.

2.2. Egg pod collection and handling

Plastic cups (diameter, 9 cm; height, 5 cm) filled with clean moist sand were placed in cages to collect egg pods when adults matured sexually. To obtain newly deposited egg pods, cups were placed only during the day time (9:00 – 16:00). As soon as a female started ovipositing, she was transferred with the oviposition cup from the large cage to a small one (28 x 12 x 28 cm) and the egg pod was collected immediately after she finished oviposition. In some cases, egg pods were collected before females deposited the foam plug to determine its effect on hatchling body color. Within 15 min after the end of oviposition, 20 eggs were usually taken from each egg pod and either washed with saline solution (0.9 % NaCl, about 40 ml x 3 times), rinsed with distilled water (about 40 ml x 3 times) and separated individually or separated individually without being washed. In some cases where only less than 40 eggs were obtained, fewer

than 20 eggs were washed or separated without being washed. In a preliminary experiment, washed eggs suffered a high rate of mortality (97.4 %; *n*=369). Thus, eggs were washed very gently not to injure eggs. To minimize a possible difference in quality of eggs at different portions of an egg pod (Papillon, 1960), eggs were sampled from the lower, middle and upper portions of each egg pod as evenly as possible. The rest eggs were kept in the broken egg pod in moist sand as controls. The treated (separated with or without being washed) eggs from each egg pod were individually kept on moist cotton in sterilized small plastic dishes (diameter, 40 mm; height, 10 mm) which were then held in an air-tight polyethylene container (20 x 28 x 10 cm) at 32℃. A small ball of moist cotton was placed in each container to avoid desiccation. The control eggs were also incubated at the same temperature. Egg hatching occurred in 2 weeks. Hatchlings were weighed by an electronic balance (Mettler, AT 201) and their body coloration was recorded after 6 hours of hatching based on the scoring system with 5 hatchling body color groups (HCGs), as previously reported by Islam *et al*. (1994a) and Maeno and Tanaka (2006): HCG 1, background color uniformly green with no black pattern; HCG 2, background color green with some black markings (not more than 30% of the body surface); HCG 3, background color green or background color almost obscured by black markings (more than 30% of the body surface), prominent femoral black stripes and light-colored eyes; HCG 4, pale background color almost obscured by black markings (60-80% of the body surface) and dark-colored eyes; HCG 5, background color entirely obscured by black markings (more than 80% of the body surface).

2.3. Rearing of hatchlings

Newly hatched green (HCG 1) and black (HCG 5) nymphs were reared either in isolation or in group (5 individuals) in Petri dishes (diameter, 9 cm; height, 2 cm) at 32℃. Hatchlings from eggs laid by crowd-reared adults were collected for experiments in two different ways: they were collected 6 – 12 hours either after hatching in group in

the oviposition cups or after hatching in isolation in small plastic dishes. Hatchlings were fed small pieces of orchard grass and cabbage leaves mixed with wheat bran which were changed daily. All nymphs were immobilized on ice for 15 min and scored on day 4 of the first stadium using the same criteria as for HCGs. The duration of the first nymphal stadium was 5 -7 days. The body coloration was again recorded on day 3 of the second stadium. In this case, nymphs were photographed according to the method by Tanaka (2003) and categorized into 3 groups based on the darkness of the body color; 1, nymphs with no black patterns; 2, nymphs with some black patterns (not more than 80% of the pronotum surface); 3, nymphs with intensive black patterns (more than 80 % of the pronotum surface).

3. Results

3.1. Effects of washing and isolation

Eggs washed with saline and separated immediately after deposition remained yellowish throughout the incubation period, whereas unwashed controls turned brownish within a day (Fig. 1). Washing caused a high rate of mortality in the eggs (Fig. 2) but most hatchlings obtained developed intensive black patterns as categorized in HCG 5 (Fig. 3). Their proportion was not significantly different from that for unwashed controls (χ^2-test; $P>0.05$). A small number of hatchlings categorized into HGC 4, 3 or 2 appeared, but no individuals that were regarded as HCG 1 were obtained in either group. Isolation of eggs immediately after deposition did not affect either the egg color (Fig. 1) or the hatching rate significantly (χ^2-test; $P>0.05$; Fig. 2). Like washing, isolation did not elicit any effect on hatchling body color at all, as the proportions of hatchlings in different HCGs were not different significantly between the separated and un-separated control groups (χ^2-test; $P>0.05$; Fig. 3). Basically the same conclusions were reached by another set of experiments in which a total of 481 eggs were washed and separated or separated without being washed (data not shown).

3.2. Effect of separation before deposition of the foam plug

Oviposition was interrupted before deposition of the foam plug for 7 egg pods. Approximately a half of eggs from each egg pod were separated immediately after the interruption of laying and the rest kept in the egg pods in moist sand as controls. The results indicated that neither the absence of foam plugs nor separation of eggs resulted in the production of black hatchlings (Fig. 4). All hatchlings obtained after separation were black and categorized into HCG 5.

3.3. Characteristics of hatchlings in different HCGs

Fig. 5 compares body weights of hatchlings in different HCGs. In all treatments and controls, apparent positive relationships were manifested between HCGs and body weights: i.e. hatchlings with darker body coloration were heavier, indicating that hatchling body color was body-size dependent (Figs. 5 and 6) and that it had probably been determined before the eggs were deposited. In the treated groups, some hatchlings were light-colored (HCG 2 or 3), but none of them were as heavy as average hatchlings (21 mg) of HCG 5.

3.4. Effects of rearing density during the first stadium on body color

To determine the effect of rearing density on body color, hatchlings of HCG 1 or 5 were reared either individually or in groups of 5 individuals. Fig. 7A compares the body coloration of nymphs at 1 or 2 days before ecdysis to the second nymphal stadium (day 4 of the first stadium). Hatchlings of either HCG 1 or 5 showed no marked change in body coloration during the first nymphal stadium under either isolated or crowded conditions. That is, light-colored hatchlings remained without any black patterns (grade 1) and dark-colored ones remained with intensive black patterns (grade 5). Some eggs were separated immediately after egg-pod deposition by crowd-reared adults. Most of them produced black hatchlings, as shown in Fig. 3, and those categorized into HCG 5 were reared either under isolated or crowded conditions. They also remained black and

did not look different from those that hatched in group and reared irrespective of the rearing density (Fig. 7A). These results indicated that isolation of eggs and hatchlings had no significant effect on the body color during the first nymphal stadium.

Crowding effects were manifested in the second stadium. Hatchlings of HCG 1 reared in isolation remained without black patterns after ecdysis to the second stadium, whereas those reared under crowded conditions developed some black patterns (Fig. 7B). Hatchlings of HCG 5 reared under isolated conditions became significantly lighter in body color at the second stadium, whereas those kept under crowded conditions remained very dark.

The above experiment did not exclude the possibility that the rearing conditions during the first 2 days of the second stadium had some influence on the body color. To test this possibility, hatchlings of HCG 5 obtained from un-separated eggs laid by crowd-reared adults were reared under isolated or crowded conditions until ecdysis to the second stadium when they were transferred to the other rearing conditions (crowded or isolated conditions) before being scored on day 2. Those nymphs which were reared under crowded conditions until ecdysis to the second stadium and then transferred to isolated conditions (n=20) were all strongly pigmented as those kept continuously under crowded conditions (n=142; data not shown). Those nymphs which were first isolated and then transferred to crowded conditions (n=40) became slightly lighter, but no significant difference was found in their proportions when compared with those (n=106) kept in isolation continuously (χ^2-test; $P>0.05$; data not shown). These results indicated that the rearing conditions during the first few days of the second stadium had no significant effect on the body color of second stadium nymphs.

4. Discussion

Recent studies conducted by the Oxford group reported that gregarious characteristics of hatchlings such as body color and gregarious behavior of *S. gregaria* were greatly influenced by a pheromonal factor derived from the accessory gland of the

female parent, as summarized by Simpson et al. (1999) from the behavioral point of view. According to their studies, this factor is produced only by gregarious females and secreted into the foam plug of egg pod (McCaffery et al., 1998). It is effective only during the first hour of deposition so that washing eggs from gregarious female adults with saline shortly after egg pod deposition can remove this factor, resulting in the production of solitarized hatchlings with lighter or green body color and solitarious behavior. Likewise, early separation of eggs has the same effects (McCaffery et al., 1998), because it was thought to prevent the pheromonal factor from reaching the eggs (Simpson et al., 1999). Ligation of the accessory glands in female adults also had a similar effect on the gregarious behavior of their hatchlings, but in this case it did not affect the body color (Hägele et al., 2000). Saline extracts of egg pods produced by gregarious female adults contain this factor and can cause presumptive solitarious hatchlings (produced by isolated-reared female adults) to develop dark color as well as gregarious behavior when applied shortly after egg-pod deposition.

The present results completely failed to reproduce the effects of washing and separation of eggs in the same species. A total of 649 eggs produced by gregarious (crowd-reared) female adults were washed with saline and rinsed with distilled water immediately after deposition to determine whether or not such treatments would produce green hatchlings. Mortality was high after washing, but most individuals obtained developed black patterns typical of gregarious hatchlings and few were light-colored, as in the unwashed counterparts. Early separation of eggs, which was suggested to be equally effective in solitarizing hatchlings (McCaffery et al., 1998), was also totally ineffective in changing hatchling body color from black to green, and most hatchlings developed intensive black patterns, as in the un-separated controls. The gregarizing factor was suggested to be present in the foam plug of egg pod (McCaffery et al., 1998; Simpson et al., 1999). In the present study, however, none of the eggs separated even before the deposition of the foam plug produced green hatchlings. These results led us to conclude that hatchling body color is not modified by washing or

separation of the eggs and that darkening of hatchlings occurs without being exposed to the foam plug factor during the egg stage.

After the above experiments, I hypothesized that hatchling body color is determined before egg-pod deposition. It has been known that black hatchlings typical of gregarious forms are heavier than green hatchlings typical of solitarious forms (Hunter-Jones, 1958). The differences in hatchling body color and body weight are caused by the crowding conditions experienced by the female parents as adults. The factor responsible for this change has not been identified, but it is produced by the female parent because the crowding effect can be exerted without males: virgin females kept in group produced eggs that gave rise to black hatchlings parthenogenetically (Hunter-Jones, 1958). If presumptive gregarious eggs produced by gregarious female adults can be modified to produce green hatchlings by washing or separation, one might expect to find some heavy hatchlings with green color in the treated group. However, no such individuals were obtained in the present study. Instead, I found a consistent positive relationship between the darkness of body color and body weight of hatchlings, as expected from the studies by Hunter-Jones (1958). Unfortunately, hatchling body weight was not measured in any of the studies by the Oxford research group (Islam et al., 1994a, 1994b; McCaffery et al., 1998; Simpson et al., 1999; Hägele et al., 2000). The present results strongly suggested that hatchling body color is determined maternally and no significant change in body color is caused by post-ovipositional treatments.

My results can hardly be reconciled also with another conclusion by the Oxford group that color changes occur according to the density experienced by the hatchlings during the first few hours following hatching in *S. gregaria* (Islam et al., 1994a). This conclusion is based on their results that hatchling body color became darker when hatchlings were kept in group during the first 6 hours. In the present study, all hatchlings were maintained under the same conditions as eggs at least until 6 -12 hours after hatching. Yet, no significant difference was found in proportions of hatchlings with

different body colors between eggs separated at deposition and those kept together in the egg pods, as described above. Therefore, hatchling body color is not likely to be modified by crowding conditions during the first few hours after hatching in contrast to the conclusion by Islam et al. (1994a). My conclusion may be strengthened by another piece of observation. That is, green hatchlings used for rearing experiments were obtained from solitarious cultures, but they were all kept undisturbed in the oviposition cup (>60 individuals per cup) at least for 6 hours before being used. However, they remained green throughout the first stadium even if they were kept continuously under crowded conditions (Fig. 7A). Crowding conditions during the first stadium influenced the darkening of the nymphs, but the effect was apparently not manifested until after ecdysis to the second stadium, as described by Injeyan et al. (1979).

The contrasting results obtained from the two laboratories seem to be difficult to explain. However, there are a few possibilities. One is a genetic difference. Although the cultures of the two laboratories originated from the same source (International Centre of Insect Physiology and Ecology, Kenya)(Lester et al., 2005; Maeno et al., 2004), they had been maintained for many years in each laboratory. This might have resulted in some genetic differences. The Oxford group used a locust saline, whereas I used a simple saline of 0.9 % NaCl. However, this difference cannot explain the fact that I also failed to produce green hatchlings by separation of the eggs. Another possibility may be associated with sampling errors. In the Oxford group, the numbers of egg pods used for experiments were small and inter-egg pod variation might have influenced their results, because single egg pods often produce a mixture of hatchlings with different body colors (Husain and Ahmad, 1936), particularly among those deposited early in the adulthood (Maeno and Tanaka, unpublished observations). For example, McCaffery et al. (1998) used hatchlings from 2-7 egg pods for each treatment to test the effects of washing and separation of eggs. In another study (Islam et al., 1994a), the number of egg pods used was not described but it was probably very small because they tested an average of only 16 nymphs per treatment. Thus, the possibility that inter-egg pod

variation caused substantial errors in their study cannot be ruled out and may also explain their sporadic results that washing of eggs from crowd-reared females had no effect on the hatchling body color in another study (Hägele et al., 2000). In the present study, I used 32 and 28 egg pods for the washing and separating experiments, respectively. I sampled eggs from different parts of each egg pod as evenly as possible for the treatments and always used the rest eggs as controls to minimize the influence of possible inter- and intra-egg pod variation.

Nevertheless, it seems difficult to explain some of the results reported by the Oxford group by assuming sampling errors alone, because the age-dependent effects of washing and separation on hatchling body color were so clearly presented (Figs. 5 and 6 of McCaffery et al., 1998). In the present study, I have not tested the effects of washing or separation of eggs on the gregarious behavior of the hatchlings. However, the role of the egg-pod foam plug factor suggested (McCaffery et al., 1998; Simpson et al., 1999) was not supported by Malual et al. (2001) who indicated C-8 unsaturated ketones as the pheromonal factor responsible for the induction of gregarious behavior in hatchlings of *S. gregaria*. The pheromonal factor suggested by the Oxford group has not been fully characterized. Its chemical identification should answer the questions raised by Malual et al. (2001) and the present study.

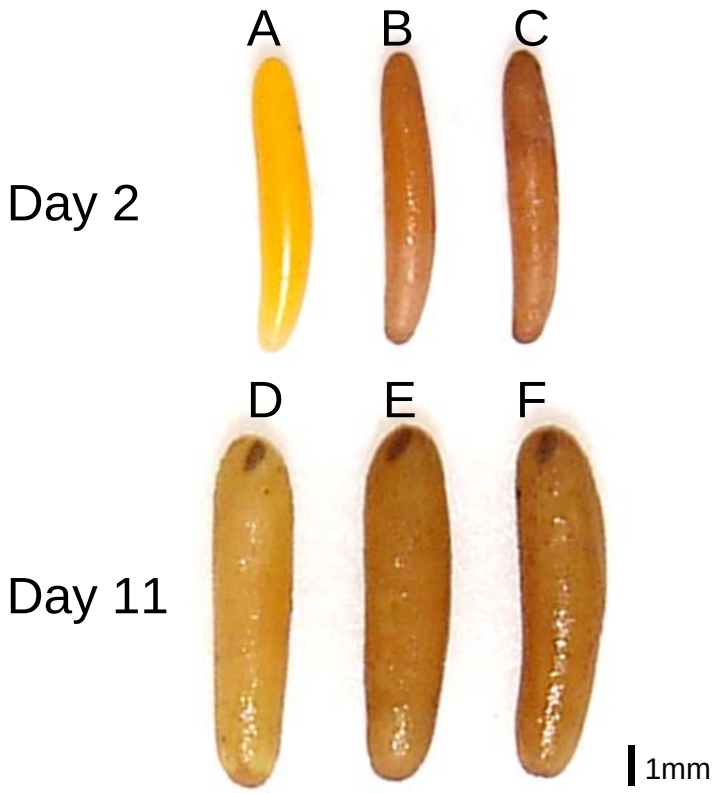

Fig. 1. Photographs showing *S. gregaria* eggs washed with saline solution (A, D), separated without being washed (B, E) and untreated (crowded, C, F) immediately after deposition of the egg pod. Eggs were incubated at 32℃ and photographs taken on days 2 and 11. Note that washed eggs remained yellowish. A dark spot in 11-day-old eggs is an embryonic compound eye.

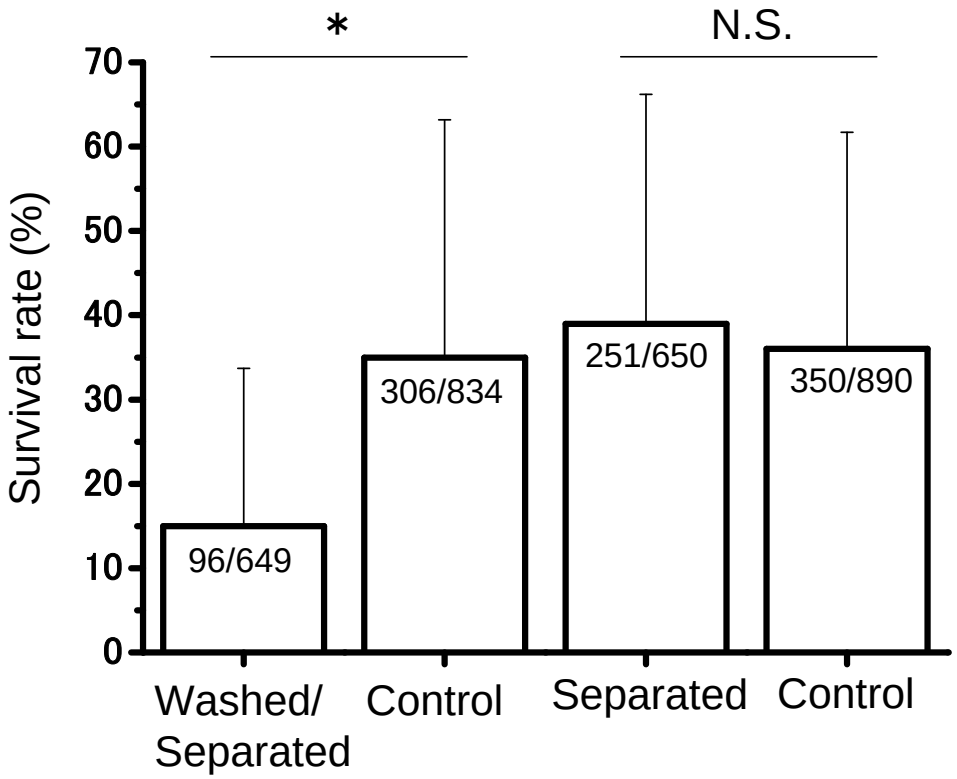

Fig. 2. Comparison of the effects of separation of eggs on survival rates with separation plus saline washing of eggs in *S. gregaria*. All eggs were laid by crowd-reared females and some of them kept without separation in the egg pods as controls. The numbers in the figure indicate the number of hatchlings obtained / that of eggs tested. Asterisks indicate a significant difference by χ^2-test at 1 %.

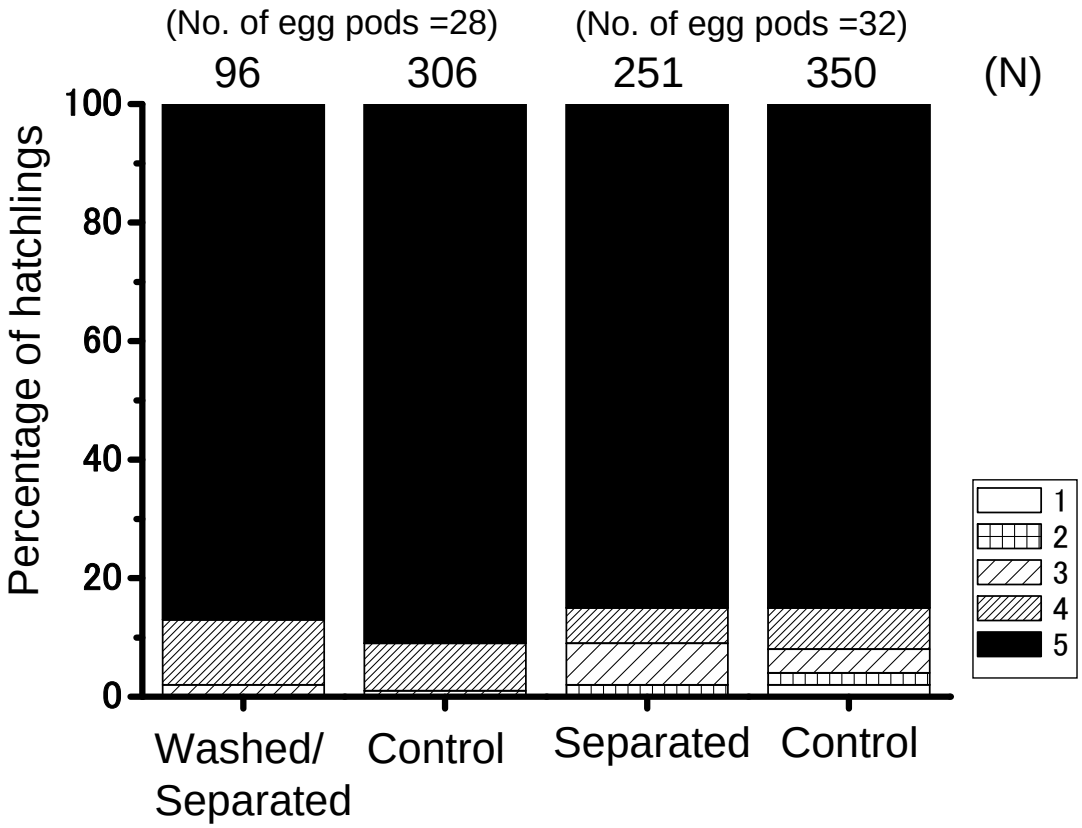

Fig. 3. Comparison of the effects of separation of eggs on hatchling body color with separation plus saline washing of eggs in *S. gregaria*. All eggs were laid by crowd-reared females and some of them kept without separation in the egg pods as controls. Hatchlings were categorized into 5 color groups based on the darkness of body color (see Materials and Methods). The numbers in the figure indicate the numbers of hatchlings obtained.

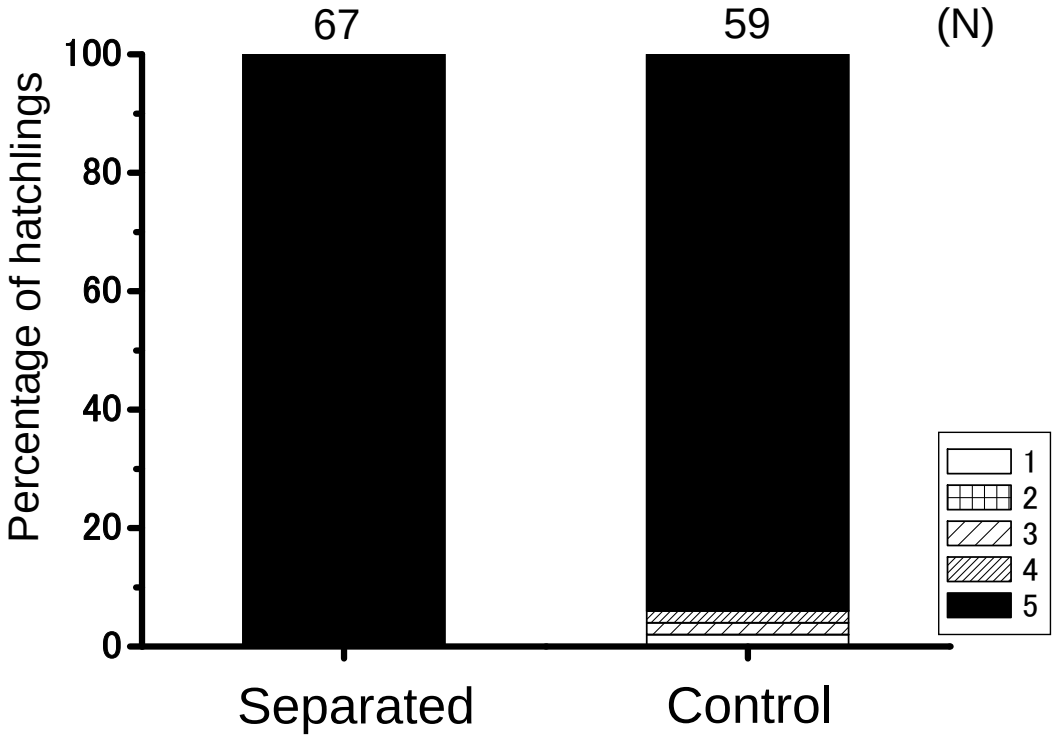

Fig. 4. Effect of separation of eggs before deposition of the foam plugs on hatchling body color in *S. gregaria*. All eggs were laid by crowd-reared females and some of them kept without separation in the egg pods as controls. Hatchlings were categorized into 5 color groups based on the darkness of body color (see Materials and Methods). The numbers in the figure indicate the numbers of hatchlings obtained.

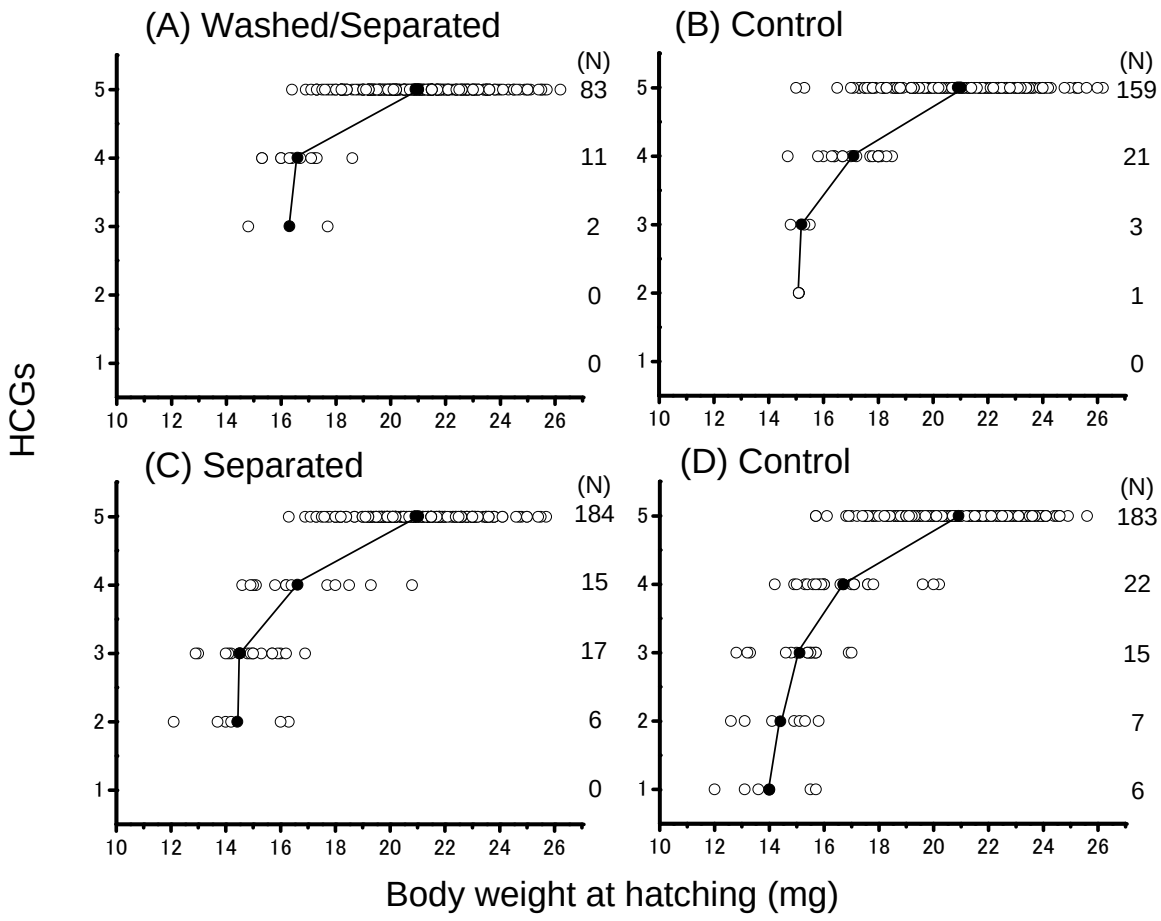

Fig. 5. Relationships between hatchling body color groups (HCGs) and body weights in hatchlings of *S. gregaria* in the experiment shown in Fig. 3. Open circles indicate individual datum points but many individuals are overlapping for HCGs 4 and 5. Closed circles indicate the means, which are connected by a line. Numbers in the figure indicate N.

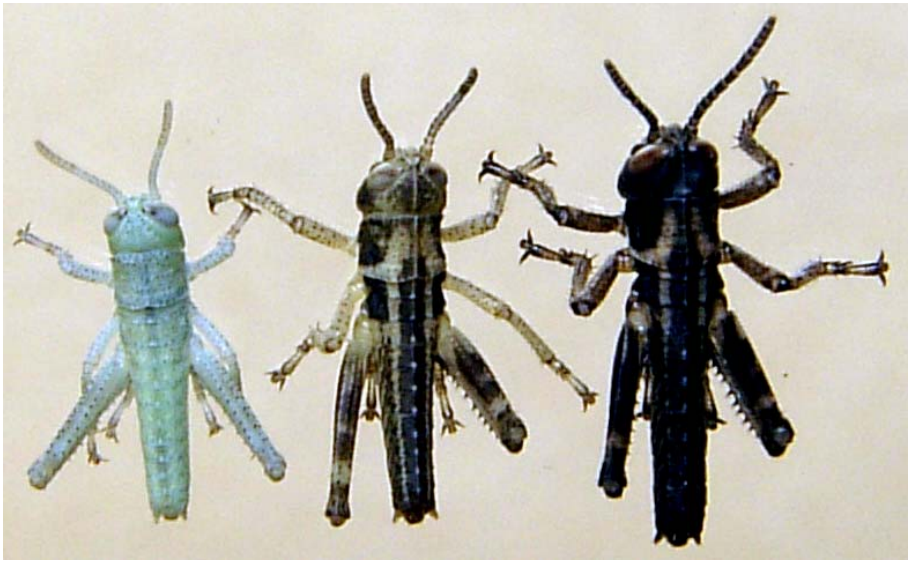

Fig. 6. Photographs of hatchlings representing HCGs 1, 3 and 5.

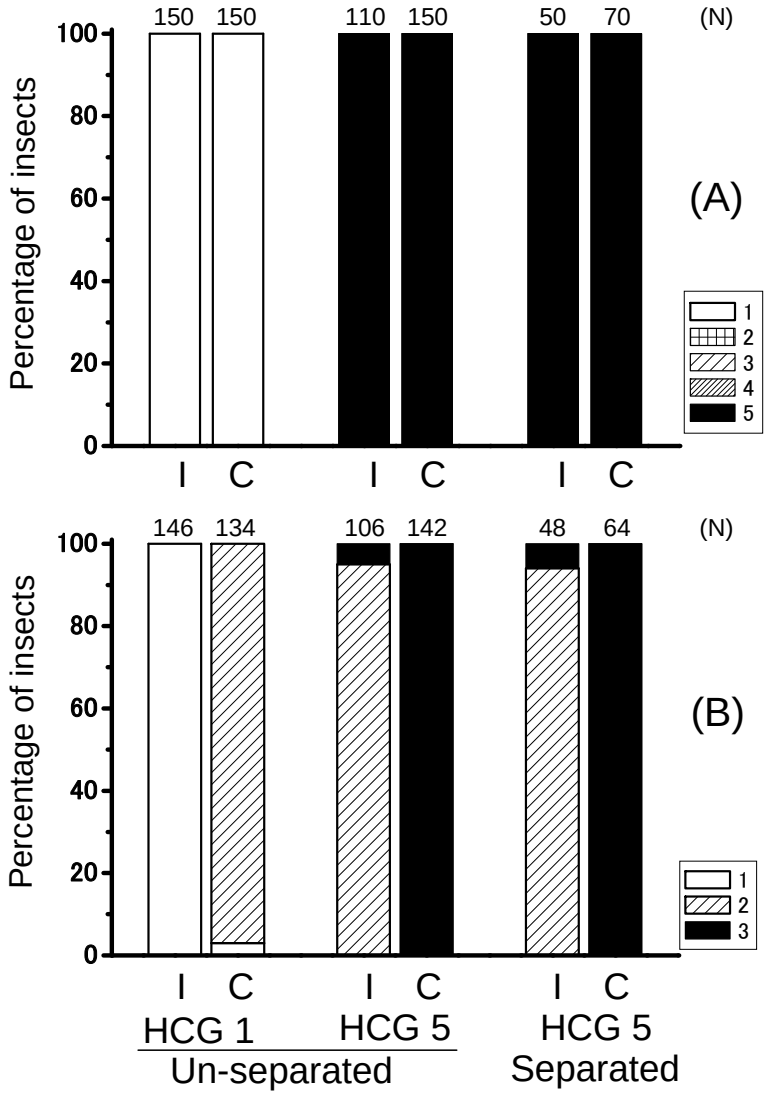

Fig. 7. Effect of nymphal rearing density on body coloration on day 4 of the first stadium (A) and on day 2 of the second stadium (B) in *S. gregaria*. Hatchlings of HCG 1 or 5 were reared either under isolated (I, 1 nymph per dish) or crowded conditions (C, 5 insects per dish). Nymphs hatched in group from eggs in the egg pod or in isolation from eggs separated on the day of deposition. Body color for first stadium nymphs was scored using the 5 grades with the same criteria for HCGs (see Materials and Methods) and body color for second stadium nymphs using 3 grades: 1, no black patterns; 2, some black patterns; 3, intensive black patterns.

Chapter 5.2.

Maternal effects on progeny body size and color in the desert locust, *Schistocerca gregaria*: Examination of a current view

Abstract

Hatchling body color and size of the desert locust, *Schistocerca gregaria*, are determined by the population density of the mothers during their reproductive period. Small green hatchlings are produced by adults at low population density (solitarious conditions) and large dark hatchlings at high population density (gregarious conditions). One claim states that a pheromonal factor secreted by gregarious mothers into foam plugs of egg pods induces darkening in hatchlings. Previous research suggests that the foam factor can be removed by separating eggs individually within 1 hour of deposition, causing presumptive gregarious eggs to hatch without darkening. The present study examined this phenomenon and a recently revised version of the foam hypothesis. Early separation was performed on eggs with a low mortality rate. The results showed that egg separation did not increase the incidence of green hatchlings. Once chorionated in the ovary, egg size remained unchanged until the second day after oviposition in either isolated or crowded locusts. This and other results suggest that the phase-dependent differences in body size and color of hatchlings are established in the ovary and that modifications by the accessory gland factor either in the oviduct or after deposition are unlikely.

1. Introduction

The desert locust, *Schistocerca gregaria*, shows phase polyphenism in response to population density (Uvarov, 1966, 1977; Pener, 1991). One of the most conspicuous changes observed in this phenomenon is body color (Faure, 1932). There are two kinds of body color polyphenisms during the nymphal stage. One kind is observed in hatchlings and the other is observed in nymphs of later nymphal stadia. In the latter, body color can change within the life of an individual in response to changes in population density. Juvenile hormone is responsible for induction of green color often expressed at low population density (Dale and Tobe, 1990; Pener, 1991; Pener and Yerushalmi, 1998) and [His^7]-corazonin is known to induce black patterns typically observed under crowded conditions (Tawfik et al., 1999; Tanaka, 2001, 2006). The mechanism controlling hatchling body color appears to be more complicated, because it involves maternal effects. Hatchlings observed at low population density (solitarious phase) are characteristically green, whereas those observed at high population density (gregarious phase) develop black patterns, although both types of hatchlings as well as intermediate types often appear from single egg pods laid by either solitarious or gregarious locusts (Hunter-Jones, 1958). Hatchling body color is not influenced by crowding or isolation during embryonic development and after hatching and remains unchanged during the first stadium (Tanaka and Maeno, 2006).

Many researchers have noticed a close correlation between body color and size of hatchlings; solitarious green hatchlings are small and gregarious ones are large. These characteristics are determined by the population density experienced by the female parents as adults (Hunter-Jones, 1958; Islam et al., 1994a). In explaining this density effect, Simpson et al. (1999) proposed three hypotheses with an emphasis on the role of male adults. However, the presence of males is not essential in this phenomenon because even virgin females produce black and large hatchlings characteristic of gregarious forms parthenogenetically when kept in groups (Hunter-Jones, 1958).

Recently, the Oxford research group of Simpson and colleagues suggested that

hatchling body color is determined after egg deposition by a water-soluble pheromonal factor produced by the accessory gland of the female parent (McCaffery et al., 1998; Simpson et al., 1999; Hägele et al., 2000). They reported that washing of presumptive gregarious eggs produced by crowd-reared females prevents darkening of the hatchlings, producing green hatchlings. Saline extracts of egg pod foam plugs contain an active factor that promotes darkening in both hatchlings from eggs of solitarious females and those from eggs of gregarious females that have been separated and washed to remove this factor. This factor was suggested to be a small (<3 kDa), hydrophilic substance (McCaffery et al., 1998; Simpson et al., 1999). The solitarizing effect was also obtained by simply separating gregarious eggs within 1 hour of deposition (McCaffery et al., 1998; Simpson et al., 1999), because the factor from the foam plug was presumably either removed or prevented from reaching the eggs by the separation. I re-examined the role of this pheromonal factor, but could not reproduce their results (Tanaka and Maeno, 2006). Instead, I found that all green hatchlings obtained in my experiments were consistently small and all black ones were consistently large regardless of exposure to the foam plug, as reported by others (Hunter-Jones, 1958). Because such differences in hatchling body size seemed extremely unlikely to occur after oviposition, I concluded that hatchling body color closely correlated with hatchling body size is determined before the eggs are laid, casting doubt on the involvement of the pheromonal factor.

Responding to my study (Tanaka and Maeno, 2006), Simpson and Miller (2007) provided two possibilities for my failure to reproduce their results. First, they suggested that the high mortality rate that I observed removed all or most eggs that would otherwise have produced green hatchlings, because they are smaller and weaker than eggs producing dark hatchlings. Second, they postulated that the eggs I analyzed might have been exposed to the accessory gland factor in the oviduct because deprivation of the ovipositing substrate during the night caused the female parents to withhold the eggs too long.

In the present study, I designed several experiments to examine the above

possibilities and the current view proposed by Simpson and Miller (2007) of the maternal effect mechanisms by which phase-dependent hatchling body color and size are determined. I first re-examined the 'green-color inducing effect' of egg separation using egg pods with a high survival rate. In this experiment, female parents were given free access to the ovipositing substrate all day long to avoid the possible occurrence of delayed oviposition. Simpson et al. (1999) suggested that the appearance of mixed green and black hatchlings from single egg pods produced by crowded locusts is caused by uneven exposure of eggs to the foam plug factor shortly after deposition. This could mean that the phase-dependent differences in hatchling size and body color are caused after exposure to this factor. I tested this hypothesis by observing the relationship between egg size at deposition and body weight of green and black hatchlings from the same egg pods laid by crowd-reared females. In this experiment, I also examined the distribution of eggs in the egg pod in relation to egg size at deposition, egg mortality and hatchling body color. Here, I present experimental evidence that indicates that both egg size and hatchling body color are determined in the ovary, and are not influenced by the accessory gland factor either in the oviduct or after deposition, which suggests that the current view of the maternal effects in this locust proposed by Simpson and Miller (2007) should be reconsidered.

2. Materials and Methods

2.1. Insects

The *S. gregaria* locust strain used in the present study was the same as that used in previous studies (Tanaka and Yagi, 1997; Maeno et al., 2004) and the rearing methods were performed as previously described by Maeno and Tanaka (2007) with slight modifications. Briefly, locusts were reared at 32°C with a photoperiod of light:dark 16:8 h at the Tsukuba laboratory. Eggs were derived from either a gregarious (crowd-reared) line that had been maintained at a density of approximately 100 individuals in large cages (42 × 22 × 42 cm) over many generations or a solitarious (isolation-reared) line in

which locusts were individually reared in small cages (28 × 15 × 28cm), except for a short period for mating (<24 hours), for more than three generations. In the present study, sexually mature crowd-reared female adults were transferred from the large cages to small ones in which each female was kept with two sexually mature males to collect egg pods. In *S. gregaria*, hatchlings from single pairs are not different in body color and weight from those produced by parents crowded for many generations (Hunter-Jones, 1958). My preliminary observations indicated that successive egg pods produced by a single female parent tend to show similar rates of egg survival. I thus picked those females that produced the first few egg pods with a high rate of egg survival, and collected egg pods for subsequent experiments. The orchard grass used to feed the insects was raised by the Field Management Section of NIAS at Ohwashi.

2.2. Collection and handling of eggs

The method for egg collection was the same as that used in a previous study (Tanaka and Maeno, 2006) except that ovipositing cups containing moist sand were placed in each cage all the time after females became sexually mature. The method of egg separation was the same as that used in a previous study (Tanaka and Maeno, 2006).

To determine if black and green hatchlings appear more frequently from the upper and lower regions of an egg pod, respectively, egg pods producing a mixture of black and green hatchlings were needed. I noticed that crowd-reared females tended to produce such egg pods early in their adulthood whereas almost all eggs laid late in their life produced black hatchlings (Maeno and Tanaka, unpublished observations). Twenty-seven egg pods obtained from crowd-reared females within 3 weeks after adult emergence were used for this experiment. Two days after deposition, eggs were carefully removed one by one starting from the bottom portion of the egg pod, held individually on moist cotton pads in small plastic dishes (diameter, 40 mm; height, 10 mm) and given a number according to their order of removal. The eggs in each egg pod were divided into three groups of equal sizes according to their position, i.e., upper,

middle and lower regions, in the egg pod. Egg length was measured at day 2 for all eggs of each egg pod by an ocular micrometer installed in a binocular microscope. My preliminary observations indicated that egg length started increasing rapidly on day 4 but did not change significantly during the first 2 days and was highly correlated with egg weight measured approximately 5 seconds after each egg was placed on a balance (R=0.939; n=200; P<0.001). I encountered difficulty in weighing individual eggs because egg weight kept decreasing on a balance as water on the egg surface evaporated. Measuring egg length is probably easier and more reliable to determine egg size than weighing eggs particularly during the first few days. Shulov and Pener (1963) reported significant changes in egg weight even during the first few days, but the differences were subtle. Because they used different eggs for measurements on different days, it is not clear if such subtle differences represented real changes in egg weight or experimental errors due to individual variation. In the present study, all eggs were observed until they hatched or died to determine which region of the egg pod had the highest frequency of green hatchlings and the highest mortality rate.

To compare egg lengths of ovarian and deposited eggs, actively reproducing female adults were allowed to deposit an egg pod and dissected 4 days later. Some of these females contained chorionated oocytes or eggs in the ovary. Ten eggs were randomly sampled from each egg pod or each pair of ovaries. The lengths of deposited eggs were measured at day 2 as described above and those from the ovarian eggs immediately after dissection.

2.3. Effect of old sand on hatchling body color

To examine the effects of old sand on hatchling body color, actively reproducing isolation-reared females were allowed to lay an egg pod into fresh sand first as a control and then lay another egg pod into old sand containing the foam material of three egg pods produced by crowd-reared females during the previous 24 hours. To avoid mixing eggs from isolation-reared females with those from crowd-reared females, crowd-reared

eggs were removed carefully from the egg pods, leaving the foam material in the old sand for the subsequent experiment. I used only those females that oviposited within 24 hours after the old sand was presented, because the pheromonal factor is assumed to be active only for a short time (Simpson and Miller, 2007). Fresh sand denotes sand that was washed thoroughly with tap water and dried at 100°C in an oven overnight. Some isolation-reared females produced green hatchlings at a low rate (<30%) from the egg pods deposited in fresh sand; such females and egg pods were not used for this experiment. Both types of sand were moistened with distilled water (ca. 15% water content) before use. The eggs deposited into the two types of sand were incubated at 32°C to compare body color of the hatchlings.

2.4. Scoring of hatchling body color

The body color of hatchlings was observed 6 – 12 hours after hatching. Hatchling body color in *S. gregaria* is not influenced by crowding conditions experienced during the first stadium (Tanaka and Maeno, 2006). Nymphs were divided into five hatchling color groups (HCGs 1 – 5) as the darkness of the color increased (Maeno and Tanaka, 2007). Hatchlings in HCG 1 are green as typically observed in solitarious forms and those in HCG 5 are almost completely black as observed in gregarious forms. Those in HCGs 2 – 4 are intermediate in color and increasingly darker.

3. Results

3.1. Effect of egg separation

From each of egg pods deposited by crowd-reared females, some eggs were removed and separated individually within 1 h of deposition and the rest eggs kept together inside the egg pod to evaluate the color of the hatchlings. The proportions of hatchlings in different color groups (HCGs 1 – 5) are shown in Fig. 1. The survival rate in different egg pods ranged from 50 to 88% (n = 9). Almost all eggs used in this experiment produced black hatchlings, and no green hatchlings were obtained. As a

result, no significant difference was found in the proportion of hatchlings in different color groups between the experimental and control groups (χ^2 test; P>0.05), which is consistent with my previous study (Tanaka and Maeno, 2006). Of the nine egg pods, four showed more than 80% hatchability, which is regarded as a high survival rate by Simpson and Miller (2007). Almost all hatchlings (n = 188) obtained from these four egg pods were black (HCG 5): no green hatchlings belonging to HCG 1 appeared regardless of whether or not they had been separated as eggs.

3.2. Egg size, mortality and hatchling characteristics of eggs in relation to their position in the egg pod

Eggs derived from crowd-reared females were isolated from the upper (1), middle (2) and lower (3) regions of the egg pods and the mean length of eggs in each egg pod region was compared (Fig. 2). The most commonly found egg pods contained various sizes of eggs that were evenly distributed among the 3 regions (63%), whereas only 11% of the egg pods had an equal distribution of eggs in the upper and middle regions that together was greater than that found in the lower region (Fig. 2B). There were no significant differences by ANOVA in the mean values for egg length among the upper (mean $\pm$ SD = 7.03 $\pm$ 0.33 mm; n = 27), middle (7.07 $\pm$ 0.34 mm; n = 27) and lower regions (7.03 $\pm$ 0.32 mm; n = 27; P>0.05)**.** Of the 27 egg pods evaluated, 8 egg pods produced HCG 1 green hatchlings at a rate ranging from 2.7 to 69.4%. Only two of these egg pods produced green hatchlings most frequently from the lower region of the egg pod. Although the sample size was small, there was no apparent tendency for green hatchlings to occur preferentially in the lower region of the egg pod (Fig. 2B).

To determine if mortality occurs more frequently in a particular region of the egg pod, survival rates were compared among the three regions of the egg pod. The analysis was performed with 10 egg pods that showed greater than 50% survival. Survival rates were similar in the upper (mean $\pm$ SD = 63.4 $\pm$ 18.2%; n = 241), middle (66.5 $\pm$ 19.4%; n = 241) and lower (67.0 $\pm$ 18.2%; n = 238) regions (χ^2 = 2.875; df = 2; P > 0.05),

indicating that there was no site-specific mortality in the egg pod. Furthermore, mean egg length measured at day 2 for un-hatched (dead) eggs (7.08 $\pm$ 0.36 mm, n = 203) was almost identical to that for hatched ones (7.11 $\pm$ 0.33 mm, n = 480; t-test; df= 681; P>0.05), indicating that there was no size-dependent mortality.

There was a highly positive correlation between egg length at day 2 and hatchling body weight (Fig. 3; R = 0.808; n = 684; P < 0.001). These data were based on egg pods producing a mixture of different hatchling body colors. Green hatchlings appeared from relatively small eggs but not from large eggs, which exclusively produced black hatchlings. This result based suggested that hatchling body color and size were closely associated with each other and were determined before day 2 of oviposition.

3.3. Comparison between ovarian and deposited eggs

To examine if egg size is modified after ovulation in the oviduct or shortly after deposition, egg length was compared between ovarian and deposited eggs obtained from solitarious and gregarious lines. As shown in Figure 4, no significant difference was found in egg length between ovarian and deposited eggs in either line (t-test; P > 0.05 each). However, both ovarian and deposited eggs from gregarious mothers were significantly longer than those from solitarious mothers (t = 17.239; df = 98; P < 0.001 for ovarian eggs: t = 17.843; df = 98; P < 0.001 for deposited eggs). Egg length remained unchanged at least through the second day of deposition once the eggs were chorionated in the ovary. Deposited eggs hatched in 2 weeks. Virtually all hatchlings from the solitarious egg pods were green and belonged to HCG 1 (96.5%; n = 115) and those from the gregarious ones were primarily black and categorized into HCG 5 (97.1%; n = 207).

3.4. Effect of contaminated sand on hatchling darkening

Isolation-reared females were allowed to lay egg pods into old sand that contained the foam material from three egg pods produced by crowd-reared females. These egg

pods were then compared in terms of the incidence of dark hatchlings with the egg pods that were laid into fresh sand singly before this trial (Fig. 5; mean ± SD = 12.7 ± 28.4%; $n = 5$): the incidence of dark hatchlings from eggs deposited in old sand did not increase (6.1 ± 13.6%; $n = 5$; t-test after arcsine transformation of the data; $P > 0.05$). Of five female adults, four laid egg pods that produced only green hatchlings irrespective of whether they had oviposited into fresh or old sand. These results indicated that the egg foam material produced by crowd-reared females had no effect on hatchling body color.

4. Discussion

Based on a series of studies by the Oxford group (Islam et al., 1994a, b; McCaffery et al., 1998; Simpson et al., 1999; Hägele et al., 2000; Simpson et al., 2005), Simpson and Miller (2007) summarized a current view of the maternal effects in *S. gregaria*. One of the most important findings was that early washing or separation of eggs without washing effectively induces green hatchlings from presumptive black hatchlings produced by crowd-reared female adults (McCaffery et al., 1998). However, my previous study (Tanaka and Maeno, 2006) showed that neither treatment induced green hatchlings in *S. gregaria*. Simpson and Miller (2007) pointed out that the high mortality rate in my previous study resulted in a failure to detect any positive effects on hatchling body color, because eggs that might have otherwise produced green hatchlings died before hatching. This possibility is based on the assumption that eggs yielding green hatchlings are smaller and less robust than those yielding black hatchlings. The present study uses highly viable eggs (50-88% survival rate), and I again confirmed that early egg separation had no effect on hatchling color**.** Furthermore, by following individual eggs and their survival, I determined that egg mortality occurred independently of egg size in this locust.

The other explanation given by Simpson and Miller (2007) for my previous results contradicting the ‘solitarizing effect’ of the foam plug (Tanaka and Maeno, 2006) concerns delayed oviposition. In a previous study (Tanaka and Maeno, 2006),

ovipositing substrate was provided only during the day. Thus, female adults did not have access to ovipositing substrate during the night, even if they were ready to oviposit. Simpson and Miller (2007) argued that 'withheld' eggs in such females might have been exposed to the active substance in the oviduct *before* oviposition, although such a phenomenon has never been documented. In the present study, female adults were provided with ovipositing sand throughout the day. Therefore, there should have been no delayed oviposition or 'withheld eggs' due to a lack of the ovipositing substrate. Nevertheless, early separation elicited no significant solitarizing effects on such eggs. This fact indicates that the delayed oviposition hypothesis does not explain the absence of the solitarizing effect of early separation or washing in this locust (current study and Tanaka and Maeno, 2006).

In *S. gregaria*, there is size variation among eggs derived from a single egg pod. Papillon (1960) observed the occurrence of a gradient in the egg pods produced by gregarious adults; the heavy black hatchlings are mainly produced by the upper and middle regions of the pod, whereas most of the light green types hatch from the lower region. Simpson et al. (1999) suggested that this variation might be due to a gradient in exposure to the pheromonal substance from the foam plug. However, in a previous study (Tanaka and Maeno, 2006), eggs separated even *before* the formation of the egg foam plug by the crowd-reared female parent consistently produced heavy black hatchlings, suggesting that the presence of such a mechanism unlikely. Shulov and Pener (1963) measured egg weights within 23 h after oviposition, and reported no significant difference in egg size between the upper and lower thirds of the egg pod, although the eggs of the middle region were slightly heavier than those from the upper or lower regions. In the present study, 26% of the egg pods exhibited the same tendency as reported by Shulov and Pener (1963), whereas only 11% conformed to Papillon's results. Most commonly, eggs of different sizes were evenly distributed in the egg pod (63%), and no significant differences were found in mean egg length among the three regions of the egg pod. A close association between body color and size of hatchlings

was observed, but no evidence indicated that green hatchlings tended to appear from the lower region of the egg pod.

Simpson and Miller (2007) erroneously listed my previous study (Tanaka and Maeno, 2006) as a study in which egg pods were collected in old sand (more than three egg pods deposited previously) rather than in fresh sand (no previous egg pod deposition). Whether one uses fresh or old sand is important, because old sand containing egg pods produced by crowd-reared females has been suggested to influence some hatchling characteristics. For example, eggs from solitarious mothers mainly produce green hatchlings, but darkening is induced if their hatchlings are deposited as eggs into old sand containing egg pods from crowd-reared females (McCaffery et al., 1998; Simpson and Miller, 2007). Because these observations are difficult to reconcile with my previous results (Tanaka and Maeno, 2006), I re-examined this phenomenon. I allowed isolation-reared females to deposit three egg pods before the test and determined the proportion of green hatchlings in those egg pods. This procedure is important because some females lay egg pods that produce many black hatchlings (>70%) even under isolated conditions. Old sand containing three egg foam plugs from crowd-reared females was completely ineffective in inducing black hatchlings in egg pods produced by isolation-reared females. These results are consistent with my conclusion, but contradict the current view by Simpson and Miller (2007) of the dark-color inducing effects of egg foam plugs.

This leads to the question of exactly where the phase-dependent body size and color of hatchlings are determined. It is well known that dark hatchlings are larger than green hatchlings in *S. gregaria,* as described in the review by Simpson and Miller (2007). According to the conclusions by Simpson and Miller (2007), hatchling body color is determined by exposure to the gregarizing factor soon after ovulation in the oviduct fluids and/or in the egg foam after oviposition. This claim suggests that the phase-dependent difference in egg size or hatchling body size is also determined either in the oviduct or after oviposition. The present results reached a different conclusion.

Comparison of egg length showed that chorionated eggs in the ovary changed little in size at least until the second day after oviposition. Because egg length at the second day of oviposition is closely correlated with body weight at hatching (Fig. 3), one can conclude that hatchling body size is determined in the ovary. In fact, I found that ovarian eggs of isolation-reared females were much smaller than those of crowd-reared females (Fig. 4). The close association between egg size and hatchling body color would lead to the conclusion that the latter is also determined in the ovary.

It should be noted that a close correlation between egg size and hatchling body coloration does not necessarily prove a causal relationship. There is a possibility that the two traits are determined by different factors, but the present study suggests that they both are determined in the ovary.

As pointed out by Tanaka and Maeno (2006), one likely explanation for the discrepancy in conclusions between the two research groups is related to the large variation in the proportion of green and black hatchlings among egg pods. According to my observations (Maeno and Tanaka, unpublished data), egg pods producing a mixture of black, intermediate and green hatchlings could occur in a somewhat systematic manner under certain conditions in *S. gregaria*. For example, most eggs obtained from crowd-reared females after deposition of several egg pods produce black hatchlings (Fig. 1), whereas those deposited during the early stage of adult life tend to produce mixed progeny (Figs. 2 and 3) and the proportion of green hatchlings ranged from approximately 3 to 70% in the present study, covering the whole range of variation obtained from gregarious egg pods after washing or separation treatments in studies by McCaffery et al. (1998). The details of this phenomenon will be published in a forthcoming paper. Therefore, special care has to be taken to avoid sampling errors due to inter-egg pod variation. In the present study, I divided eggs from each egg pod into two and allotted them to experimental (early separation) and control groups, as performed in a previous study (Tanaka and Maeno, 2006). On the other hand, the Oxford group (Islam et al., 1994a,b; McCaffery et al., 1998; Hägele et al., 2000)

apparently adopted a different method, and the possibility that their results were influenced by inter-egg pod variation cannot be ruled out. Another possibility is a genetic difference between the strains used by the two laboratories, as pointed out by Tanaka and Maeno (2006) and Simpson and Miller (2007).

From these results, I conclude that hatchling body size and color are determined in the ovary and it is unlikely that these modifications occur either in the oviduct or after egg deposition in my strain of *S. gregaria*.

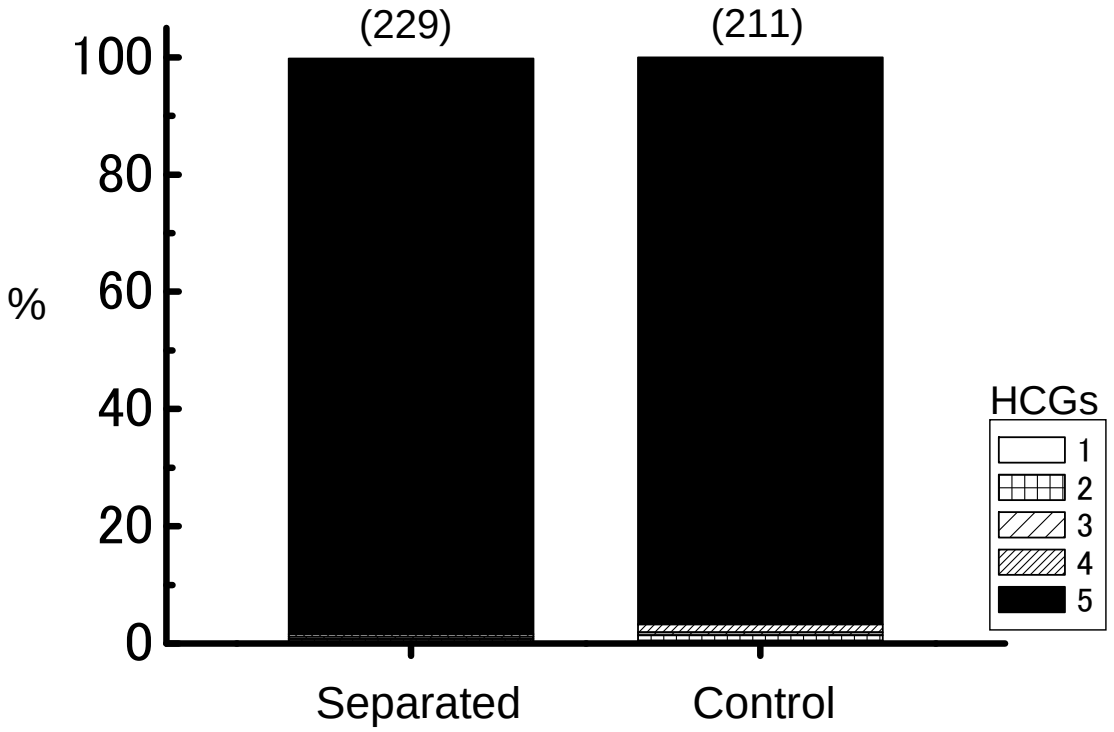

Fig. 1. Effect of egg separation on hatchling body color in eggs laid by crowd-reared female *S. gregaria* adults. Each of nine egg pods was divided into two groups, which were either separated individually (separated) within 1 hour of deposition or kept together in the egg pod (control). Hatchlings were categorized into five color groups (HCGs 1 – 5) based on the darkness of body color (see Section 2.4). The numbers in parentheses indicate *n*.

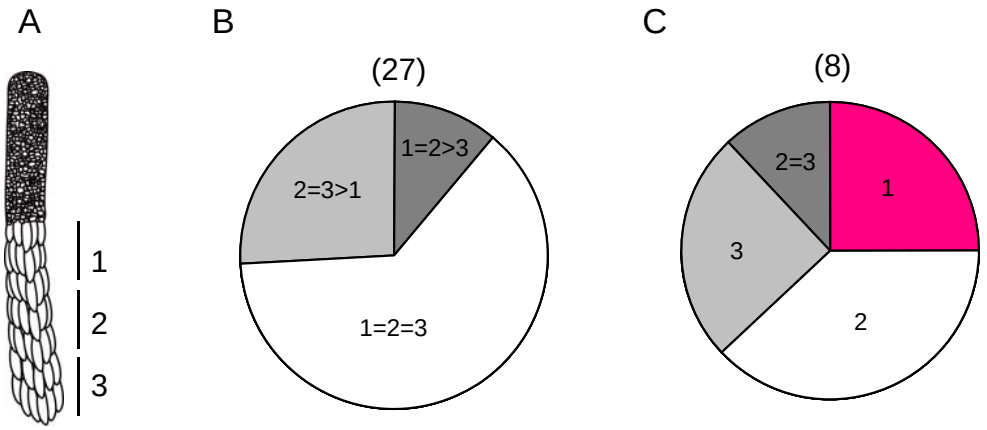

Fig. 2. Variations in egg size and the region in which green hatchlings appear most frequently in egg pods laid by crowd-reared female *S. gregaria* adults. A, Illustration of an egg pod with the upper (1), middle (2), and lower (3) regions indicated; B, comparison of mean egg length among the three regions of the egg pod; C, region in the egg pod that yielded the highest frequency of green hatchlings. The numbers in parentheses indicate the numbers of egg pods used in the experiment.

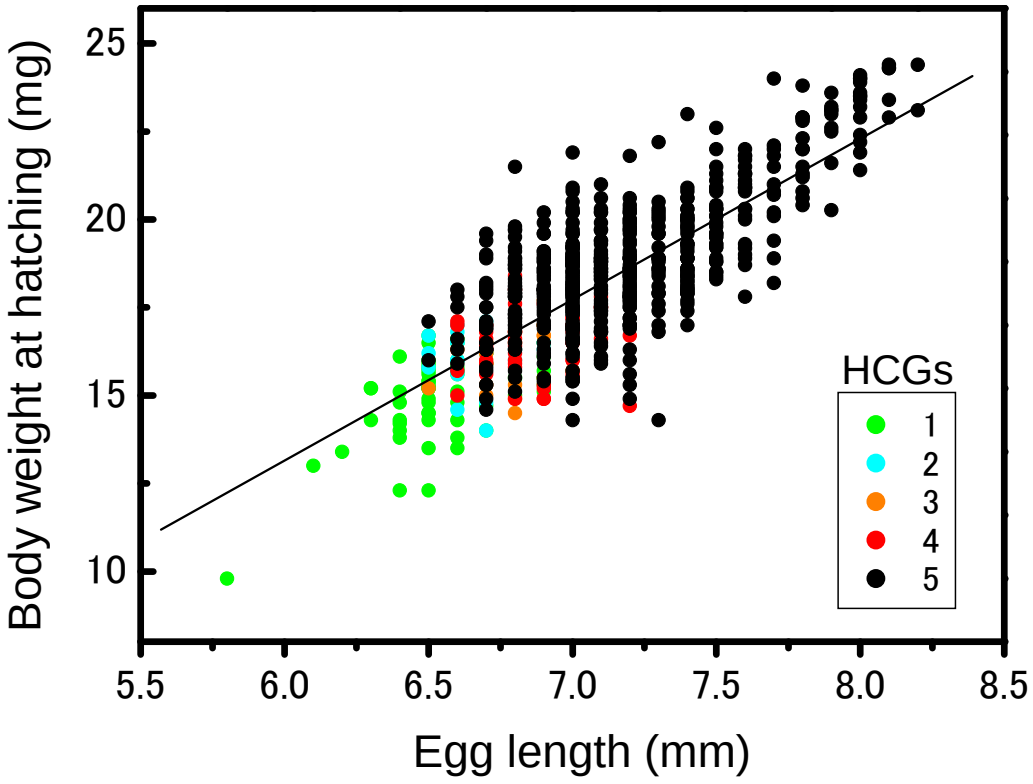

Fig. 3. Relationship between individual egg lengths at day 2 of deposition and hatchling body weights in *S. gregaria* (n = 684). All green hatchlings (n = 60) appeared from small eggs. The data were based on eggs derived from the experiment in Fig. 2.

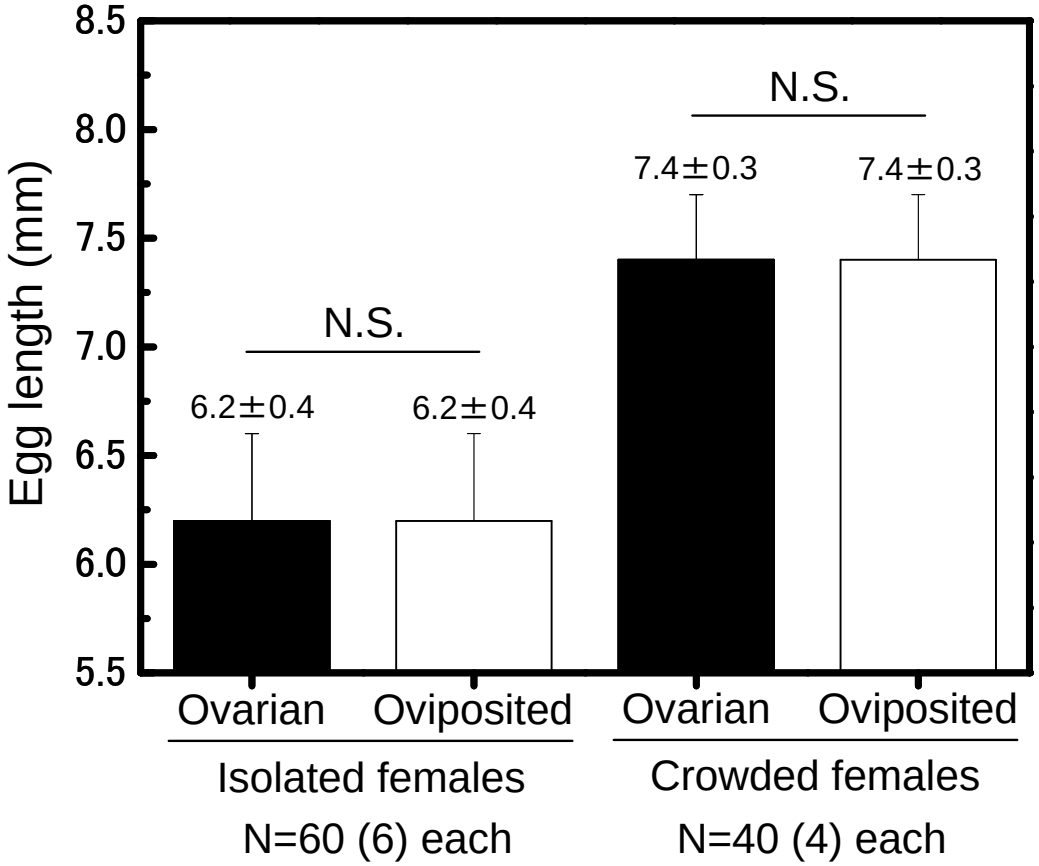

Fig. 4. Comparison of egg lengths between ovarian (black) and deposited (white) eggs from isolation-reared and crowd-reared *S. gregaria* females. N.S. indicates no significant difference at 5% by *t*-test. The numbers in parentheses indicate the number of female adults used in this analysis.

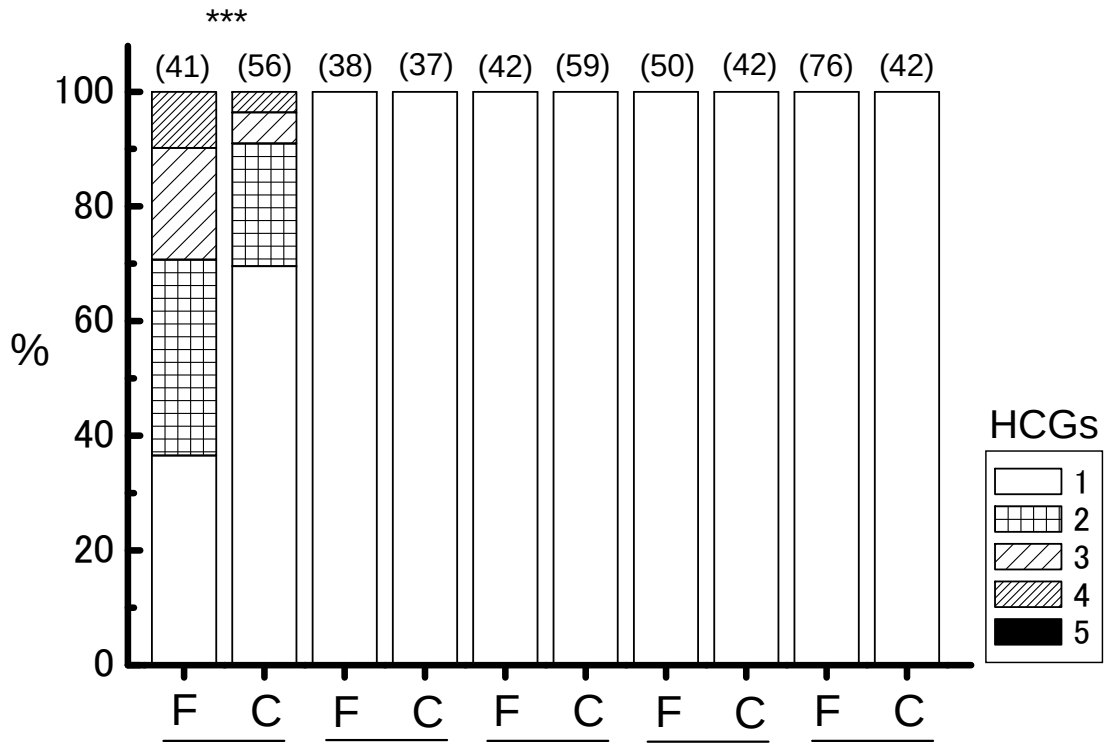

Fig. 5. The effect of fresh and old ovipositing sand on hatchling body color in *S. gregaria*. Five isolation-reared female adults were allowed to lay an egg pod into fresh sand (F) and another egg pod into old sand containing the foam material from three egg pods deposited by crowd-reared female adults (C). The crowd-reared eggs were removed from the egg pods before the test to avoid mixing the isolation- and crowd-reared eggs. N, number of hatchlings scored. ***, $P<0.001$ (χ^2 test).

Chapter 6

Maternal effects on progeny size, number and body color in the desert locust, *Schistocerca gregaria*: density- and reproductive cycle-dependent variation

Abstract

The effects of rearing density and mother's age on the progeny size, number and coloration were investigated in the desert locust, *Schistocerca gregaria*. Isolated-reared females deposited smaller but more eggs than crowd-reared females. The former produced smaller and more eggs with their age, whereas the latter showed a tendency to produce larger and fewer eggs. A similar tendency was also obtained from virgin females. It was found that the first egg pod produced by each crowd-reared female contained significantly smaller and more eggs than did the subsequent egg pods. The former often produced many green hatchlings (0–100%) characteristics of solitarious forms, whereas the egg pods deposited after the first one predominantly produced black hatchlings typical of gregarious forms. Adults were highly sensitive to a shift in rearing density and quickly modified the quality and quantity of their progeny depending on the density encountered. The number of eggs per pod was influenced not only by the mother's rearing density but also by the grandmother's one. The present results demonstrated that the characteristics of progeny are influenced not only by the crowding conditions experienced by the mother and the grandmother but also by the mother's reproductive cycle.

1. Introduction

Locusts show density-dependent phase polyphenism in behavioral, morphological and physiological characteristics (Uvarov, 1966, 1977; Pener, 1991). For example, phase-dependent differences are observed in hatchling characteristics such as body color, size and behavior (Uvarov, 1966; Pener, 1991). In the desert locust, *Schistocerca gregaria* Forskål, solitarious hatchlings are typically green and small, whereas gregarious hatchlings are dark and large. The former are relatively inactive compared with the latter (Faure, 1932; Husain and Ahmad, 1936; Hunter-Jones, 1958; Stower, 1959; Uvarov, 1966). The large body size of gregarious hatchlings is likely to be adaptive at high population density, because they not only show a higher tolerance to desiccation and fasting than small hatchlings of solitarious forms (Albrecht and Blackith, 1960) but also grow to larger adults (Maeno and Tanaka, submitted).

Hatchling characteristics are influenced by the crowding conditions experienced by the female parent during the adult stage in *S. gregaria* (Faure, 1932; Hunter-Jones, 1958). Although the fact that solitarious hatchlings are smaller than gregarious ones is well known, the underlying mechanism controlling their size and body color has not been well understood. Based on a series of studies (Islam et al., 1994; McCaffery et al., 1998; Simpson et al., 1999; Hägele et al., 2000), the Oxford research group proposed a hypothesis that hatchling body color is determined after oviposition by exposure to a gregarizing factor in the egg foam in *S. gregaria*. According to their hypothesis, this gregarizing factor is a small (<3 kDa) hydrophilic substance secreted by gregarious female adults into foam plugs of egg pods. Because it is water soluble, it can be removed from the eggs by washing with water within 1 hour of deposition, causing presumptive gregarious eggs laid by crowd-reared females to produce green hatchlings typical of solitarious forms (McCaffery et al., 1998). In a previous study, however, I re-examined the role of this pheromonal factor, but could not reproduce their results (Tanaka and Maeno, 2006). Instead, I reached a different conclusion that hatchling characteristics have already been determined prior to oviposition (Tanaka and Maeno,

2006). Simpson and Miller (2007) then modified their hypothesis by including a possibility that hatchling body coloration could be determined after ovulation in the oviduct fluids under certain conditions. However, a more recent study demonstrated that the phase-dependent difference in egg size, which is closely correlated with hatchling size and coloration, occurs in the ovary (Tanaka & Maeno, submitted). In the present study, I discovered a new aspect of phase-dependent variation in maternal effects on progeny characteristics, which might explain why the above discrepancy had been brought about.

In *S. gregaria*, a mixture of green, intermediate and black hatchlings often appeared from egg pods laid by either solitarious or gregarious females (Faure, 1932; Husain and Ahmad, 1936; Hunter-Jones, 1958; Bouaïchi et al., 1995; McCaffery et al., 1998; Tanaka and Maeno, 2006). It seems reasonable to assume that such a variation is caused by the length and intensity of crowding stimuli the female parent receives from other individuals including her mating partner. However, I noticed that adult density alone could not fully explain the variation in proportions of different hatchlings, because such a variation occurs even without changing adult density. In this study, I observed how the variations in progeny size, number and body color were brought about in relation to the adult age and reproductive cycles.

While the phenomenon that adult density influences the progeny size and number in locusts is well known (Norris, 1952; Uvarov, 1966; Injeyan and Tobe, 1981; Pener, 1991), most studies have been conducted under constant density conditions by keeping locusts either in isolation or in a group. In the present study, I changed the rearing density at or after adult emergence and observed the effects on the progeny characteristics. As a result, I found that females of *S. gregaria* were highly sensitive to rearing density during the adult stage and modified the quality and quantity of their progeny rapidly. The stimulus perceived by the females appears to be neither sex specific nor species-specific. Furthermore, I observed that the number of eggs produced was influenced by the density experienced not only by the mother but also by the

grandmother. The present paper describes and discusses the results of these observations.

2. Materials and methods

2.1. Insects and rearing conditions

The *S. gregaria* colony used in the present study has been described (Tanaka and Yagi, 1997; Maeno and Tanaka, 2004). Nymphs and adults were kept in groups of approximately 100 individuals in large cages (42 × 22 × 42 cm) or in isolation in small cages (28 × 15 × 28 cm) at 32 ± 1 ℃, LD 16:8 h and 40-70% relative humidity in a well ventilated room, as described previously (Maeno et al., 2004). They were fed fresh leaves of orchard grass and cabbage together with wheat bran. A gregarious line had been maintained for more than 20 generations, and a solitarious line was established from the gregarious colony by rearing nymphs and adults individually in small cages except for a short period for mating (Maeno and Tanaka, 2007). All experiments were carried out with 3rd and 4th solitarious generations and with >20th gregarious generations. The *L. migratoria* colony used in the present study was described previously (Maeno et al., 2004).

2.2. Effect of the number of males on progeny size

In *S. gregaria*, pairing of a female with a single male induces crowding effects on the progeny that are as strong as rearing her with many males (Hunter-Jones, 1958). To confirm this phenomenon with my strain, I determined how many males would be required for a female to perceive crowding by adding 1, 2 or 10 sexually mature males to isolated-reared females in small cages. For comparison, the effect of hetero-specific males was also tested using two *Locusta migratoria* males for each isolated-reared *S. gregaria* female. All females were mated once (<24 h) and allowed to lay 3 egg pods under isolated conditions before males were introduced to them. Then, another 3 egg pods were collected from each female to analyze the crowding effects on the lengths of

eggs deposited. Some females were not added with any males and continuously kept under isolated conditions as controls. Egg length was measured for eggs of the 3rd to 5th egg pods produced by each female using an ocular micrometer installed in a microscope 2 day after deposition. A total of 10 eggs were randomly chosen from each egg pod and placed on a piece of moist filter paper (9 cm diameter) to avoid desiccation before measurements.

2.3. Egg pod collection and measurements of egg size and egg number

Females of a gregarious line were marked individually with white paint (Pentel, EZL31-W, Japan) at adult emergence. They were kept together with males in a group of about 100 individuals in a large cage during the first 12 days of adult life. Then, females were removed from the large cage and held individually in small cages with two sexually mature males to obtain egg pods from individual females. To determine the role of males or mating on the progeny size and number, some females were reared individually both as nymphs and adults in small cages to collect eggs without mating. Twenty females reared under crowded conditions were held together in a medium sized cage (28 x 18 x 30 cm) to maintain their virginity and egg pods produced were collected every day. Females of *S. gregaria* lay eggs without mating and some eggs develop parthenogenetically (Hamilton, 1955; Hunter-Jones, 1958). Plastic cups (diameter, 9 cm; height, 5 cm) filled with clean moist sand were placed in cages to collect egg pods. Egg pods collected during the first 2 months after adult emergence were incubated at 32 ± 1 ℃. Egg length was measured 2 days after deposition as described above and the number of eggs of each egg pod was also counted at that time. After measurements, eggs were returned to moist sand and incubated at the same temperature until hatching.

2.3. Scoring of hatchling body color and body weight

The body color of hatchlings was observed 6 – 12 hours after hatching. Hatchling body color in *S. gregaria* is not influenced by the crowding conditions experienced

during the embryonic stage and first nymphal stadium (Tanaka and Maeno, 2006). Nymphs were divided into 5 hatchling color groups (HCGs) based on the method of Maeno and Tanaka (2007): body color is green without dark spots in HCG 1, increasingly darker in HCGs 2-4 and almost entirely black in HCG 5. After body color was scored, hatchlings were weighted individually using an electronic balance (METTLER AT201, Japan).

2.4. Effects of a change in adult density on the progeny

To investigate the effects of a change in adult density on the progeny size, number and coloration, female adults kept reared under isolated conditions were transferred to crowded conditions and *vice versa*. All females were reared under crowded conditions during the nymphal stage. As will be described below, egg pods deposited early in the adult life produced a mixture of green and black hatchlings. Therefore, each isolated-reared female was allowed to deposit 3 egg pods before she was crowded with 2 sexually mature. Crowd-reared females were kept in a large group (ca. 100) during the first 25 days during which they started laying eggs actively. Females were then transferred to small cages individually with 2 sexually active males. Each female was allowed to deposit one egg pod before she was isolated by removing the 2 males from the cage. Because these females had started ovipositing in the large cage, the number of eggs deposited by each female before the test was unknown, and the egg pods produced by each female during the test were called x + 1, x + 2, x + 3 and so on according to the order of deposition.

2.5. Effects of nymphal density on progeny

To investigate the effects of nymphal density on the progeny size and number, hatchlings of HCG 5 (typical gregarious hatchlings) were reared under either isolated- or crowded conditions and upon adult emergence females were kept individually in small cages to collect egg pods in isolation except for <1 day for mating with a male.

3. Results

3.1. Effect of the number of males on egg size

Fig. 1 compares egg lengths in the egg pods produced before and after adding different numbers of males to ovipositing females of a solitarious line. Some females were kept without males as controls. Mean egg length for egg pods produced by isolated controls was approximately 6.4 mm on average and remained almost constant in the successive egg pods (Fig. 1). On the other hand, after one *S. gregaria* male was introduced, the eggs produced started increasing in length compared with the controls (Scheffe's test; $P<0.05$). However, an addition of more than one male did not cause any further increase in egg length (Scheffe's test; $P>0.05$). A similar crowding effect was also elicited by an addition of 2 males of *L. migratoria*. These results confirmed the observations by Hunter-Jones (1958) that pairing of a female with a single male induces crowding effects on the progeny that are as strong as rearing her with many males, and suggested that a similar crowding effect was obtained even from a different species.

3.2. Effects of phase and age

Egg length and number per pod are compared between a solitarious (isolated-reared) and gregarious (crowd-reared) line (Figs. 2A and B). Mean egg length for the solitarious line (6.4±0.3 mm; n=485) was significantly shorter than that for the gregarious line (7.2±0.3 mm; n=575) (t-test; t=37.719, df=1058; $P<0.001$; Fig. 2A). In the former, egg size remained relatively constant throughout the adult stage, but a statistically significant negative correlation was found between egg length and mother's age at deposition (r= -0.1291; n=485; $P<0.01$). In the gregarious line, on the other hand, females produced relatively small eggs during the early stage of adult life, and egg length tended to increase with the mother's age at deposition (r=0.292; n=575; $P<0.001$). Egg pods laid by gregarious females within 30 days after adult emergence contained relatively small eggs as well as large ones, ranging from 6.1 to 8.0 mm in length

(n=374). This range overlapped that for the solitarious line (5.6-7.1 mm, n=259). After 30 days of adult emergence, eggs laid by gregarious females did not show any significant tendency to change their length with the mother's age at deposition (r= 0.085; n=201; $P>0.05$). A phase-dependent difference was also observed in mean numbers of eggs per pod (Fig. 2B). Egg pods laid by solitarious females had significantly more eggs (89.9±21.9; n=485) than those laid by gregarious females (78.6 ±15.1; n=575; t-test; t=-9.852, df=1058; $P<0.001$; Fig. 2B). The number of eggs per pod slightly increased with the mother's age at deposition in the solitarious line (r=0.1080; n=485; $P<0.05$), whereas a significant negative correlation was found between the two variables in the gregarious line (r= -0.136; n=575; $P<0.01$). These results suggested that egg pods laid early in the adult stage tended to contain somewhat different properties of eggs from those laid later in the adult stage.

To investigate the role of mating or males in determining the characteristics of the progeny, females from solitarious and gregarious lines were continuously kept virgin under isolated and crowded conditions, respectively, after adult emergence. The temporal patterns of changes in egg size were similar between egg pods produced by virgin (Figs. 2C) and mated females (Fig. 2A), although a significant correlation between egg length and mother's age at deposition was found only under crowded conditions (r= -0.631; n=63; $P<0.001$) (Fig. 2C).

A significant density-dependent difference was also found in numbers of eggs per pod in virgin locusts (mean ± SD=88.0 ± 24.6 eggs under isolated conditions; 71.7 ± 19.2 eggs under crowded conditions; t=-4.157, df=125; $P<0.001$), but no significant correlation was found between the number of eggs and mother's age at deposition under either isolated or crowded conditions (Fig. 2D). When eggs produced by mated and virgin females were compared, no significant difference was observed in egg length and number under isolated conditions. Under crowded conditions, on the other hand, virgin females produced significantly fewer but larger eggs than did mated females (t-test; $P<0.05$ each).

Hatchlings obtained from fertilized eggs in the above experiment were categorized into 5 grades based on the darkness of the body. Fig. 3 summarized the pooled data for every 10 day period after adult emergence. Most hatchlings of the solitarious line were green in body color (HCG 1), whereas black hatchlings (HCG 5) were predominant in the gregarious line, although green, intermediate and black hatchlings appeared from both lines. In the solitarious line, the frequency of black hatchlings belonging to HCG 5 was the highest in the first 10-day ovipositing period (32.8 %; n=259 egg pods; range 0 – 100 %; Fig. 3A), and tended to decline rapidly with the age of the female parents (Fig. 2A). In the gregarious line, the majority of hatchlings were dark (HCG 5), but substantial numbers of green hatchlings belonging to HCG 1 also appeared and their incidence was the highest in the first 10-day ovipositing period (15.6 %; n=123 egg pods; range 0 – 100 %).

Body weights were significantly heavier in hatchlings of HCG 5 (mean $\pm$ SD=16.6 $\pm$ 1.2 mg, n=100) than those of HCG 1 (13.2 $\pm$ 1.2 mg, n=100; t=-20.464; df=198; P<0.001) in the solitarious line. The corresponding values in the gregarious line (20.0 $\pm$ 1.9 mg, n=100 for HCG 5; 13.7 $\pm$ 1.2 mg, n=100 for HCG 1) were also different significantly from one another (t=-28.210; df=198; P<0.001). Although hatchlings of HCG 1 from the two lines were almost identical (P>0.05), those of HCG 5 were significantly heavier in the gregarious line than in the solitarious line (t=15.658; df=198; P<0.05).

From eggs deposited by virgin females under crowded conditions, 3 hatchlings appeared parthenogenetically. They were all black (HCG 5).

3.3. Progeny characteristics as a function of reproductive cycles

The above experiments were based on the egg pods produced by a total of 328 adult females. Some females produced only 1 or 2 egg pods before they died, and others produced more. Therefore, it was difficult to determine if the patterns of changes observed in the above experiment were formed by individual females or different

females each showing a fixed pattern. To clarify this problem, egg length and number as well as hatchling coloration were compared among 5 consecutive egg pods produced by the same female adults (Fig. 4). In the solitarious line, no significant difference was found in egg size or number per pod among the 5 egg pods (ANOVA; $P>0.05$; Figs. 4A and B). In the gregarious line, on the other hand, mean egg length was significantly smaller in the first egg pods than in the other egg pods ($P<0.05$; Fig. 4D). By contrast, the number of eggs per pod was significantly larger in the first egg pods ($P<0.05$; Fig. 4E). Hatchling body coloration also showed a pattern of changes similar to what was observed in the above experiment (Figs. 4C and F). That is, the number of hatchlings in HCG 5 was generally small in the solitarious line, but their incidence was significantly higher in the first egg pods than that for the hatchlings from the second egg pod onward (Scheffe's test after arcsine transformation of data; $P<0.05$). It should be noted that the incidence of black hatchlings ranged from 0 to 100 % among the first egg pods of the solitarious line (n=34 egg pods). In the gregarious line, black hatchlings were predominant, but their incidence was relatively small (53 %) in the first egg pods compared with 86 – 90 % in the second egg pods onward (ANOVA after arcsine transformation of data; F=18.283; df=4, 240; $P<0.05$). The incidence of green hatchlings belonging to HCG 1 amounted 15.4 % on average with a range from 0 to 100 % (n=53 egg pods) among the first egg pods of the gregarious line. These results suggested that the first egg pods of either line had a strong tendency to produce substantial numbers of hatchlings with a body color that was not expected from their phase.

3.4. Effects of a change in adult density

Because the first egg pods produced by solitarious females contained some black hatchlings (Fig. 4C), isolated-reared females were allowed to deposit three egg pods and then added with two males to create crowding conditions, whereas other females were continuously kept in isolation as controls. In this experiment, only those females which

produced less than 30 % of black hatchlings in the first egg pod were used. Eggs produced under isolated conditions were relatively small and their size remained almost constant in the successive egg pods (Fig. 5A). However, after males were introduced, the females started producing significantly large eggs in the next (4^{th}) oviposition (ca. 4 days later) compared with the controls (t-test; $P<0.001$). Mean length of their eggs progressively increased over successive egg pods and became almost constant in the 5^{th} egg pod onward, the 2^{nd} oviposition after the addition of males. Mean egg length (7.2 ± 0.3 mm; n=12 egg pods) obtained from the 7^{th} egg pods produced by the crowded solitarious females was not significantly different from that from egg pods laid by gregarious females in Fig. 2A (t-test; $P>0.05$), indicating that an isolated-reared female could switch over her egg size from the one typical of solitarious forms to the one typical of gregarious forms. A significant effect of crowding was also observed in the number of eggs per pod (Fig. 5B). Isolated-reared females decreased the number of eggs per pod gradually after being crowded. However, compared with isolated controls a significant reduction was not observed until the 6^{th} egg pods, indicating that three ovipositions were required to attain a significant change in the number of eggs per pod.

The production of green hatchlings (HCG 1) was also greatly influenced by a change in adult density (Fig. 5D), while females kept in isolation continued to produce high proportions of green hatchlings (Fig. 5C). After males were introduced, the proportion of green hatchlings rapidly decreased, and the proportion of black hatchlings increased instead (Fig. 5D). The latter became predominant in the 5^{th} egg pods, the 2^{nd} oviposition laid after being crowded.

A reversed change in adult density was tested on crowd-reared females. In this experiment, actively reproducing crowd-reared females were transferred from the large cage to small cages where each female was kept with two males. Some of them were isolated after producing one egg pod by removing the males, whereas the other females were continuously reared with two males as crowded controls. The latter continued to lay relatively large eggs over the 4 successive egg pods (Fig. 5E). On the other hand,

those isolated after producing one egg pod reduced egg lengths significantly in the next egg pod (x + 2 in Fig. 5E) and continued to produce smaller eggs over successive egg pods. However, mean egg length reached by the last (x + 4) egg pods was still longer than that obtained from solitarious controls in Fig. 2A (t-test, $P<0.001$). The number of eggs was significantly increased in the first egg pods (x + 2) after isolation and no further significant increase was detected by continuous isolation (Fig. 5F). The mean number of eggs in the last egg pods was 91.3 eggs (SD= $\pm$13.3, n=14), which was not significantly different from the value observed for solitarious egg pods in Fig. 2B (t-test; $P>0.05$). Body coloration of hatchlings also showed a remarkable response to isolation of their mother (Fig. 5H). While most control eggs produced black hatchlings (Fig. 5G), those deposited after isolation produced considerably fewer black hatchlings and the proportion of green hatchlings rapidly increased instead. These results suggested that crowd-reared females were highly sensitive to isolation and modified the egg size and number as well as hatchling body coloration of their progeny rapidly.

3.5. Effects of nymphal density on the progeny

To determine the effects of nymphal density on the reproductive traits, black hatchlings (HCG 5) obtained from a gregarious line were reared either under isolated or crowded conditions and then kept in isolation after adult emergence except for a short period for mating with a male. For comparison, green hatchlings (HCG 1) obtained from a solitarious line were reared under isolated conditions and their eggs obtained under isolated conditions were compared with those obtained from the above two groups. As shown in Fig. 6A, irrespective of the phase at hatching and nymphal density, females isolated as adults produced relatively small eggs and no significant difference was observed among the three groups. Egg pods contained more eggs when produced by females from a solitarious line than when produced by females from a gregarious line (Fig. 6B). In the latter, nymphal density brought about no significant difference in the number of eggs per pod (Fig. 6B). These results suggested that the density

experienced by the mothers as nymphs had no effect on the egg size and number per egg pod but the density experienced by the grandmothers had some effect on the latter trait.

4. Discussion

Maternal crowding influences progeny size and number in *S. gregaria*. As reported previously (Norris, 1952; Hunter-Jones, 1958 Uvarov, 1966; Injeyan and Tobe, 1981; Pener, 1991), the differences in these characters between solitarious (isolation-reared) and gregarious (crowd-reared) locusts are remarkable. The present study demonstrated new aspects of phase-dependent variation in progeny characteristics through maternal effects. That is, egg size is positively correlated with the age of the mother at oviposition, while the number of eggs per pod shows a negative correlation with it in a gregarious line. On the other hand, these traits exhibit an opposite tendency in a solitarious line, although the correlation coefficient was low for either trait (Fig. 2A and B).

A similar tendency is observed in egg size among egg pods from crowded virgin females, indicating that the variation in egg size is dependent on the rearing density of the female adults but independent on mating or the presence of males. Like mated females, virgin females produced significantly fewer eggs under crowded conditions than under isolated conditions, but showed no significant correlation between the number of eggs per pod and the age of the mother at oviposition. The difference in results between mated and virgin females might have been related to the difference in rearing conditions for crowd-reared females, small sample sizes used and/or some male factor.

By keeping each of crowd-reared mated female adults with 2 males, it was revealed that they deposited significantly smaller eggs in the first egg pod than in the subsequent egg pods (Fig. 4D) which contained larger eggs similar to those typically observed in a gregarious line (Tanaka and Maeno, submitted). Thus, the positive correlation between egg length and age of the mother at oviposition observed in Figure

2A is likely to have been caused by the variation in age at first oviposition among individuals. Inversely correlated with egg size was the number of eggs per pod. In the solitarious line, neither egg size nor egg number per pod showed a significant variation over successive egg pods (Fig. 4). However, the first egg pods produced by some solitarious females contained larger eggs than egg pods laid later, so a negative correlation was found between egg length and age of the mother at oviposition. The absence of a significant variation seems reasonable in view of the fact that the production of heavy and black hatchlings was larger in the first egg pod than in the subsequent egg pods but the differences were rather small (>10%; Fig. 3C).

Probably, the most important finding in this study is that solitarious females, which are known to produce mainly light and green hatchlings (Uvarov, 1966), laid significant numbers of eggs producing heavy and black hatchlings during the early stage of reproductive period compared with the values observed during the later stages. On the other hand, gregarious females, which primarily produce heavy and black hatchlings (Uvarov, 1966), tended to produce substantial numbers of light and green hatchlings from eggs laid early in the adult life. These phenomena have not been reported for *S. gregaria*, although the occurrence of a mixture of green and black hatchlings from single egg pods has been repeatedly reported (Husain and Ahmad, 1936; Hunter-Jones, 1958). By observing the first 5 successive egg pods deposited by the same females, it was demonstrated that the first egg pod produced large numbers of black hatchlings compared with the corresponding values for the subsequent 4 egg pods in the solitarious line. A similar tendency was also observed in the gregarious line. In this case, however, it is green hatchlings that appeared in relatively high proportions in the first egg pods compared with the subsequent egg pods.

The underlying mechanism causing female locusts to produce such hatchlings in the first egg pod is unknown. There is a possibility that the enhanced proportions of black hatchlings in the solitarious line have been caused by the social stimuli each female had received from the male during the mating trial. Although the duration of

pairing was brief in the present study (<24 hours), the above possibility cannot be ruled out completely. In the gregarious line, however, some physiological mechanism other than crowding stimuli from males seems to be responsible for the increased production of green hatchlings, because their mothers had been kept with males constantly after adult emergence.

As mentioned earlier, the Oxford research group (Simpson and Miller, 2007) hypothesized that hatchling body color is determined by exposure to a gregarizing factor from the female accessory glands soon after ovulation in the oviduct fluids and/or in the egg foam after oviposition. Eggs obtained from crowd-reared females produced a high proportion of green hatchlings when washed or separated without washing shortly after deposition (McCaffery et al., 1998). In some experiments (McCaffery et al., 1998), they observed that eggs from isolation-reared females produced a high proportion of black hatchlings when deposited into old sand contaminated with egg pods from crowd-reared females. Because some of these experiments were carried out using first egg pods, there is a possibility that their results were influenced by inter-egg pod variation. As described above, the proportion of black hatchlings from first egg pods laid by isolation-reared females varies from 0 to 100% and that of green hatchlings from those laid by crowd-reared females also showed a full range of variation. In their studies using only egg pods produced after the first ones, they failed to confirm the role of the accessory glands in the control of hatchling body color (Hägele et al., 2000). Furthermore, recent studies (Tanaka and Maeno, 2006, submitted) testing their hypotheses completely failed to reproduce their results when only egg-pods deposited late in the adult stage were used, and provided evidence to suggest that progeny characteristics including hatchling size and coloration are determined in the ovary.

The present study demonstrated that female adults of this species remain sensitive to a shift in rearing density and change progeny characteristics relatively rapidly in *S. gregaria*. In this case, females display a maximum response to even an addition of a single male, confirming the results of Hunter-Jones (1958). It was also shown that they

respond similarly to an addition of hetero-specific males. Isolated female adults changed egg size significantly in the first egg pods after being crowded with males, and produced large eggs in the second egg pod onward as typically observed for continuously crowded females. The number of eggs per pod was also changed after the shift in rearing density, although a significant reduction compared with continuously isolated controls was not detected until the 3rd egg pod. The proportion of black hatchlings also increased rapidly after the mothers were crowded with males, whereas it remained at a low level in isolated controls. A reversed shift in rearing density resulted in similar responses, though in an opposite direction. In these experiments, a shift in rearing density was given to females on the day of oviposition and the next oviposition occurred approximately 4 days later on average. According to my preliminary observations, when actively reproducing isolated-reared females were exposed to crowding 1 or 2 days before deposition of another egg pod, darkening was not induced in the hatchlings of that egg pod (Maeno and Tanaka, unpublished). These observations may suggest that there is a critical stage of oocyte development after which maternal crowding does not affect the progeny characteristics. Alternatively, there is a minimum length of crowding or isolation required to elicit its influence on the progeny. Research to test these hypotheses is currently underway.

Finally, it was demonstrated that maternal density as adults is important in the determination of progeny characteristics in *S. gregaria*, but that as nymphs is not (Fig. 6), confirming the observations by Hunter-Jones (1958). Interestingly, the number of eggs per pod was found to be affected by the rearing density of their grandmothers when a total of 658 egg pods were used for the comparison in the present study, although a statistically significant difference was not detected in another comparison using a small sample size (Fig. 5B). This grandmother effect may be explained by a density-dependent variation in the number of ovarioles. In locusts, solitarious females have more ovarioles than gregarious ones, and the number of ovarioles per individual is determined by the population density of the female parent (Uvarov, 1966; Injeyan and

Tobe, 1981). Therefore, the difference in hatchling property (HCG 1 or 5) of the parental generation, which is determined by the density of grandmother's population density, is likely to produce a difference in their egg producing capacity, although egg size is determined only by the crowding conditions of the female parents as adults.

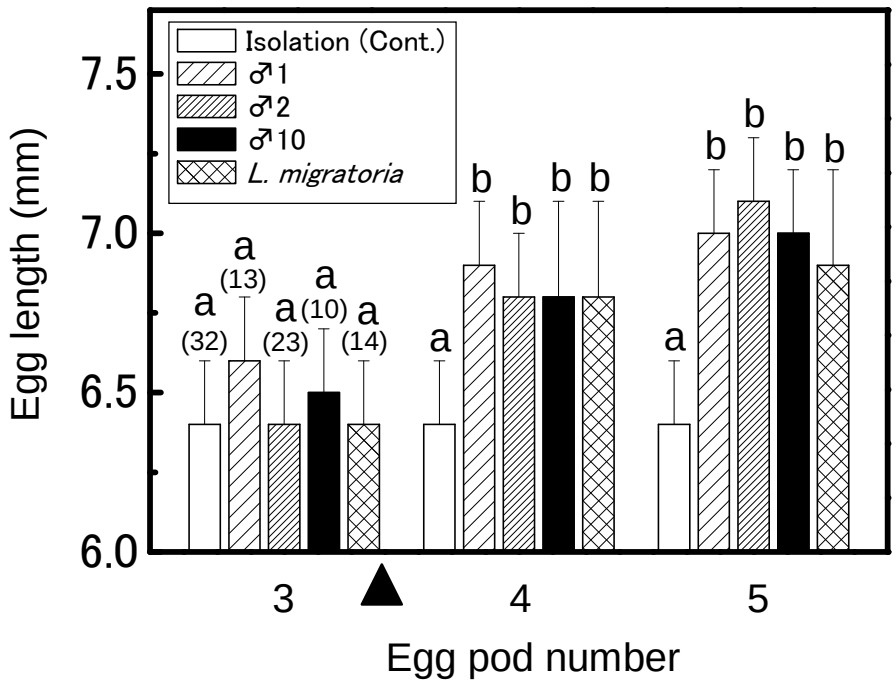

Fig. 1. Effect of the number of males added to isolated *Schistocerca gregaria* females on lengths of eggs produced by the latter. Females obtained from a crowd-reared colony were isolated after adult emergence, allowed to lay 3 egg pods after mating for a short time and crowded with 1, 2 or 10 males of *S.gregaria* or 2 males of *L. migratoria*. Some females were continuously kept in isolation as controls. Different letters above the bar indicate significant difference at 5% by Scheffe's test in each egg pod number. Numbers in the parenthesis indicate *n*.

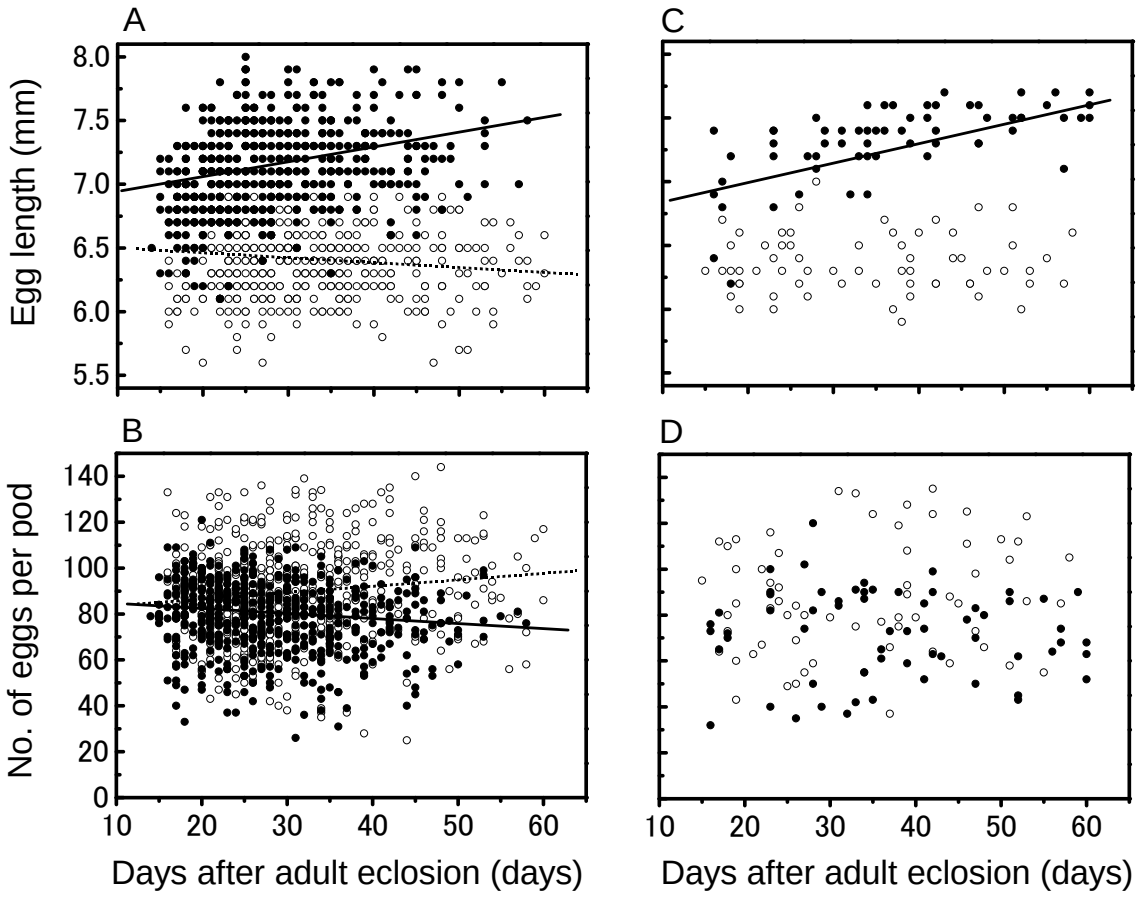

Fig. 2. Temporal variation in egg length (A and C) and egg number per egg pod (B and D) of mated (A and B) and virgin females (C and D) of *Schistocerca gregaria* under isolated (○ and ⋯) and crowded (● and -) conditions. Isolated females were derived from a solitarious line and crowded females from a gregarious line.

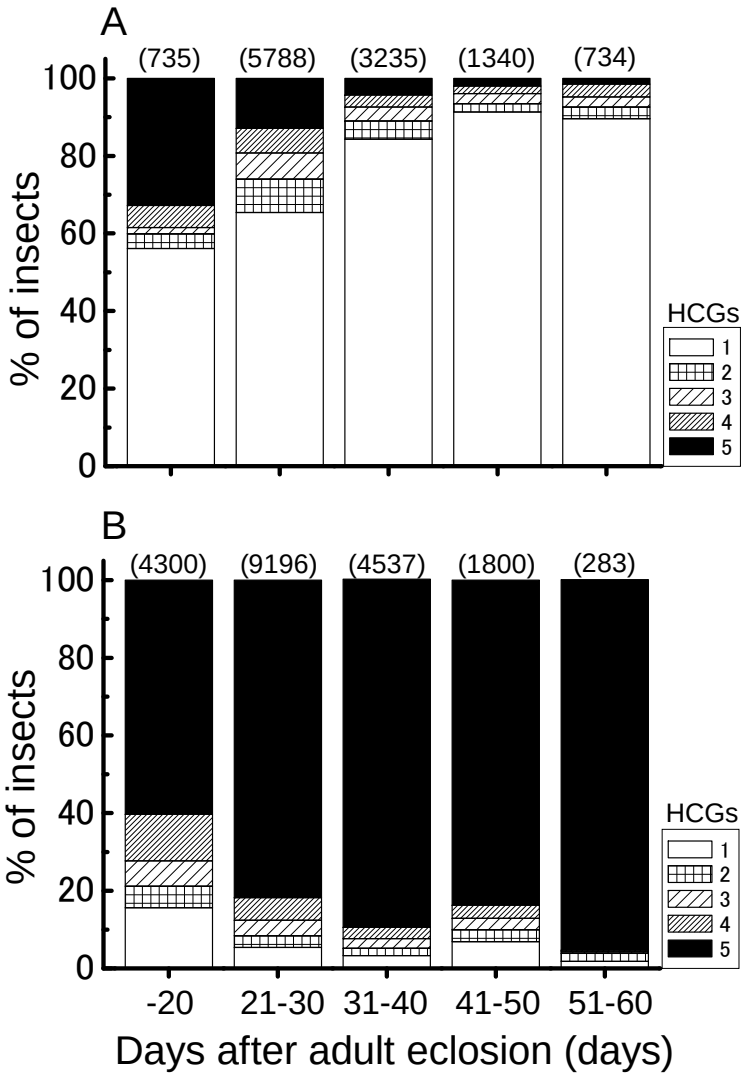

Fig. 3. Percentages of hatchlings with different HCGs produced by mated females of a solitarious line under isolated conditions (A) and those of a gregarious line under crowded conditions (B) in *Schistocerca gregaria*. Numbers in the parenthesis indicate *n*.

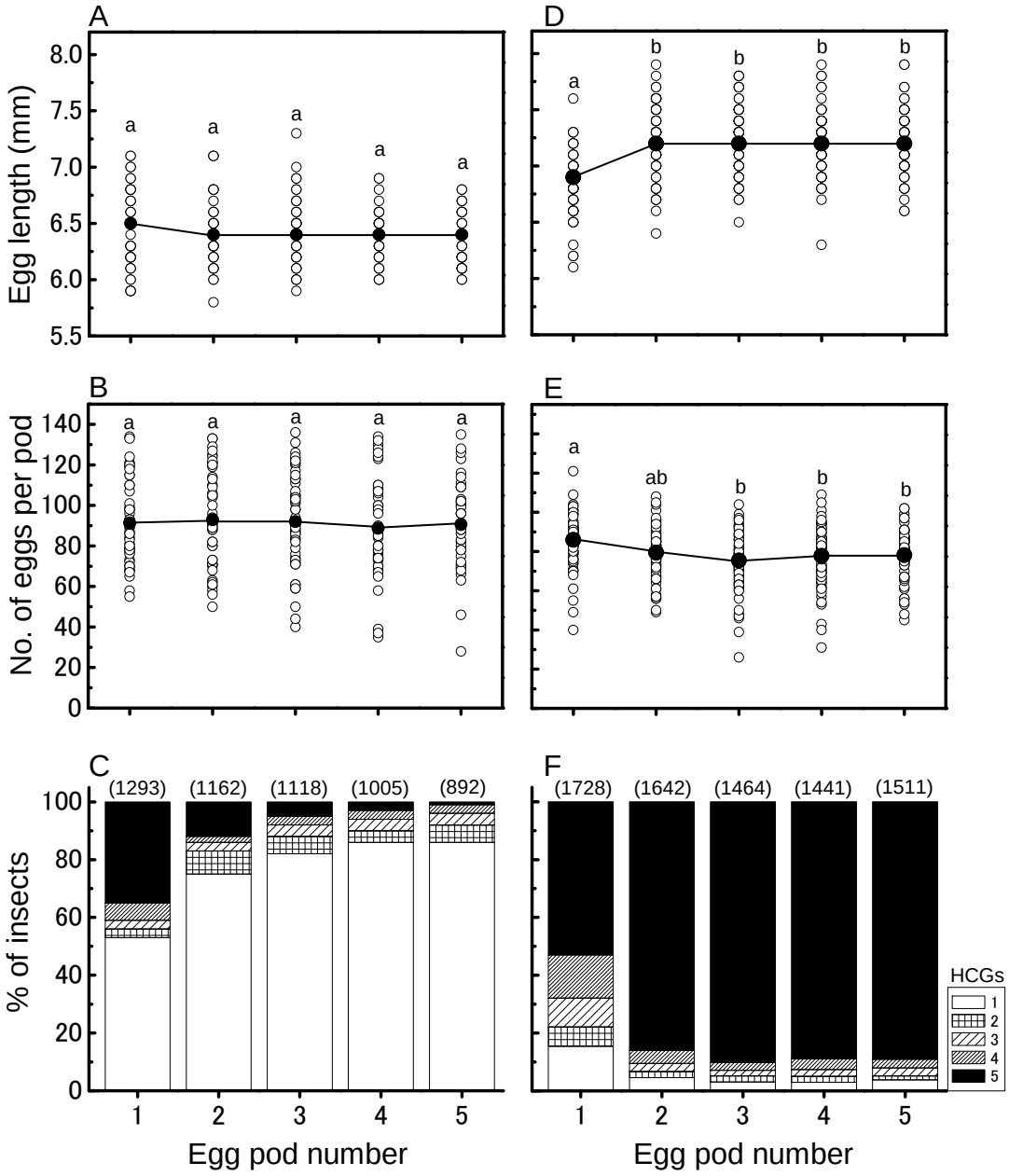

Fig. 4. Changes in progeny characteristics in the first 5 egg pods produced by isolated *Schistocerca gregaria* females of a solitarious line (A-C) and crowded ones of a gregarious line (D-F). Each of isolated females was allowed to mate with a male once before ovipositing. Crowded females were reared in a group of approximately 100 individuals in a large cage until day 12 of adult emergence and then transferred to small

cages where each female was kept with 2 males in a cage to collect egg pods from individual females. Open circles indicate individual datum point and closed ones indicate means, which are connected by a line. Egg pods were obtained from 34 females in isolated females and 77 from crowded females.

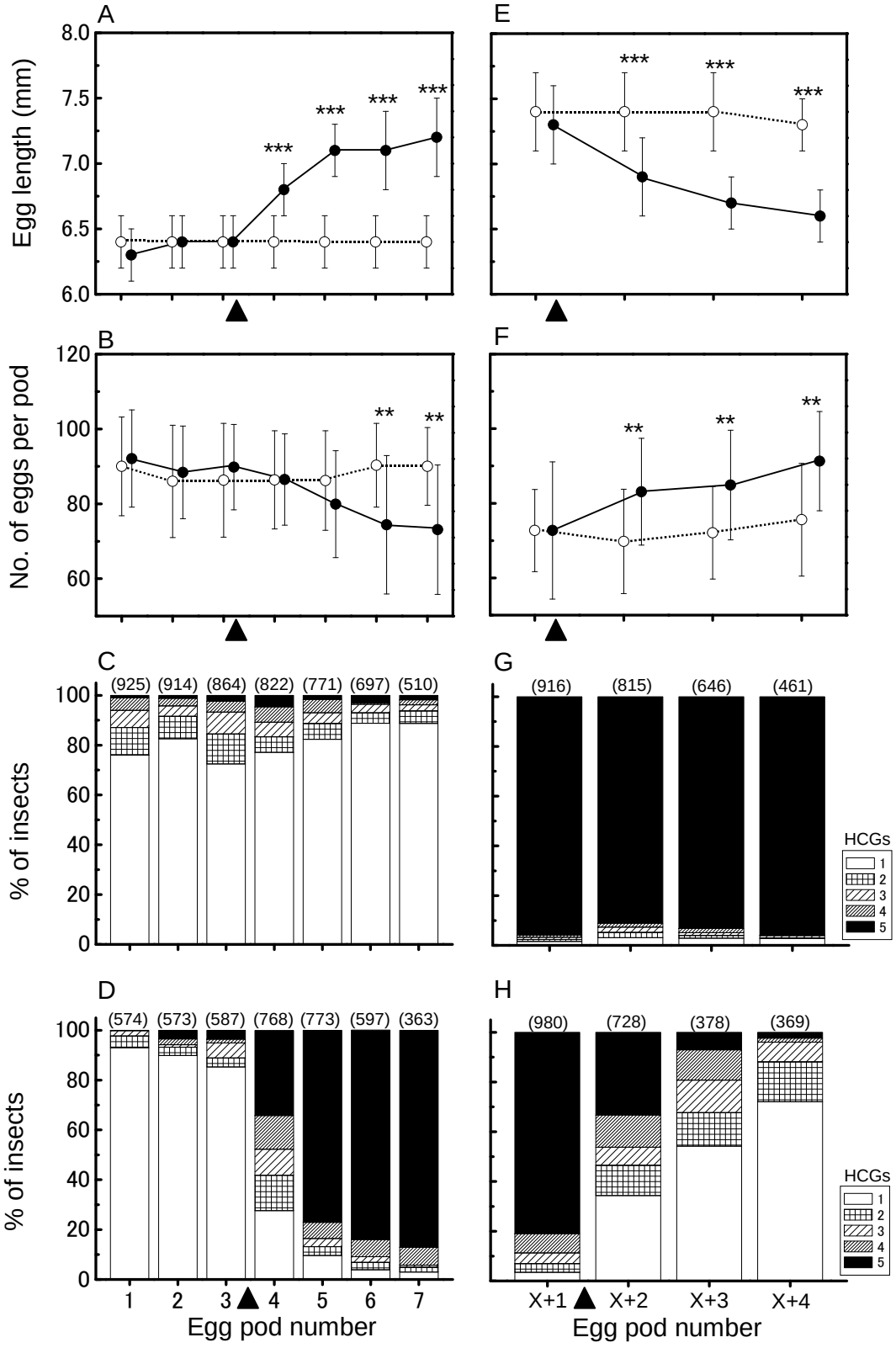
A
E
Egg length (mm)
8.0
7.5
7.0
6.5
6.0
***
B
F
No. of eggs per pod
120
100
80
60
**
C
G
% of insects
(925) (914) (864) (822) (771) (697) (510)
(916) (815) (646) (461)
HCGs
1
2
3
4
5
D
H
(574) (573) (587) (768) (773) (597) (363)
(980) (728) (378) (369)
1 2 3 4 5 6 7
X+1 X+2 X+3 X+4
Egg pod number

Fig. 5. Effects of a change in adult density on progeny characteristics of *Schistocerca gregaria.* Females were either transferred from isolated to crowded conditions (A-D) or from crowded to isolated conditions (E-H) and their progeny characters including egg length, egg number per egg pod and hatchling coloration were observed. In the group transferred from isolated to crowded conditions, females obtained from a gregarious line were kept in isolation after adult emergence except for a short period for mating and 23 females were then exposed to crowding by adding 2 males in each cage, while 32 females were kept in isolation as isolated controls. In the group transferred in the reversed direction, females obtained from a gregarious line were reared in group of ca. 100 individuals for 25 days after adult emergence and then transferred to small cages where each female was kept with 2 males. After collecting one egg pod from each female, 27 females were exposed to isolation by removing the males from the cages, while 24 females were continuously kept with males as crowded controls. Open circles indicate control individuals and closed ones treated ones in A, B, E and F. Hatchlings obtained were categorized into 5 different body color categories (HCGs 1 -5) in C, D, G and H. For explanation, see *2.3*. Sample sizes decreased as females grew older because some females died. Asterisks above a pair of circles between treatments and controls indicate a significant difference by t-test. **, $P<0.01$; ***, $P<0.001$. Numbers in the parenthesis indicate *n*.

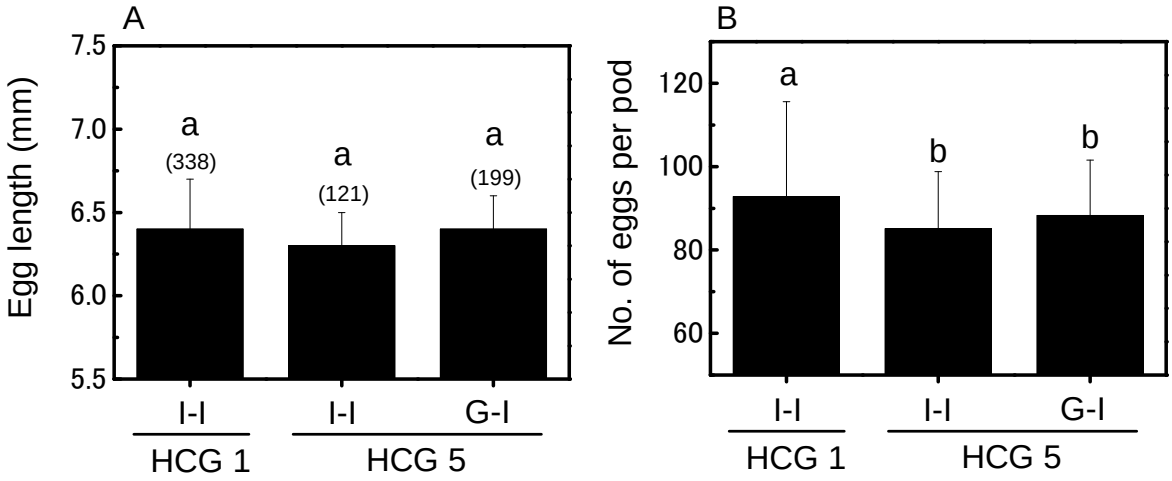

Fig. 6. Effects of nymphal density on mean egg length (A) and egg number per egg pod (B) of *Schistocerca gregaria*. Hatchlings of HCG 1 (from a solitarious line) were reared under isolated conditions and those of HCG 5 (from a gregarious line) were reared under either isolated or crowded conditions. All adults were then kept in isolation except for a short period for mating and allowed to lay eggs. Different letters above the bar indicate significant difference at 0.05% by Scheffe's test. Numbers in the parenthesis indicate *n*.

General discussion

Regulation of nymphal body color in relation to hatchling characters

Locusts show body-color polyphenism that is influenced by genetic and environmental factors, especially crowding (for reviews, see Rowell, 1971; Fuzeau-Braesch, 1985; Dearn, 1990; Pener, 1991; Tanaka, 2001, 2006). The present study investigated the genetic and environmental control of nymphal body color in *S. gregaria*.

There are two kinds of body color polyphenisms during the nymphal stage in *S. gregaria*. One kind is observed in hatchlings and the other is observed in nymphs of later stadia. During the present study, I noticed that some hatchlings were distinctively lighter in color than other hatchlings in a laboratory stock. By selecting for such individuals, it was found that the lighter body color was a recessive trait controlled by a simple Mendelian unit. Because these hatchlings develop reddish brown patterns, I called them the reddish brown (RB) strain. At NIAS, we have been maintaining an albino strain of this locust that was originally derived from a laboratory in Poland. This albinism was also known to be a recessive trait (Hunter-Jones, 1957). I made crossing experiments among these two genetic strains and a normal strain to determine how nymphal body color was genetically controlled. As a result, it was revealed that at least two genes are involved in the expression of normal black patterns of gregarious hatchlings in *S. gregaria*. One gene, tentatively designated as pigmentation gene *P* or *p*, determines whether locusts become pigmented or not, and the other gene, designated as melanization gene *M* or *m*, determines the degree of melanization. The melanization gene expresses brownish patterns in the hatchlings if it is recessive, whereas black (normal) patterns are manifested when it is dominant. In the albino strain, hatchlings are greenish in color and fail to express dark colors even under crowded conditions. In this strain, the pigmentation gene is recessive, but the melanization gene is dominant. Therefore, in a cross between the albino and RB strains, F1 hatchlings develop normal phenotype with black patterns.

At present, the exact function of the pigmentation gene is unknown, but it might be

related to the expression of enzymes responsible for pigment synthesis or that of corazonin receptor systems, because injections of a huge dose (50 ng) of the dark-color inducing neuropeptide ([His^7]-corazonin) induced a slight darkening in some of the injected albino locusts (Yerushalmi et al., 2000). The melanization gene might be related to the production of [His^7]-corazonin, because injection of this peptide into RB nymphs made the latter as dark as normal nymphs. However, there is a possibility that such manipulation simply induced a large amount of black pigment in the cuticle that made the underlying reddish brown color invisible. Apparently, further studies are necessary to determine the genetic and biochemical mechanisms controlling the hatchling body color.

Late stadium nymphs of *S. gregaria* change body coloration in response to population density (for reviews, see Rowell, 1971; Fuzeau-Braesch, 1985; Dearn, 1990; Pener, 1991; Tanaka, 2001, 2006). The present study demonstrated that both body color at hatching and rearing density during nymphal development influence the body coloration at the last nymphal stadium in *S. gregaria*. Last-stadium nymphs with typical solitarious or gregarious body coloration appear when they have the phase-specific body coloration at hatching as well.

To express suitable body coloration matching their habitat, the above-mentioned response could contribute to increase survival rate in *S. gregaria*. At low density, completely cryptic body color matching their habitat background color on which they live could be required for locusts, because predators might easily find prey with body color contrasting to their habitat (Isely, 1938). It is thought that both hatchlings derived from the solitarious and gregarious phase retain the ability to assume camouflaged body color under low density conditions. On the other hand, it is often argued that gregarious body coloration is an aposematic signal (Key, 1957; Sword, 2002). However, there is a possibility that conspicuous black and yellow or orange coloration in *S. gregaria* might provide an advantage to be cryptic in their habitats (Gillett & Gonta, 1978). This hypothesis may be consistent with the occurrence of black patterns with a different

background color in other acridids such as *L. migratoria* (Faure, 1932), the American grasshopper, *Schistocerca americana* (Tanaka, 2004a) and an acridids, *Nomadacris japonica* (S. Tanaka, unpublished) that are not known to consume toxic plants. The significant of gregarious body coloration should be examined in more detail.

Regulation of growth and reproduction in relation to hatchling characters

S. gregaria changes developmental and reproductive performance depending on population density (Uvarov, 1966, 1977; Pener, 1991). Despite the fact that this locust is potentially the economically most harmful insect pest in the world, few reliable data are available about phase-dependent differences in duration of nymphal development and the information is not consistent. Most studies were conducted without considering the variation in the number of nymphal stadia. Crowding, particularly in late-stadium nymphs, can easily cause a shortage of food, and special care is required to avoid a secondary effect of crowding. In the present study, I analyzed locusts with five and six nymphal stadia separately and changed the grass twice a day for late-stadium nymphs to ensure that they had food *ad libitum* throughout nymphal life. The present results indicated that small hatchlings typical of solitarious forms grew faster under crowded conditions than under isolated conditions at the expense of the final body size. On the other hand, larger hatchlings typical of gregarious forms also grew faster under crowded conditions than under isolated conditions, but without becoming smaller as adults. Thus, under isolated conditions, large hatchlings grew faster but emerged as larger adults than did small hatchlings except for some individuals of the latter group that underwent extra molting. Under crowded conditions, large and small hatchlings grew at a similar rate, but the former became larger adults than the latter. Small hatchlings showed a trade-off between development time and body size at maturation, but this constraint was avoided by large hatchlings. The feature of the latter could be important for this locust which often undergoes outbreaks. In solitarious forms, hatchlings are small and take a long time to mature (with low developmental rates) but can attain a large adult body size. At

low population density at which food is less likely to be limiting, large adult body size rather than rapid growth may be more important in terms of fitness. Conversely, in gregarious forms, hatchlings are large and grow rapidly. These characteristics are likely to be adaptive under crowded conditions, because large hatchlings are more tolerant to desiccation and fasting than small ones (Albrecht & Blackith, 1960), and rapid development would reduce the time of exposure to predators. Indeed, large hatchlings rarely undergo extra molting even when reared in isolation. With increased nymphal growth efficiency, gregarious locusts can accomplish both rapid growth and large adult body size by evading the trade-off by which solitarious locusts are constrained.

The present study confirmed that gregarious locusts produce larger eggs than solitarious locusts (Uvarov, 1966). Interestingly, with increased body size of the female parents, egg size tends to increase in gregarious forms, whereas it tends to decrease slightly in solitarious forms. A negative correlation between progeny size and female size is rare (Fox *et al.*, 2000). It is possible that under non-competitive conditions at low population density, selective pressure has favored solitarious females to increase the number of eggs at the expense of individual egg size. The production of smaller eggs by larger females in isolation-reared locusts might be an adaptive response because the environment in which large adults occur is likely to be more favorable for nymphal growth compared with an environment where small adults occur. This would effectively lead to a reduction in the amount of investment to each egg without lowering hatchling survival and a greater investment in egg production. At high population density, on the other hand, large body size in hatchings is likely to increase fitness, as mentioned above. Because of such differences in selective pressure between the two phases, a trade-off between egg size and number, which is clearly shown when data for the two phases are combined, may become less obvious within the solitarious forms and non-significant within the gregarious forms.

In conclusion, locusts exhibit phase-dependent and body size-dependent differences in various developmental and reproductive characteristics. Unlike the

solitarious forms in which development and reproduction are constrained by a trade-off, the gregarious forms have acquired capacities to grow faster without reducing the final body size and to produce more and larger eggs as the body size of the female parent increases. The latter is achieved by increasing the egg-developing capacity relative to body size. Crowding seems to serve as a stimulating signal for locusts to express a set of gregarious characteristics that contribute to rapid population growth during outbreaks.

Mechanism of maternal effects on progeny characters

Based on a series of studies by the Oxford group (Islam et al., 1994a, b; McCaffery et al., 1998; Simpson et al., 1999; Hägele et al., 2000; Simpson et al., 2005), Simpson and Miller (2007) summarized a current view of the maternal effects that gregarious characteristics of hatchlings such as body color and gregarious behavior of *S. gregaria* were greatly influenced by a pheromonal factor derived from the accessory gland of the female parent. Their key observation was that early washing or separation of eggs without washing effectively induces green hatchlings from presumptive black hatchlings produced by crowd-reared female adults (McCaffery et al., 1998). However, the present study (Chapter 5.1) showed that neither treatment induced green hatchlings in *S. gregaria*.

According to Simpson and Miller (2007), whether one uses fresh or old sand is important, because old sand containing egg pods produced by crowd-reared females has been suggested to influence some hatchling characteristics. The present study re-examined this phenomenon and demonstrated that old sand containing three egg foam plugs from crowd-reared females was completely ineffective in inducing black hatchlings in egg pods produced by isolated-reared females. These results are consistent with my conclusion, but contradict the current view by Simpson and Miller (2007) of the dark-color inducing effects of egg foam plugs.

The above results lead to the question of exactly where the phase-dependent body size and color of hatchlings are determined. It is well known that dark hatchlings are

larger than green hatchlings in *S. gregaria*. Simpson and Miller (2007) suggested that hatchling body color is determined by exposure to the gregarizing factor soon after ovulation in the oviduct fluids and/or in the egg foam after oviposition. However, such differences in hatchling body size seemed extremely unlikely to occur after oviposition. Comparison of egg length showed that chorionated eggs in the ovary changed little in size at least until the second day after oviposition. Because egg length at the second day of oviposition is closely correlated with body weight at hatching, one can conclude that hatchling body size is determined in the ovary. In fact, it was found that ovarian eggs of isolation-reared females were much smaller than those of crowd-reared females. Thus, from these results it was concluded that hatchling characteristics are determined before the eggs are laid.

The present study revealed new aspects of phase-dependent variation in progeny characteristics through maternal effects. That is, solitarious females, which are known to produce mainly light and green hatchlings (Uvarov, 1966), laid significant numbers of eggs producing heavy and black hatchlings during the early stage of reproductive period compared with the values observed during the later stages. On the other hand, gregarious females, which primarily produce heavy and black hatchlings (Uvarov, 1966), tended to produce substantial numbers of light and green hatchlings from eggs laid early in the adult life. These phenomena have not been reported for *S. gregaria*, although the occurrence of a mixture of green and black hatchlings from single egg pods has been repeatedly reported (Husain and Ahmad, 1936; Hunter-Jones, 1958). By observing the first 5 successive egg pods deposited by the same females, it was demonstrated that the first egg pod produced large numbers of black hatchlings compared with the corresponding values for the subsequent 4 egg pods in the solitarious line. A similar tendency was also observed in the gregarious line. In this case, however, it is green hatchlings that appeared in relatively high proportions in the first egg pods compared with the subsequent egg pods.

These findings may explain the discrepancy in conclusions between the Oxford

and our research groups. As mentioned earlier, the Oxford research group (Simpson and Miller, 2007) hypothesized that hatchling body color is determined by exposure to a gregarizing factor from the female accessory glands soon after ovulation in the oviduct fluids and/or in the egg foam after oviposition. Eggs obtained from crowd-reared females produced a high proportion of green hatchlings when washed or separated without washing shortly after deposition (McCaffery et al., 1998). In some experiments (McCaffery et al., 1998), they observed that eggs from isolation-reared females produced a high proportion of black hatchlings when deposited into old sand contaminated with egg pods from crowd-reared females. Because some of these experiments were carried out using first egg pods, there is a possibility that their results were influenced by inter-egg pod variation. The proportion of black hatchlings from first egg pods laid by isolated-reared females varies from 0 to 100% and that of green hatchlings from those laid by crowd-reared females also showed a full range of variation. In their studies using only egg pods produced after the first ones, they failed to confirm the role of the accessory glands in the control of hatchling body color (Hägele et al., 2000). Furthermore, the present studies (Chapter 5.1 and 2) testing their hypotheses completely failed to reproduce their results when only egg-pods deposited late in the adult stage were used, and provided evidence that progeny characteristics including hatchling size and coloration are determined in the ovary.

The underlying mechanism causing female locusts to produce such hatchlings in the first egg pod is unknown. There is a possibility that the enhanced proportions of black hatchlings in the solitarious line have been caused by the social stimuli each female had received from the male during the mating trial. Although the duration of pairing was brief in the present study (<24 hours), the above possibility cannot be ruled out completely. In the gregarious line, however, some physiological mechanism other than crowding stimuli from males seems to be responsible for the increased production of green hatchlings, because their mothers had been kept with males constantly after adult emergence.

The present study demonstrated that female adults of this species remain sensitive to a shift in rearing density and change progeny characteristics relatively rapidly in *S. gregaria*. In this case, females display a maximum response to even an addition of a single male, confirming the results of Hunter-Jones (1958). It was also shown that they respond similarly to an addition of hetero-specific males. Isolated female adults changed egg size significantly in the first egg pods after being crowded with males, and produced large eggs in the second egg pod onward as typically observed for continuously crowded females. The number of eggs per pod was also changed after the shift in rearing density, although a significant reduction compared with continuously isolated controls was not detected until the 3rd egg pod. The proportion of black hatchlings also increased rapidly after the mothers were crowded with males, whereas it remained at a low level in isolated controls. A reversed shift in rearing density resulted in similar responses, though in an opposite direction. In these experiments, a shift in rearing density was given to females on the day of oviposition and the next oviposition occurred approximately 4 days later on average. According to my preliminary observations, when actively reproducing isolated-reared females were exposed to crowding 1 or 2 days before deposition of another egg pod, darkening was not induced in the hatchlings of that egg pod (Maeno and Tanaka, unpublished). These observations may suggest that there is a critical stage of oocyte development after which maternal crowding does not affect the progeny characteristics. Alternatively, there is a minimum length of crowding or isolation required to elicit its influence on the progeny. Research to test these hypotheses is needed to understand this phenomenon.

The endocrine mechanisms controlling progeny characteristics have been studied in locusts, although little is known about the hormonal control (for reviews, see Dale and Tobe, 1990; Pener and Yerushalmi, 1998; Tanaka, 2001, 2006). Most researches on this topic have centered on the role of juvenile hormone (JH), which is biosynthesized and released by the corpora allata (CA) and serves as an important hormone for development and reproduction in locusts and other insects. The most widely accepted

hypothesis is that the small green hatchlings characteristic of solitarious forms are induced by an elevated JH titer (Cassier, 1965; Cassier and Papillon, 1968; Tobe and Dale, 1990; Pener, 1991; Applebaum et al., 1997). However, these results were not reproduced by another study in *S. gregaria* (Injeyan et al., 1979). Therefore, the role of JH in controlling progeny characters still needed to be re-examined in locusts. Most previous studies investigating the effects of JH did not consider the effects of reproductive-cycle, which was found to be an important factor affecting progeny characters in the present study. It is possible that locusts treated with JH during the early stage of adult life may produce eggs earlier than untreated counterparts. Because eggs laid early in the adult life tend to be smaller than those laid later as observed in the present study, the mean egg size might become smaller in the former than in the latter even if JH does not affect egg size directly. Experiments to test this possibility should be carried out in the future.

The present study has revealed that crowd-reared locusts start producing smaller eggs after isolation (Chapter 6). If JH has solitarizing effects on progeny characteristics, crowded locusts would produce smaller eggs after JH treatments just like isolated gregarious locusts. This is another possibility to be tested in the future.

Although much information has accumulated about locust phase polyphenism, we know relatively little about its underlying mechanisms. Some of the information that had been widely accepted turned out to be wrong after this study. One of the most mysterious phenomena in locust phase polyphenism is phase accumulation in which maternal inheritance dealt with in this study is known to be involved. According to the present results, the ovary appears to be the site where progeny characteristics are determined in response to changes in population density experienced by female locusts. The present study has provided foundation to explore the biochemical and molecular mechanisms involved in this phenomenon.

Summary

1. The desert locust, *Schistocerca gregaria,* shows density-dependent phase polyphenism in behavioral, morphological and physiological traits. Female locusts modify progeny quality and quantity depending on the population density experienced as adults. This thesis consists of 6 chapters: the first three chapters focus on the physiological adaptations of *S. gregaria* to crowding and the last three concern with the mechanism of maternal effects on the progeny characters.

2. Sexual behavior of *S. gregaria* was investigated for a gynandromorphy. The information obtained was used to establish an experimental system to produce hatchlings with different phase-dependent characteristics (Chapter 1). The gynandromorph observed had a mixture of male and female morphological characteristics. By presenting sexually mature adults to this gynandromorph, it was found that this individual was attracted to normal females but it was recognized as a female by normal males. This observation suggests that the gynandromorph might have had a female-specific pheromone.

3. I investigated how phase-dependent differences in hatchling body coloration would influence the body-color polyphenism at a late nymphal stage at different population densities in *S. gregaria* (Chapter 2). Under isolated conditions, the background body color was either greenish or brownish. Most individuals were greenish and the highest percentage of brownish insects was obtained from hatchlings with the darkest body color. Under crowded conditions, the background color was yellow or orange and the percentage of yellowish nymphs tended to decrease when they were darker at hatching. These results indicated that the background color of last-stadium nymphs was influenced not only by the rearing density during nymphal development but also by the body color at hatching and the latter exerted its influence more strongly under crowded conditions than under isolated conditions. The intensity of black patterns

differed depending on the body colors at hatching and subsequent rearing density. Most isolated-reared nymphs exhibit few or no black patterns, but nymphs with some black patterns also appeared, particularly among those that had been dark at hatching. Under crowded conditions, the black patterns became more intensive when they were darker at hatching. These results indicated that the body coloration of hatchlings and rearing density influenced the intensity of black patterns at the last-nymphal stadium, and that the former had a significant impact especially under crowded conditions. Therefore, last-stadium nymphs with typical solitarious or gregarious body coloration appeared when they had the phase-specific body coloration at hatching as well. The present results demonstrated that both body color at hatching and rearing density during nymphal development influenced the body coloration at the last- nymphal stadium.

4. The effects of hatchling body size on several developmental and reproductive traits were examined under different rearing densities to elucidate the physiological responses to crowding (Chapter 3). The results indicated that small hatchlings typical of solitarious forms grew faster under crowded conditions than under isolated conditions at the expense of the final body size. On the other hand, larger hatchlings typical of gregarious forms also grew faster under crowded conditions than under isolated conditions, but without becoming smaller as adults. Thus, under isolated conditions, large hatchlings grew faster but emerged as larger adults than did small hatchlings except for some individuals of the latter group that underwent extra molting. Under crowded conditions, large and small hatchlings grew at a similar rate, but the former became larger adults than the latter. Small hatchlings showed a trade-off between development time and body size at maturation, but this constraint was avoided by large hatchlings. Phase-specific as well as body size–dependent differences were also detected in reproductive performance. As adult body size increased, females of a solitarious line produced more but slightly smaller eggs, whereas those of a gregarious line produced more and larger eggs. Total egg mass per pod was larger in gregarious

forms than in solitarious forms on average. A trade-off between egg size and number was shown by a solitarious line but not by a gregarious line that produced relatively large eggs with similar numbers of eggs per pod. These results suggested that phase transformation involves not just a shift of resource allocation but also an enhanced capability expressed in response to crowding.

5. The genetic control of phase-specific body color polymorphism was studied using two genetic mutants and a normal strain of *S. gregaria* (Chapter 4). A reddish-brown (RB) mutant found in a laboratory colony developed reddish brown patterns rather than black patterns in the nymphal stage. Reciprocal crosses between the RB mutant and normal strains indicated that the RB phenotype was recessive to the normal phenotype and controlled by a simple Mendelian unit. Reciprocal crosses between the RB mutant and another mutant (albino) produced only normal phenotypes in the F1 generation. In the F2 generation, the normal, RB and albino phenotypes appeared in a ratio of 9:3:4, indicating that two Mendelian units, which control the appearance of dark body color and the intensity of melanization under crowded conditions, may be involved in the regulation of body coloration. To test a hypothesis that the appearance of reddish-brown patterns in the RB strain is due to a reduced concentration of the dark-color inducing hormone, [His^7]-corazonin, the hormone was injected into nymphs of the mutant. This treatment, which is known to be ineffective in inducing dark color in albino nymphs, produced a dose-dependent darkening in RB nymphs, some of which became indistinguishable from normal individuals by appearance. These results may suggest that the RB mutant gene regulates the degree of melanization, possibly through controlling the production and/or release of [His^7]-corazonin under crowded conditions.

6. The mechanism controlling the body color of hatchlings was studied for *S. gregaria* (Chapter 5.1). A pheromonal factor secreted by gregarious female adults into

the foam plugs of egg pods has been suggested for a decade to cause darkening in their progeny. I re-examined the role of this maternal factor by washing or separating eggs at deposition. Eggs produced by crowd-reared female adults were washed with saline or separated individually without being washed immediately after deposition and the body color of the hatchlings from them was compared with that from the eggs unwashed and kept in the egg pod until hatching. Most hatchlings were dark and no significant difference was found in the proportions of dark- and light-colored hatchlings between the treatments and controls. Likewise, eggs separated before the foam plug deposition produced dark-colored hatchlings as in the un-separated controls. These results demonstrated that neither washing nor separation of eggs at deposition affected the hatchling body coloration. The variation in hatchling body color was correlated closely to the body weight at hatching, indicating that hatchling body color had been determined maternally. Green hatchlings reared under crowded conditions remained green until the second stadium at which black patterns were induced. It was concluded that body color at hatching has been determined maternally and crowding during the first nymphal stadium influences nymphal body color but its effect is not manifested until the second stadium. This study casts doubts on the presence of the pheromonal factor recently suggested.

Simpson and his colleague (2007) wrote a review article in which two possibilities were pointed out to explain the differences between their results and ours. One of these possibilities was that our eggs used in the experiments had a high rate of mortality which excluded all green hatchlings, thus leaving only black hatchlings. The other possibility was that our eggs had been exposed to the active compound in the oviduct because they were withheld in the mother's body too long. The latter happened because the mother was deprived of ovipositing substrate during the nighttime, according to Simpson and Miller (2007). Therefore, I tested these possibilities and evaluated their revised version of the foam hypothesis based on the results obtained in this study (Chapter 5.2). Early separation was performed on eggs with a low mortality rate. The

results showed that egg separation did not increase the incidence of green hatchlings. Once eggs were chorionated in the ovary, egg size remained unchanged until the second day after oviposition in either isolated or crowded locusts. This and other results suggested that the phase-dependent differences in body size and color of hatchlings are established in the ovary and that modifications by the accessory gland factor either in the oviduct or after deposition are unlikely.

The results obtained from Chapter 5 cast doubt on the validity of the revised version of the foam hypothesis proposed by Simpson and his colleague. This leads to the question of exactly where the phase-dependent body size and color of hatchlings are determined. The results in Chapter 5.2 suggested that the determination of hatchling characteristics occurs in the ovary rather than in the oviduct or after oviposition Simpson and Miller (2007) claimed.

7. Corresponding to these studies, to investigate how a mixture of green and black hatchlings appears even from egg pods produced by solitarious or gregarious females, I investigated the effects of rearing density and mother's age on the progeny size, number and coloration in *S. gregaria* (Chapter 6). Isolated-reared females deposited smaller but more eggs than crowd-reared females. The former produced gradually smaller and more eggs with their age, whereas the latter showed a tendency to produce larger and fewer eggs as the mother grew older. A similar tendency was also obtained from virgin females, indicating that mating or males are not important in this phenomenon. It was found that the first egg pod produced by each crowd-reared female contained significantly smaller and more eggs than did the subsequent egg pods. The former often produced many green hatchlings (0–100%) characteristics of solitarious forms, whereas the egg pods deposited after the first one predominantly produced black hatchlings typical of gregarious forms. This discovery not only revealed a new aspect of phase polyphenism in locusts but also provided an explanation for the discrepancy in results between the Oxford research group and ours (Chapter 5). Adults were highly sensitive

to a shift in rearing density and quickly modified the quality and quantity of their progeny depending on the density encountered. The number of eggs per pod was influenced not only by the mother's rearing density but also by the grandmother's. The present results demonstrated that the progeny characters are influenced not only by the crowding condition experienced by the mother and the grandmother but also by the mother's reproductive cycle and age.

8. In conclusion, the evidence presented in this study revealed that developmental and reproductive performance is greatly influenced by the hatchling characters and crowding conditions. As a whole, gregarious (crowd-reared) hatchlings do better than solitarious (isolated-reared) ones particularly under crowded conditions. The highly efficient developmental and reproductive performance of gregarious forms seems to contribute to rapid population growth during outbreaks. The progeny characteristics are determined or pre-determined in the ovary of the mother according to the population density experienced during her reproductive period, but not by the foam factor suggested for another strain of the same species. The discovery of the age- and reproductive cycle-dependent variation in progeny characters suggests that inter-egg-pod variation needs to be considered when one studies underlying the mechanism controlling the progeny characters.

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